CXXXIX. LIPASE AND ITS ACTION.

I. THE SYNTHETIC ACTION OF PANCREATIC LIPASE IN THE SYSTEM: OLEIC ACID-GLYCEROL-WATER-DISSOLVED LIPASE.

By ERNEST ALEXANDER SYM.

From the Biochemical Laboratory, Medical Faculty, Warsaw University.

(Received May 20th, 1930.)

THE place of action of the enzyme in the macroheterogeneous two-phase system oleic acid-pancreatic lipase in aqueous glycerol solution may be located in the three following regions—the aqueous phase, the oleic acid phase, and the phase interface. Whilst it is generally accepted that the reaction of hydrolysis of fats takes place at the water-fat interface, no exact experimental evidence in support of this view has been adduced. Further, the opinion has been expressed [Rona and Bien, 1914; Mellanby and Woolley, 1914] that the lipases of blood and of pancreatic juice differ from that extracted from organs in that they act in homogeneous systems in the aqueous phase. Willstätter [Willstätter *et al.* 1923] considers that the enzymic hydrolysis of fats takes place in a microheterogeneous system, and on this hypothesis he bases his theory of the adsorptive action of activators.

The object of the researches described in the present paper was definitely to determine the place of action of pancreatic lipase, in order to be able to explain both the kinetics of reactions catalysed by this enzyme and the mechanism of the action of its activators.

EXPERIMENTAL.

The reaction of the synthesis of olein was selected as the most convenient for the study of the problems outlined in the introduction. The advantages of this reaction for the purpose in question consist, firstly, in that it proceeds, to a certain extent, according to the law of mass action, in contradistinction to the opposite reaction of hydrolysis of oil, which is considerably inhibited at p_H 5.7 (that of the glycerol extracts of pancreas), hydrolysis not exceeding 10 %. Secondly, the p_H of the medium undergoes only very inconsiderable fluctuations during the period of synthesis; the introduction of a new factor in the form of buffer solutions is thus obviated. Finally, the addition of substances activating lipase is unnecessary, as the enzyme remains active in this system for months without change. It was for

this reason that Willstätter's standard solutions, containing buffers and activators, and used for the quantitative determination of the activity of preparations of lipase, were not considered suitable for our experiments, from which it was desired as far as possible to exclude all non-essential factors.

The preparation of lipase used was not as highly purified as were those of Willstätter, so that impurities accompanied the enzyme and may probably have taken part in the reaction. The results obtained with our crude lipase were, however, quite uniform, and no difficulties were encountered in their interpretation. The enzyme was prepared in the following way.

Fresh pig's pancreas was obtained from the slaughter-house, fatty tissue was removed, and the residue passed three times through a mincing machine. The pulp so obtained was triturated in 100–200 g. portions with acetone and introduced into a 10-litre flask, to which a fivefold weight of acetone was then added, and the whole left 4 hours with occasional shaking. The acetone was then filtered off, a fresh portion of acetone was added to the residue, and the whole operation was twice repeated. After the last portion of acetone had been filtered off, the residue was twice extracted with a five-fold volume of ether, after which it was spread in a thin layer on filter-paper and allowed to dry. The well-dried pulp was next ground to a fine powder, the powder was passed through a fine-meshed sieve, and the product was ground in a mortar with 16 times its weight of 88 % glycerol, after which the suspension was left 4 hours at 37°. Water was now added in such quantity as to dilute the glycerol to about 75 %, and the suspension was filtered. The filtrate contains dissolved lipase.

Merck's oleic acid was used in our experiments. Oleic acid was determined by titration with 0.1 *N* alcoholic sodium hydroxide solution, using *o*-cresolphthalein as indicator. The quantity of acid found is given per 1000 g. of emulsion, the weight and not the volume of samples of which was determined, as being considerably more accurate.

The amount of ester synthesised per 1000 g. of emulsion was determined by hydrolysis using 0.1 *N* alcoholic sodium hydroxide solution, to which one-third of its volume of water was added before each determination. The excess of alkali remaining after hydrolysis was determined by titration with 0.1 *N* hydrochloric acid solution.

1. *Effect of changes in the phase interface.*

The following experiments were performed with the object of determining the influence of changes in the surface of contact between the aqueous phase and oleic acid on the velocity of synthesis and on the definitive equilibrium state. Three vessels of different cross-section, viz. (a) 19.5 cm.², (b) 62.8 cm.² and (c) 399.00 cm.², were taken, and to each 282.38 g. of oleic acid and 73.0 % glycerol lipase extract to a total volume of 500 cc. were added. The lipase extract was poured carefully down the walls of the vessel containing oleic acid, in order to avoid emulsification. In this way the liquid phases in all

three vessels were delimited by a sharply defined boundary. The vessels were then kept at 37°.

The amount of ester formed was determined at various intervals of time by taking a sample (2–3 g.) from the oleic acid layer, which was previously carefully stirred, in order to ensure homogeneity. The weighed sample was then titrated with alcoholic sodium hydroxide. The quantity of ester formed is given in millimols per 1000 g. of fatty layer, and is calculated from the difference between the original content of oleic acid, viz. 3546 millimols per 1000 g., and that found at any given time during the reaction, with the introduction of a correction for the increase in the mass of the fatty phase due to progressive replacement of part of its oleic acid by larger molecules of ester. As a result, the sum of free and combined oleic acid in 1000 g. of the fatty phase diminishes progressively as the reaction proceeds. The ester content of a number of samples of mixtures close to the equilibrium point was determined both by titration and by hydrolysis, and an empirical correction was derived from the difference between these two values. Thus 5 g. of such a mixture were found to contain 11.7 millimols of free acid, *i.e.* 2340 millimols per 1000 g. The difference between this and the original value of 3546 millimols would give an apparent value of 1206 millimols of combined oleic acid. Hydrolysis gave, however, a value of only 1140 millimols, giving a total oleic acid content of 3480 millimols. The difference of 66 millimols represents about 5 % of the apparent oleic acid content, and this correction was applied in all cases.

Together with the above three systems, a fourth, of the same composition, continually shaken during the time of duration of the reaction, was taken. Microscopic examination showed that this system is an emulsion of the water-in-oil type. The area d of the phase interface was in this case calculated from the number and the average diameter of the drops of the aqueous phase, with the application of a correction due to the presence in each drop of aqueous phase of 2–3 drops of fatty phase. The approximate value of the area d was calculated with an accuracy sufficient for our purpose to be 356,000 cm.² or 35.6 m.² The degree of esterification was determined on the whole emulsion, calculated per 1000 g. of emulsion, and the appropriate value was calculated per 1000 g. of fatty phase, in order to render possible comparison with the results obtained from the first three systems. In this case, the correction

Table I. *Velocity of synthesis using different contact surfaces.*

Millimols of combined oleic acid per 1000 g. of fatty phase														
Exp.	Surface cm. ²	Days										K	Remarks	
		1	2	7	15	21	23	30	45	60	77			108
a	19.5	—	—	7	22	—	72	84	—	160	451*	—	—	—
b	62.8	—	—	15	68	—	145	215	—	367	463**	—	—	—
c	399.0	—	—	59	339	—	597	755	—	998	1076	1140	1.75	—
d ₁	356 × 10 ³	139	238	—	1110	1224	—	1243	1246	—	—	—	1.55	Calculated for oil phase
d ₂	356 × 10 ³	88	150	—	698	770	—	782	884	—	—	—	—	Calculated per 1000 g. emulsion

Calculated for oil phase
Calculated per 1000 g.
emulsion

applied in unshaken systems is obviously unnecessary. The results obtained for the four systems examined are given numerically in Table I and graphically in Fig. 1.

As a control, oleic acid was left in a vessel of about 100 cm.² cross-section at 37°, in contact with a glycerol solution of the same strength as that used in the above experiments. At the end of 2 months, no reaction had taken place.

It will be seen from Table I that the velocity of reaction has increased fourfold from the 7th to the 15th day in the systems *a*, *b* and *c*, and still continues rapidly to rise on the 23rd day, after which it becomes practically constant. The velocity curves of these systems are, as may be seen from Fig. 1, of the autocatalytic type.

Fig. 1. Influence of surface on velocity of esterification. *a*. 19.5 cm.² *b*. 62.8 cm.²
c. 399.0 cm.² *d*. 356 × 10³ cm.²

During the first three weeks of reaction a greyish-white membrane formed at the phase interface, which grew in thickness and cracked into fragments; such fragments were found suspended in the aqueous phase and also at the bottom of the oily phase.

Experiments carried out on systems *a* and *b* after the completion of reaction showed that active lipase was almost exclusively present in the membrane and its fragments (Table II). These experiments on the partition of active lipase after 108 days of action were carried out in the following way. The aqueous phase was separated from the supernatant oily phase; portions of both phases were then filtered, and the following four systems were examined: (1) filtered aqueous phase—oleic acid; (2) unfiltered aqueous phase—oleic acid; (3) filtered oily phase—74 % glycerol; (4) a 74 % glycerol suspension of the membranes remaining after filtration of the aqueous phase previously washed with acetone—oleic acid. The proportion of oily to aqueous phase was in all cases the same as in the original systems; the reaction mixtures, which each weighed 100 g., were shaken for 6 days at 37°. The results are given in millimols of combined oleic acid per 1000 g. of emulsion. It will be seen from Table II that the filtered aqueous and oily phases are practically free

from lipase, which is present in the unfiltered solution and in the membranes themselves.

Table II. *Distribution of lipase.*

Millimols of combined oleic acid found in the following systems.

	Days			
	1	2	4	6
1. Filtered aqueous phase + oleic acid	15	18	18	19
2. Aqueous phase with membranes + oleic acid	31	—	82	—
3. Oleic acid phase without membranes + 74 % glycerol	11	—	10	—
4. Same as 3, with addition of membranes	50	112	—	—

The acid content of the aqueous phase amounted at the commencement of Experiment *a* to 31 millimols per 1000 g., and at the end to 93 millimols, a gain of 62 millimols; the respective p_H values were 6.5 and 4.6.

The fragments of membrane originating from the aqueous layer, examined under a magnification of 80, exhibited a granular structure, unaffected by the action of acetone or water; Sudan yellow III stains these membranes only very feebly. Those fragments derived from the oily layer contain numerous globules, stained strongly by Sudan, and soluble in acetone or ether, but not in water; such a membrane assumes an identical appearance to the former membranes after ether or acetone extraction. The growth of the membrane on the phase interface, which may conveniently be observed with the aid of a microscope, lasts for weeks. The membrane gives distinct Millon and biuret reactions, and only a very feeble Molisch reaction.

In Experiment *b* (Table I), on the 60th day, marked by two asterisks, most of the aqueous layer was carefully removed by a pipette, leaving a layer of about 1 cm. in thickness, together with the membrane. As can be seen from the curve, the further progress of the reaction was in no way affected by this operation. Whilst during the 30 previous days the quantity of oleic acid combined amounted to 152 millimols, an average daily amount of 5.1 millimols, the amount of synthesis during the subsequent 17 days was 96 millimols, or 5.6 millimols *per diem*, *i.e.* synthesis was even to a certain extent increased. It follows from this result that the lower, aqueous layer, containing only a very small quantity of fragments of membrane, played no part in the catalysis of the reaction.

The contents of the vessel containing reaction mixture *a* were on the day marked by an asterisk in Table I transferred, after previous removal of the upper two-thirds of the oily layer, to a different vessel of a cross-section of 64.2 cm.², *i.e.* practically the same as that used in Exp. *b*. The combined oleic acid content was found 17 days later to be 291 millimols per 1000 g. oil layer, an average daily amount of 17.1 millimols. Had not two-thirds of the oily phase been removed, this same quantity of ester would have presumably been dissolved in a volume three times as great as that actually present; the concentration of combined oleic acid per 1000 g. of oily layer would then have been only one-third of the value found, *viz.* $17.1/3 = 5.7$ millimols, a value

very close to that actually found in the preceding experiments. This is shown in Fig. 1, where the dotted line connecting curve *a* to *b* represents synthesis after transference to a different vessel. A similar process was found later to take place also in emulsions.

It is not quite clear how it is that in these experiments, involving the destruction of the membrane, the synthetic action of lipase remains unimpaired. The fragments of membrane, being specifically lighter than water, rise to their former position at the oil-water interface, but contact can in no case be as close as when the membrane was intact.

Table III. *Influence of area of surface of contact on velocity of synthesis.*

Millimols of combined oleic acid per cm. ² per day.						
Exp.	No. of days					Area of contact cm. ²
	1	7	15	23	30	
<i>a</i>	—	0.05	0.075	0.160	0.14	19.5
<i>b</i>	—	0.04	0.072	0.100	0.12	62.8
<i>c</i>	—	0.02	0.057	0.065	0.06	399.0
<i>d</i> *	39 × 10 ⁻⁵	—	—	—	—	—

* Determinations made only on 1st day, as later data would not be comparable with the others.

Table III gives the number of millimols of combined oleic acid per day per cm.² of phase interface. For the first three systems during the interval between the 7th and the 15th day of reaction a certain proportionality appears to exist between the velocity of reaction and the area of the surface of contact, although these surfaces vary as 1 : 3 : 20. The mean velocity of reaction is about 0.1 millimol/cm.² day, *i.e.* about 6×10^{19} molecules of oleic acid undergo combination. Since the number of molecules of oleic acid occupying 1 cm.² of the oil-water interface is about 2×10^{14} [Langmuir, 1916], it follows that the surface film of oleic acid undergoes esterification 3×10^5 times per day, or about 8.5 times per second. It is probable that diffusion plays an important part in the velocity of this process of replacement of the surface film.

In the experiment with emulsion, the velocity of synthesis per unit of surface is about 250 times smaller than where small contact surfaces exist. Here the constituent molecules of the surface film are replaced only once every 100 seconds.

Whilst in Exps. *a*, *b* and *c* equilibrium was not attained, it may from the shape of the curves be surmised that these would, were sufficient time to be allowed, asymptotically approach curve *d*. The equilibrium constant, $K = [\text{acid}]/[\text{ester}]$, is for experiment *d* 1.55, and for *c* 1.75. These values of *K* do not agree with those obtained in other experiments (see p. 1271) using similar concentrations of glycerol; the reason for this discrepancy is that in these systems the proportion of aqueous to acid phase was relatively too small; hence the formula for *K*, which assumes excess of glycerol and water, is not strictly applicable.

We would, in the light of the experimental data obtained, explain the

course of reactions *a*, *b* and *c* in the following way. During the first 7 days, when the lipase was for the greater part dissolved in the aqueous phase, it was not to any great extent in contact with the oily phase. For this reason, the velocity of reaction was three times smaller than during the subsequent period. The acceleration observed after the first week is thus connected with the formation of the membrane containing lipase and forming at the oil-water interface, and it is for this reason that the velocity curve has a shape similar to that of an autocatalysed reaction.

In order to find the connection between velocity of reaction and area of the phase interface in emulsions, a number of experiments were carried out, in which the weight of the aqueous phase was in all cases kept constant (55 g.) but that of the oily phase was varied from 5 to 30 g. The aqueous phase was a 74.7 % aqueous glycerol extract of lipase. Flasks containing these reaction mixtures were shaken for 5 hours at 37°, whereby an oil-in-water emulsion was obtained. The radius of the drops of oleic acid was about the same in all systems, and amounted on the average to about 25 μ . 1 g. of oleic acid gave about 18×10^6 drops, each of which weighed, therefore 562×10^{-7} mg. The surface of each drop amounted to 75×10^{-4} mm.², so that the surface developed by 1 g. of oleic acid amounted to $18 \times 10^6 \times 75 \times 10^{-4}$ mm.² or 0.14 m.² About 10^{14} molecules of oleic acid are present in each globule of the emulsion. Certain of the systems were left 10 days at 37° in order to

Table IV. *Influence of amount of oleic acid on velocity of reaction.*

All systems shaken for 5 hrs. at 37°.

Exp.	Aqueous phase g.	Oleic acid g.	Total weight of system g.	Area of interface m. ²	Millimols oleic acid combined in 1 g. emulsion	Millimols oleic acid combined in whole system	$K = \frac{[\text{acid}]}{[\text{ester}]}$
1	55	30	85	4.2	0.147	12.5	1.28
2	55	20	75	2.8	0.168	12.6	1.27
3	55	10	65	1.4	0.19	12.3	1.30
4*	55	5	60	0.7	0.20	12.0	1.28

* Velocity of reaction determined in 1.5 hrs., and amount of synthesis calculated for 5 hrs.

allow them to attain equilibrium. The initial velocity of reaction is, in our opinion, a better index of the activity of lipase than is that obtaining in its more advanced stages, when the retarding influence of the reaction of hydrolysis already makes itself felt, and when the velocity of synthesis ceases, as a result, to be uniform.

The results, given in Table IV, show that the velocity of synthesis does not depend on the relative amount of oleic acid taken, and hence on the surface of contact of the oil and water phases, and the same applies to the equilibrium constant. These results are in disagreement with those described for Experiments *a*, *b* and *c*, in which the surface of contact was relatively small.

Order of reaction. The reaction, if bimolecular, should proceed according to the equation $\frac{k_1 + k_2}{2.303} = \frac{1}{t} \cdot \frac{\log Ka}{Ka - (1 + K)x}$; this reaction would be reversible in

the presence of excess water and glycerol. A system consisting of 80 g. of lipase extract containing 73.6 % of glycerol was shaken for 7 days at 38°, samples being taken from time to time for the determination of combined oleic acid, expressed in Table V as millimols per 1000 g. of emulsion.

Table V. *Order of reaction.*

Time hrs.	(a - x)	x	$K = [\text{ester}]/[\text{acid}]$	$\frac{k_1 + k_2}{2.303}$
0	472	0	—	—
3	444	28	—	0.021
15.4	367	105	—	0.020
26.3	331	141	—	0.019
420	310	162	—	0.016
∞	268	204	0.761	—

It will be seen from the figures given in the last column of Table V that the above equation agrees fairly well with experiment; the law of mass action is thus applicable to this reaction, in spite of the fact that it takes place in a heterogeneous system. It follows that the velocity of diffusion does not determine the velocity of the reaction in question.

2. *Influence of the relative amount of aqueous phase present on the initial velocity of reaction and on the value of the equilibrium constant $K = [\text{acid}]/[\text{ester}]$.*

All the systems used in the following experiments contained the same weight (20 g.) of oleic acid, but different amounts of 74.7 % glycerol extracts of pancreas. The flasks were shaken vigorously for 2 mins. to produce an emulsion, and were then shaken gently for 4.5 hrs. at 38°. The number of drops of oleic acid, estimated with the aid of a Thoma-Zeiss cell, was practically the same in all systems (see Table VI), so that the phase interface in all cases was approximately the same, viz. about 2.8 m.² Thus the phase interface, the quantity of oleic acid, and the concentration of the constituents of the aqueous phase are the same in all systems studied, but the amount of these constituents is varied. The effect of thus varying the quantity of enzyme taken on the initial velocity of synthesis and on the equilibrium constant is studied by determining the number of millimols of combined oleic acid per g. of emulsion after 4.5 hrs. of action.

Table VI. *Effect on velocity of reaction of varying the quantity of aqueous phase.*

Systems shaken for 4.5 hrs. at 38°.

System	Oleic acid g.	Aqueous phase g.	Wt. of whole system g.	Millimols combined oleic acid per g. emulsion	Millimols combined oleic acid per g. aqueous phase	No. of globules in 1 cc. emulsion	No. of globules in 1 g. emulsion	No. of globules in whole system	K
1	20	20.2	40.2	0.116	0.230	10×10^6	9×10^6	36×10^8	1.27
2	20	36.0	56.0	0.144	0.224	7.5×10^6	6.3×10^6	35×10^8	1.12
3	20	72.8	92.8	0.175	0.222	4.5×10^6	3.8×10^6	35×10^8	1.10
4	20	108.0	128.0	0.155	0.184	3.3×10^6	2.8×10^6	35×10^8	1.15

The following conclusions may be drawn from the results obtained (cf. Table VI). The initial velocity of reaction is to a certain extent proportional to the weight of aqueous phase taken, as is shown by the values obtained for the ester content of the entire system. For the aqueous phase alone, however, the velocity of reaction falls with increase in its volume. The value of the equilibrium constant $K = [\text{acid}]/[\text{ester}]$ does not on the whole vary with variations in the volume of aqueous extract taken, as glycerol and water are in all cases present in excess. In system 1, however, in which the relative concentration of these constituents is smallest, K differs from that of the other systems.

3. *Effect on the velocity of reaction and value of the equilibrium constant of varying the concentration of glycerol.*

22.5 g. portions of 74.7 % glycerol extract containing lipase were mixed with equal weights of solutions of glycerol at various concentrations. Whilst the volumes of these systems, and hence the concentration of enzyme in them, are not strictly equal, yet the difference between the volumes of the systems does not exceed 2 cc. in the extreme cases, and this difference has no practical effect on the results obtained for the action of the enzyme. 20 g. of oleic acid were added to all systems, which were shaken for 22 hrs. at 38°, at the end of which time the concentration of combined oleic acid in millimols per 1000 g. emulsion was determined, whilst the equilibrium constant,

$$K = [\text{acid}] [\text{glycerol}] / [\text{ester}] [\text{water}],$$

was determined at the end of 2 weeks. In this way, the effect of varying the concentration of water and of glycerol, whilst keeping the concentration and quantity of enzyme, as well as the interphase surface, and the quantity of aqueous and fatty phases, constant, was studied.

The initial velocity of reaction was calculated from the formula

$$\frac{k_1 + k_2}{2.303} = \frac{1}{t} \cdot \log \frac{K_2 a}{K_2 a - (1 + k_2) e},$$

where e is the concentration of ester. Since the quantity of all substrates taken is determined per 1000 g. of emulsion, we have not in our case to deal with concentrations in the strict sense of the word. For comparative purposes, however, the above formula, based on the law of mass action, may be applied; the error due to the above discrepancy does not, in any case, exceed 4 %.

The results given in Table VII show that the reaction is markedly accelerated by increasing the concentration of glycerol, and that the same factor shifts the equilibrium point of the reaction in the direction of synthesis. A comparison of the equilibrium constants, K , obtained shows that the reaction does not, over the range of concentrations taken, follow the law of mass action. The values given in the last column show that glycerol, at the concentrations taken, does not activate lipase, and that the increase in the initial velocity of reaction is due only to the shifting of the equilibrium point.

Table VII. *Influence of concentration of glycerol on velocity of reaction.*

Exp.	Conc. of glycerol %	Millimols combined oleic acid per 1000 g.		Millimols combined oleic acid per 1000 g. whole system	K_1	$K_2 = \frac{[\text{ester}]}{[\text{acid}]}$	$\frac{k_1 + k_2}{2.303}$	Remarks
		glycerol	water					
1	62.3	6.8	2.1	52.5	12.6	0.257	0.0064	—
2	66.3	7.2	1.9	64.5	9.5	0.44	0.0050	—
3	72.4	7.8	1.5	100.5	8.3	0.61	0.0065	—
4	76.7	8.3	1.3	147	6.0	1.07	0.0069	—
5	82.4	9.0	0.98	180	5.9	1.54	0.0077	—
6	88.4	—	—	197	—	2.32	0.0074	Calc. comparatively from adsorption Exp. 3, Table X

4. *Effect on the velocity of reaction and the equilibrium constant of varying the concentration of enzyme.*

It was here desired to establish whether proportionality exists between the concentration of lipase and the initial velocity of reaction, as has been found to hold for many other enzymes. With this object, 55 g. portions of mixtures in different proportions of lipase extract and of solutions of the same concentration of glycerol (74.7 %) were taken, and shaken for 6 hrs. at 38° with 20 g. of oleic acid, after which combined oleic acid, expressed in Table VIII as millimols per 1000 g. emulsion $[e]$, was determined. The equilibrium constant K was determined at the end of 2 weeks of reaction, at which time, however, system 4 was still far from the equilibrium state.

Table VIII. *Influence of concentration of enzyme on velocity of reaction and on K .*

Exp.	Glycerol extract taken g.	74.7 % glycerol solution added g.	Relative lipase concentration $[E]$	Millimols combined oleic acid per 1000 g. emulsion $[e]$	e/E	K
1	55	0	1	156	156	1.15
2	41.25	13.75	0.75	111	149	1.09
3	27.5	27.5	0.5	71	142	1.03
4	13.75	41.25	0.25	36	144	—

The results, given in Table VIII, show that the initial velocity of reaction is directly proportional to the relative concentration of enzyme, whilst the equilibrium constant is independent of this value.

ADSORPTION OF LIPASE ON OLEIC ACID.

40 g. portions of lipase extract were shaken at a definite temperature and for a fixed time with oleic acid, and the systems were then centrifuged 10 mins. The upper layer of oleic acid, of a jelly-like consistency, was then removed with a spatula, whilst the lower, aqueous layer was removed with a pipette. The lipase content of 20 g. of the aqueous layers was then determined by adding 5 g. of oleic acid, and determining velocity of synthesis (v_2)

as usual. Percentage adsorption of lipase was evaluated by comparison with the corresponding velocity v_1 found in a similar system with fresh lipase extract.

A microscopic examination of the oleic acid layer after centrifuging showed that this differed in no way from the whole emulsion except in the greater number of globules per unit volume; in particular, the dimensions of the individual globules were unchanged.

1. *Connection between quantity of oleic acid and percentage adsorption of lipase.*

These experiments were performed on lipase extracts containing 77 % of glycerol, at p_H 5.8. The systems were shaken for 10 mins. at 18°, and centrifuged for 10 mins. The results, given in Table IX, show that practically the entire

Table IX. *Influence of relative quantity of oleic acid taken on the degree of adsorption.*

40 g. of 77 % glycerol lipase solution shaken for 10 mins. at 18° with different quantities of oleic acid, system centrifuged for 10 mins., and 5 g. oleic acid added to 20 g. of aqueous layer.

Exp.	Oleic acid taken g.	Time t hrs.	v_2 millimols	v_2/t	$(v_1 - v_2) \cdot 100$
					v_1 %
1	10	5	22.1	4.4	91
2	10	20	95	4.7	90
3	4	4	106	26	49
4	1.2	4	152	38	26
5	Comparative system (20 g. original extract, 5 g. oleic acid)	3.7	$v_1 = 188$	$v_1/t = 51$	0

lipase is removed from solution by shaking with 10 g. of oleic acid. The relationship between the quantity of oleic acid taken and percentage sorption is shown graphically in Fig. 2.

2. *Velocity of adsorption, and influence of temperature on the adsorption equilibrium.*

40 g. portions of lipase extract containing 72 % of glycerol were shaken with 1.2 g. of oleic acid at different temperatures and for different lengths of

Table X. *Velocity of adsorption and influence of temperature on the degree of adsorption.*

All flasks contain 40 g. of 72 % glycerol lipase solution and 1.2 g. oleic acid; time of reaction 6 hrs.

Exp.		v_2	v_2/t	$(v_1 - v_2) \cdot 100$
				v_1
1	Shaken 20 mins. at 18°	168	28.0	22
2	" 5 " 18°	157	26.0	27
3	" 1 " 18°	161	27.0	25
4	" 20 " 28°	148	24.7	31
5	" 20 " 38°	150	25.0	30
6	" 1 " 38°	148	24.7	31
7	Comparative system: 20 g. original lipase extract, 5 g. oleic acid	$v_1 = 215$	39	0

time, after which the systems were centrifuged for 8 mins., and lipase determined as before in the aqueous layer.

The results, given in Table X, show that the velocity of adsorption was too great to be measured under our conditions, as equilibrium was already attained after shaking for 1 min. and centrifuging for 8 mins. Percentage adsorption of lipase was slightly greater at higher than at lower temperatures.

3. *Activity of adsorbed lipase.*

In order to investigate the activity of adsorbed lipase, two systems were prepared, containing respectively 40 g. and 20 g. of lipase extract containing 72 % glycerol and 10 g. of oleic acid. Both systems were shaken for 10 mins., and centrifuged for 10 mins., after which 10 g. of the upper layers were carefully transferred to two flasks, each containing 40 g. of 88.4 % glycerol. These flasks were then shaken for 4 hrs. at 38°. In this way, two emulsions were obtained, each containing 40 g. of aqueous glycerol and about 10 g. of oleic acid. The previous experiments showed that the degree of adsorption should in both cases amount to over 90 %, whence it follows that the concentration of lipase in the first system should be twice as great as in the second.

Table XI. *Activity of adsorbed lipase.*

Exp.	Time <i>t</i> hrs.	<i>v</i> ₂ millimols	<i>v</i> ₂ / <i>t</i>
1	4	216	54
2	4	117	29

The results, given in Table XI, show that the velocity of reaction in the first system is 1.86 times as great as in the second. It follows from this that adsorbed lipase is not thereby inactivated, and that the initial velocity of reaction is proportional to the concentration of adsorbed lipase.

The equilibrium constant in Exp. 1 was at equilibrium 2.3.

4. *Effect on degree of adsorption of keeping the emulsions at different temperatures for 48 hrs.*

In the experiments described at the beginning of this paper it was found that the velocity of reaction increased during the second week, giving to the velocity curve the appearance of that of an autocatalysed reaction.

The experiments described in this section had as their object to ascertain whether, in those cases in which total adsorption of lipase does not take place, the remainder in solution is not later also deposited in some other way on the surface of the oleic acid globules. Emulsions of 1.2 g. of oleic acid in 40 g. of lipase extract (72 % glycerol) were left in separating funnels for 48 hrs. at different temperatures. At the expiration of this period the oleic acid globules had collected in the upper layers of the system, and samples of the lower, aqueous layer were analysed as usual for lipase. No inactivation of lipase can have taken place in this time, as our first experiments had shown that lipase, in membrane form, retains its activity even after 3 months.

Table XII. *Second period of sorption.*

Exp.	Temp.	Duration of emulsion	Time <i>t</i> hrs.	$\frac{v}{t}$ Combined oleic acid millimols	$\frac{v}{t}$	% elimination of lipase from solution
1	18°	20 mins.	4	110	27.5	23
2	18°	48 hrs.	4	99	25	31
3	38°	20 mins.	4	104	26.0	27
4	38°	48 hrs.	4	47	11.8	67
5	Comparative system		4	144	36.0	0

The results, given in Table XII, show that the further removal of lipase from solution, after the first, rapid process of adsorption, takes place comparatively slowly, and that its progress is to a large extent dependent on temperature. Thus sorption of lipase during this second period amounts at 18° to $31 - 23 = 8\%$, whilst at 38° the difference is $67 - 27 = 40\%$, *i.e.*, the globules of oleic acid remove during this second period five times as much lipase at 38° as at 18°.

5. *Connection between relative quantity of oleic acid and percentage sorption of lipase during the second period of elimination from solution.*

The experiments described in this section were made in the same way as those of the previous section, except that the quantity of oleic acid was varied, and that the lipase extract here contained 77 % glycerol. For comparison with the first period of adsorption, the results obtained are compared in Table XIII with those of Table IX, and are also shown graphically in Fig. 2.

Table XIII. *Connection between relative quantity of oleic acid taken and extent of elimination of lipase during the second period of adsorption.*

Exp.	Adsorbent	% adsorption, duration of emulsion		
		1st period	2nd period	
		20 mins.	24 hrs.	48 hrs.
1	1.2 g. oleic acid	26	49	65
2	2.5 "	—	—	91
3	4 "	49	—	—
4	5 "	—	82	96
5	10 "	91	98	99

The results obtained (Table XIII) show that the removal of lipase from solution during the second period is due to some factor other than adsorption; this process proceeds very slowly, and is dependent on temperature.

Combining these results with those obtained in the first experiments described in this paper, it might be concluded that the second period consists in the formation at the phase interface of lipase-containing membranes.

Since in all subsequent kinetic experiments we used such quantities of oleic acid that practically the whole of the lipase present was adsorbed during the first period, one can readily comprehend why it was that in these later experiments the velocity of reaction was not proportional to the area of the

phase interface, and that the autocatalytic type of reaction observed in the first experiments did not recur in the subsequent ones.

Fig. 2. Relation between percentage adsorption and time.
I. 20 mins. II. 24 hrs. III. 48 hrs.

6. *Reversibility of adsorption of lipase.*

40 g. portions of lipase extract (72 % glycerol) were shaken for 10 mins. with 4 g. portions of oleic acid, and the systems were then centrifuged. The lipase content of the lower aqueous layer was determined as described above in order to determine adsorption, which amounted to 43.3 % (Exp. 2, Table XIV). This value is derived in the following way: the entire lipase of the system, when shaken with 10 g. of oleic acid, gives a value for combined oleic acid of 144 millimols/1000 g. emulsion (Exp. 1). Percentage adsorption would therefore be equal to $100(144 - 81.7)/144 = 43.3$, where 81.7 represents the number of millimols of synthesis obtained in Exp. 2.

The oleic acid layer was transferred to a fresh 40 g. portion of lipase extract, again shaken for 10 mins., and centrifuged, and the lipase content of the aqueous layer determined; the difference between the first and the second values would represent elution, which amounted to 32 % (Exp. 3, Table XIV). Since 81.7 millimols represents the activity of unadsorbed lipase, it follows that that of adsorbed lipase would be given by $144 - 81.7$ millimols. Since eluted lipase accounted for a synthesis represented by 19.9 millimols, percentage elution would be given by $100 \times 19.9/(144 - 81.7) = 32$ %; consequently adsorption in this system is 68 %.

In order to ascertain whether the definitive adsorption equilibrium is the same whether the lipase is present originally exclusively in solution or in adsorption, glycerol solutions (40 g.) containing an amount of lipase equal to that adsorbed by oleic acid in the above experiment (44 %) were shaken with

4 g. of oleic acid, and percentage adsorption determined as before (Exps. 4 and 5). The value of 59 % adsorption is obtained as follows: combined oleic acid in system 5 amounts to 24.3 millimols, whilst where the whole of the lipase acted 59.7 millimols were combined. Hence percentage adsorption is given by $100 (59.7 - 24.3)/59.7 = 59$.

Table XIV. *Elution of lipase.*

Exp.	Conditions	Combined oleic acid millimols per 1000 g. emulsion	% adsorption	% elution
1	<i>Activity of total lipase.</i> Comparative system to 2. 10 g. oleic acid shaken 4 hrs. with 40 g. lipase extract at 38°	144	—	—
2	<i>Activity of unadsorbed lipase.</i> 4 g. oleic acid shaken with 40 g. lipase extract, centrifuged, and 10 g. fresh oleic acid shaken with 40 g. aqueous layer 4 hrs. at 38° ...	81.7	43.3	—
3	<i>Activity of eluted lipase.</i> 4 g. oleic acid shaken with 40 g. lipase extract, centrifuged, oleic acid layer shaken with 40 g. 72 % glycerol solution, centrifuged, and 10 g. oleic acid added to aqueous layer, then shaken 4 hrs. at 38°	19.9	68	32
4	Same as Exp. 1, but concentration of enzyme 44 % that of Exp. 1	59.7	—	—
5	Same as Exp. 2, but concentration of enzyme 44 % that of Exp. 2	24.3	59	—

Initial percentage adsorption was calculated from the initial velocities of reaction in Exps. 1 and 2, corresponding respectively to the activity of total lipase and to that of residual, unadsorbed lipase. Percentage adsorption from solutions containing 44 % of the amount of lipase present in Exp. 1 was evaluated from the velocities of reaction in Exps. 4 and 5, corresponding to 1 and 2. Finally, percentage elution (32 %) was determined from Exps. 1, 2 and 3.

It follows from Exps. 3, 4 and 5 that adsorption is only partially reversible, since where the enzyme was at the commencement entirely in adsorption, 68 % remained, whilst where the same quantity was at first entirely in solution, only 59 % underwent adsorption. Percentage adsorption is greater at lower than at higher concentrations of lipase, being respectively 59 and 43.3 %.

DISCUSSION.

Lipase acts at the phase interface in the system lipase-glycerol extract-oleic acid.

When the two phases are in contact with one another, at the commencement of the reaction lipase disappears from the aqueous phase and accumulates at the phase interface. This process takes place in two ways. The first of these is of the nature of adsorption, as follows from its rapid completion and its comparative independence of temperature. Lipase so adsorbed is

active, and a proportionality exists between the quantity of adsorbed lipase and the amount of synthesis occurring.

The second process of accumulation of lipase depends probably on the formation of a membrane at the phase interface, as is shown by the experiments dealing with small surfaces, as well as by the slow disappearance of lipase from solution, and by the dependence of this process on temperature.

The area of contact of the two liquid phases has a noticeable effect on the velocity of reaction only where this area is comparatively small, and, consequently, comparatively large quantities of lipase are accumulated on a small surface. In such cases such factors as diffusion and the time necessary to form the membrane may undoubtedly affect the velocity of reaction. We can, by comparing the results obtained from experiments on adsorption with those found for experiments on the influence of the area of the surface of contact, roughly estimate what is the smallest area of contact necessary in order to obtain total adsorption of lipase during the first period. The first experiment on adsorption showed that using four parts of lipase extract to one of oleic acid, adsorption is practically quantitative. 1 g. of oleic acid develops an area of 0.14 m.² in emulsions, whence it follows that using similar quantities of aqueous extract to those taken in the surface experiments, *i.e.* about 220 g., we should need to add 55 g. of oleic acid, with a surface of 7.7 m.², in order to obtain quantitative adsorption of lipase in the first period of elimination from aqueous solutions. It follows, therefore, that in Exp. *a* (*cf.* p. 1267) only 1/200th part of the lipase present was adsorbed during the first period, whilst the remainder gradually accumulated during the process of membrane formation.

On the assumption that in Exp. *d* (p. 1267) the whole of the lipase present is active, the percentage of active lipase in Exp. *c* (p. 1267) may be estimated in the following way. During the second week of reaction (Exp. *c*) about 35 millimols of acid were combined daily, whilst in Exp. *d* 193 millimols of acid were converted into ester on the first day of reaction. It hence follows that only one-fifth of the amount of active lipase in Exp. *d* was present in Exp. *c*.

SUMMARY.

An examination of the system oleic acid-aqueous glycerol-pancreatic lipase yielded the following results.

1. Lipase undergoes sorption at the oil-glycerol interface in two ways. The first is a process of adsorption which is completed within a few minutes, and the velocity of which is independent of temperature. The second process consists in the formation of a membrane at the phase interface; this process takes place five times as rapidly at 38° as at 18°. The whole of the lipase remaining in solution after the completion of the first rapid process of sorption is concentrated in this membrane by the end of a few days. The membrane gives the Millon and the biuret reactions, as well as a feeble Molisch reaction.

2. The adsorption of lipase on oleic acid is partly reversible.

3. Only lipase present at the phase interface is active; for this reason, in systems in which the phase interface is smaller than that necessary to remove the entire lipase present by the first process of adsorption, the velocity of reaction is to a certain extent proportional to the area of the phase interface. Above this limiting value the area of the surface of contact does not affect the velocity of synthesis.

4. The reaction of synthesis of olein gives results in agreement with a bimolecular reaction. It would, therefore, appear that the reaction in question follows the law of mass action, although it takes place in a heterogeneous system.

5. Where the quantity of aqueous phase is varied, keeping the contact-surface, the amount of oleic acid and the concentration of lipase constant, the initial velocity of reaction increases for the whole system with the amount of aqueous phase taken. The number of millimols of combined oleic acid per g. of aqueous phase falls, however, with increase in volume of the latter phase.

6. The effect of increasing the concentration of glycerol in the aqueous phase is to accelerate reaction and to shift the equilibrium point in the direction of synthesis. The reaction does not, over the range of concentrations taken in these experiments, follow the law of mass action.

7. The initial velocity of reaction, but not the equilibrium constant, is directly proportional to the concentration of enzyme in the aqueous phase.

The author desires to express his sincere gratitude to Professor St. J. Przytycki for his unfailing advice and assistance during the prosecution of this research, as well as to Dr R. Truszkowski.

REFERENCES.

- Langmuir (1916). The fundamental properties of liquids; quoted from Rideal: Surface chemistry, p. 72 (Cambridge, 1926).
Mellanby and Woolley (1914). *J. Physiol.* **48**, 287.
Rona and Bien (1914). *Biochem. Z.* **59**, 100.
Willstätter, Waldschmidt-Leitz and Memmen (1923). *Z. physiol. Chem.* **125**, 93.