XXXVII. MOLECULAR CONSTITUTION AND ACCESSIBILITY TO ENZYMES.

THE EFFECT OF VARIOUS SUBSTANCES ON THE VELOCITY OF HYDROLYSIS BY PANCREATIC LIPASE.

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(Received March 1st, 1929.)

INTRODUCTION.

It has been shown by Quastel and Wooldridge [1928] that in the case of the dehydrogenating enzymes of the bacterial cell, the question of accessibility to the active centres is closely bound up with the molecular constitution of the substance under consideration.

It was suggested by Dr Quastel that it would be instructive to investigate a so-called soluble enzyme, *i.e.* one presumably not capable of being considered part of a cellular interface, and to find whether any substances possessing a particular molecular structure, and other than the natural substrates, are adsorbed at its surface, using the same general criterion, *viz.* that, if the substance is adsorbed, it will compete with the substrate for the available surface and diminish the velocity of the normal reaction.

It followed from the active centre hypothesis that a number of substances characterised by the possession of a particular type of structure should be adsorbed by (or capable of combining with) each enzyme or centre; but out of these substances only a certain number can be activated to undergo the chemical reaction. Substances which are adsorbed but not activated will compete for the active centre with those which are activated and hence bring about a diminution in the velocity of reaction of the latter. The facts found with the dehydrogenating enzymes supported these conclusions, but it was desirable to determine whether a study of a hydrolytic enzyme would also give confirmatory results. The investigation to be described therefore was primarily taken up to examine the active centre hypothesis from a study of hydrolytic enzymes.

The lipases were selected for study as they are not specific to anything like the same degree as the carbohydrases, and it should therefore be expected that a larger number of compounds would be accessible. Nevertheless in this connection Armstrong [1904] showed that for the sugar-splitting enzymes substances could be found which, though not attacked by the enzyme, inhibited its action on substrates, thus α -methylgalactoside, which is unattacked by lactase, inhibits the hydrolysis of lactose by that enzyme. The peptidases

also show specificity only of a limited degree, but lipases possess the advantage that the substrates are much more readily obtained in a state of purity.

Method and apparatus.

The aim of the experiments being to observe the effects of added substances on the velocity of hydrolysis, and not in any way on the total amount hydrolysed either up to an equilibrium or in any given time, all such methods of measurement as incubation for a standard time were unsuitable, and it was necessary to find a method by which the actual velocity at any moment could be readily calculated.

For this purpose the gasometric method was considered the most suitable. The principle of this method, in which the extent and velocity of liberation of acid in a system is measured by observing the output of carbon dioxide from a carbonic acid-bicarbonate buffer, is due to Warburg [1923], who employed it in the investigation of lactic acid formation in tissues. It was first applied to the study of lipases by Rona and Lasnitzki [1924], who used a micro-method carried out in ordinary Barcroft manometers. Both cups contained Ringer's solution containing bicarbonate, and the air was displaced by a gas mixture containing a fixed percentage of carbon dioxide. Enzyme, in their case diluted serum or tissue slices, was added to each cup and substrate to only one. By this means the time-course of the hydrolysis could be accurately followed.

For the present work the apparatus used by Dann and Quastel [1928] for the study of the rate of carbon dioxide production in fermentation was employed. By means of this apparatus, velocities of carbon dioxide output up to 1 cc. in 30 or 40 seconds can be measured with reasonable accuracy.

The chief advantage of this method, in addition to the fact that the actual velocity can be followed, is that the hydrogen ion concentration is automatically kept constant, as free acid is never able to accumulate in the system under the experimental conditions. The hydrogen ion concentration of a system carbonic acid-bicarbonate is given by $[H]^+ = K \frac{[H_2CO_3]}{[HCO_3^-]}$ or approximately by $K \frac{\text{free } CO_2}{\text{combined } CO_2}$ or $K \frac{\text{acid}}{\text{salt}}$, where K is the first dissociation constant of carbonic acid. If the concentration of the salt initially is sufficiently great, the amount decomposed makes a negligible difference, and the numerator term remains the same also to a first approximation so long as the partial pressure of the carbon dioxide remains constant. Since an actual halving of the concentration of salt, leading to a doubling of the actual hydrogen ion concentration, would only increase the p_H by 0.3, which would not materially affect the activity of the enzyme over the range employed, it will be seen that the error from this source is small. The largest error is found to be that due to irregularity in the speed of the stirring mechanism. The speed of the stirrer must be maintained at a value high enough to make the water surface a steep funnel, otherwise the liquid tends

to become supersaturated with carbon dioxide. In such circumstances not only is the rate of evolution of gas not constant, but also the mean rate is not comparable with the normal, since the ionic equilibrium controlling the p_H is disturbed.

The actual value of the p_H could be deduced from the equation, but is more conveniently found by experimental determination. To obtain any desired value for this quantity either the numerator or the denominator may be varied. Under the technique of Rona and Lasnitzki referred to, the p_H was adjusted by varying the percentage of CO_2 in the gas mixture filling the apparatus. The amount of extra CO_2 liberated in the experiment was of the order of 5 to 10 micro-mols only and did not affect the concentration. In the method here described, however, large volumes of liberated CO_2 are dealt with, and successive amounts of 5 cc. are, after measurement, expelled from the apparatus. A gas mixture is therefore impossible, and the whole air in the apparatus must always be displaced by CO_2 before the start of the experiment. For this reason the bicarbonate concentration had also to be much greater in order to obtain a p_H of the order of 7, in fact the concentration required was about $M/7$. This concentration however was found to have no deleterious effect on the enzyme.

Preparation of the enzyme.

Nearly all the experiments were carried out with pancreatic lipase of the pig. A dry preparation of pig's pancreas, free from fat, was prepared by drying with acetone and ether after the procedure of Willstätter and Waldschmidt-Leitz [1923]. The dry preparation appears to keep indefinitely if stored in the ice-chest. Aqueous extracts were made as desired, employing 16 cc. distilled water for each gram and shaking intermittently for 3 to 4 hours at room temperature and centrifuging. Such extracts can be kept for a week without toluene if regularly replaced in the ice-chest after use; and under such treatment their activity, after falling during the first 12 hours, remains constant for about 5 days. Subsequently the activity rapidly falls off, but samples in this stage were always discarded.

Experimental procedure and types of result.

The contents of the reaction vessel, which held about 70 cc., were made up of 40 cc. $M/4$ sodium bicarbonate solution, 25 cc. distilled water and 5 cc. of the solution containing the enzyme. The bicarbonate solution was usually saturated with washed CO_2 from a cylinder, but in any case the whole mixed contents were saturated with the gas during the replacement of the air in the apparatus by CO_2 , the gas passing through the liquid from the outlet tap at the bottom. The substrate, generally 1 cc., was added last, the taps being then closed to leave the capillary leading to the burettes the only outlet for gas, and the apparatus placed in the bath, the water in which had been

already adjusted to the correct temperature, and the stirrer started. The bath was maintained at a temperature of 30°.

The apparatus was left for 5 minutes or more to attain equilibrium, the approximate time being known from experiments in which the substrate was not added until pressure equilibrium was obtained. In any case readings were discarded before the point at which a constant velocity of gas evolution was first observed.

It was found that linear curves for the hydrolysis could be obtained over considerable periods with this technique. The actual extent was found to vary markedly from substrate to substrate. The most satisfactory results were obtained when using ethyl butyrate as substrate, the linear course being followed up to 40 or 50 % hydrolysis. The phenomenon is probably attributable to the properties of the products of hydrolysis, the alcohols more particularly than the fatty acid salts. Thus, while ethyl butyrate and other

Fig. 1.

x x x x Triacetin.

⊙ ⊙ ⊙ ⊙ Ethyl butyrate.

ethyl esters maintain a linear course up to 50 % hydrolysis as stated, glycerides give a much shorter period and butyl and amyl esters fail to maintain a linear course at all. In correlation with this, butyl and amyl alcohols cause the greatest inhibition when added to the system, although the observed difference between glycerol and ethyl alcohol is not very noticeable. Fig. 1 shows the different course followed by the hydrolyses of ethyl butyrate and of triacetin. It will be observed that in the latter case the linear course does not persist longer than 10 minutes, while no deviation is to be noticed at all in the case of the ethyl butyrate.

Rona and Lasnitzki also state that the linear course of hydrolysis of tributyrin in their micro-technique persisted only up to the liberation of 2.5 micro-mols of butyric acid or about 7.5 % hydrolysis. That this is not due to the products of partial hydrolysis, dibutyryn and monobutyryn, having a greater affinity for the enzyme than the tributyrin itself, is easily shown. Although the velocity of hydrolysis of these compounds by pancreatic lipase

is much smaller than the velocity of hydrolysis of tributyrin, their addition to a system of tributyrin and enzyme does not cause a fall in the velocity. This was shown by Terroine [1910] and was confirmed by the writer for triacetin and monoacetin.

Hydrolysis of mixtures of substrates.

The above case is merely a general example of the study of mixtures of substrates. Where such mixtures were used in these experiments, it was found in nearly all cases that the velocity of hydrolysis was equal to the velocity of hydrolysis of the more rapidly hydrolysed constituent taken singly. Thus the addition of ethyl butyrate to the apparatus while triacetin was being hydrolysed led to no alteration in the velocity of gas evolution, but triacetin added to the system hydrolysing ethyl butyrate increased the velocity to that observed with triacetin alone. The following figures represent parts of a pair of such experiments:

A. Triacetin added to ethyl butyrate.

1 cc. CO ₂ in	1' 25"	1' 26"	1' 32"	*	56"	59"	57"	59"
2 cc. "	2' 55"	3' 0"	3' 7"	*	1' 54"	1' 59"	1' 55"	1' 57"
3 cc. "	4' 25"	4' 37"	4' 48"	*	2' 53"	2' 58"	2' 49"	2' 57"
4 cc. "	5' 57"	6' 17"	6' 24"	*	3' 46"	3' 55"	3' 46"	3' 56"

B. Ethyl butyrate added to triacetin.

1 cc. CO ₂ in	38"	36"	38"	*	39"	38"	39"
2 cc. "	1' 15"	1' 12"	1' 17"	*	1' 17"	1' 16"	1' 17"
3 cc. "	1' 55"	1' 49"	1' 56"	*	1' 54"	1' 55"	1' 54"
4 cc. "	2' 32"	2' 27"	2' 35"	*	2' 32"	2' 34"	2' 30"

* represents point of addition.

It is known however from the results of other workers that this phenomenon is not an unalterable rule. Thus Willstätter, Kühn, Lind and Memmen [1927], investigating the so-called latent period in the hydrolysis of ethyl mandelate by liver esterase, found that it was due to the presence of traces of ethyl phenylglyoxylate, the ester of the corresponding keto-acid, which they concluded was adsorbed much more strongly than the mandelate but hydrolysed more slowly. This compound is however an ester of a keto-acid, and the results to be described suggest that another phenomenon may be involved here.

It is now recognised that the velocity of hydrolysis of an ester by lipase is the result of two factors, the affinity of the enzyme for the substrate (represented by the Michaelis constant), and the velocity of decomposition of the enzyme-substrate complex. There is no reason to suppose that the relation between these should be the same for all substrates, and indeed it is not. Thus, of several pairs of optical isomers, that one which separately is less rapidly hydrolysed is found to be preferentially attacked in the optically inactive form. This phenomenon has been particularly studied by Willstätter, Kühn and Bamann [1928] and by Rona and Itolsohn-Schechter [1928].

The point to be stressed is that in no case is the velocity of hydrolysis of a mixture of substrates greater than that of either separately, that is to say,

there is no evidence that there is more than one enzyme concerned in the hydrolysis of all substrates.

EFFECT ON THE VELOCITY OF HYDROLYSIS OF COMPOUNDS CHEMICALLY
UNRELATED TO THE REACTANTS.

The main object of this work was, however, to investigate whether compounds quite other than any possible reactants, *i.e.* neither esters, acids, nor alcohols, could influence the rate of hydrolysis by occupying part of the active surface of the enzyme.

Since the grouping common to all esters is —CO.O— , it is reasonable to consider that attachment of the ester to the active centre is achieved by some part of this group, either the carbonyl group or the ethereal oxygen atom. Ketones and ethers were therefore the first types of compounds investigated.

Effect of ketones.

The first ketone tried was acetone, but the inhibition in this case is very small. Increasing the length or weight of one of the chains, however, causes a great increase in the inhibiting powers. Thus methyl propyl ketone gives the following figures (see also Fig. 2):

Substrate: ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO ₂ in	56"	55"	53"	56"	*	1' 45"	1' 32"	1' 51"	1' 45"	1' 53"	1' 44"
2 cc. "	1' 53"	1' 55"	1' 53"	1' 53"	*	3' 42"	3' 23"	3' 42"	3' 31"	3' 44"	3' 40"
3 cc. "	2' 53"	2' 56"	2' 54"	2' 51"	*	5' 33"	5' 28"	5' 36"	5' 22"	5' 35"	5' 39"
4 cc. "	3' 44"	3' 48"	3' 54"	3' 46"	*	7' 7"	7' 17"	7' 25"	7' 3"	7' 16"	7' 24"

* 1 cc. substance added.

It is to be noted that all ketones give a true equilibrium with the substrate, and a linear course of hydrolysis in the mixture.

Acetophenone (methyl phenyl ketone) gives even greater inhibitions, thus, as seen from the following table, 0.1 cc. added to the normal quantity of reagents in the apparatus gives an inhibition of 33 %. (Inhibitions are expressed, after Rona, by the value $\frac{V_0 - V}{V_0} \times 100$ %, where V_0 = initial, V = final velocity.)

Substrate: ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO ₂ in	36"	34"	35"	*	51"	47"	51"	52"
2 cc. "	1' 10"	1' 8"	1' 10"	*	1' 46"	1' 41"	1' 45"	1' 44"
3 cc. "	1' 47"	1' 46"	1' 47"	*	2' 39"	2' 35"	2' 40"	2' 35"
4 cc. "	2' 24"	2' 23"	2' 22"	*	3' 34"	3' 28"	3' 37"	3' 27"
5 cc. "	2' 59"	3' 0"	2' 57"	*	4' 28"	4' 24"	4' 40"	4' 24"

* 0.1 cc. acetophenone added.

Quantitative relationships. Competitive adsorption.

The inhibitions obtained with acetophenone were so large that this substance was selected in order to work out the quantitative relationship. As stated above all the substances produce a stationary state or point of equilibrium, the velocity attaining and remaining at a constant value. The actual velocity depends on the relative concentrations of the two substances.

If the inhibitor acts in the way postulated, viz. is adsorbed by the enzyme in exactly the same way as is the substrate, then the inhibitor and substrate will compete for the available surface of enzyme. The following reasoning can then be applied.

At concentrations of the two substances greater than the saturation concentration (which can be experimentally found for the substrate and is known to be much lower than the experimental value), if c_1 and c_2 be the concen-

Fig. 2. Effect of methyl propyl ketone on hydrolysis of ethyl butyrate.

trations of the substrate S and inhibitor I respectively, and k_1 and k_2 their respective affinity constants, then $\frac{k_1 c_1}{k_1 c_1 + k_2 c_2}$ represents the fraction of the enzyme remaining active for purposes of hydrolysis. The velocity is directly proportional to this, so that V/V_0 is equal to this fraction. But the inhibition is defined

$$h = \frac{V_0 - V}{V_0} = 1 - \frac{k_1 c_1}{k_1 c_1 + k_2 c_2} = \frac{k_2 c_2}{k_1 c_1 + k_2 c_2}.$$

If for c_2/c_1 is written n , which is thus the number of mols of I per mol of S , and K for k_1/k_2 , we have $h = \frac{n}{K+n}$. By this means K represents the value of n when the inhibition is 50 %.

Experimental values of h were found for several different relative concentrations of substrate and inhibitor and plotted. A theoretical curve was drawn for a value of K approximating to the mean of the values obtained. Fig. 3 shows the extent of agreement between the theoretical and observed values.

It was possible to obtain several points in a single experiment. V_0 was determined and a certain amount, 0.1 cc. in most experiments, of acetophenone then added through the top tap. When this V was satisfactorily obtained, that is when the velocity of gas evolution had become constant, a further quantity of the inhibitor or a further quantity of substrate could be added in the same way, and further points obtained. When additional substrate was given to the system containing a certain quantity of inhibitor a rise in the velocity was obtained. This in itself was sufficient to show that the

inhibitor did not act through any deleterious effect on the enzyme, but merely as a competitor with the substrate.

The extent of inhibition was moreover shown by experiment to be independent of the amount of enzyme present, but a function exclusively of the quantity here designated as n , that is the ratio of the concentrations of inhibitor and substrate. The two experiments quoted demonstrate this, the initial velocities which are proportional to the enzyme concentrations being approximately 2 to 3:

Substrate: ethyl butyrate.

A.									
1 cc. CO ₂ in	51"	51"	*	1' 8"	*	1' 30"	*	1' 41"	46" * 50"
2 cc. "	1' 43"	1' 43"	*	2' 17"	*	2' 15"	*	2' 37"	37" *
3 cc. "	2' 36"	2' 35"	*	3' 28"	*	3' 2"	*	3' 36"	36" *
4 cc. "	3' 31"	3' 24"	*	4' 41"	*	3' 49"	*	4' 34"	34" *
5 cc. "	4' 25"	4' 15"	*	5' 53"	*	4' 37"	*	5' 35"	35" *
						5' 24"	*	6' 31"	31" *
						6' 14"	*	7' 29"	29" *
						6' 58"	*	8' 25"	25" *
						7' 43"	*	9' 23"	23" *
Inhibitions=(i) 25 %; (ii) 43 %; (iii) 55 %.									
B.									
1 cc. CO ₂ in	33"	33"	*	40"	42"	*	53"	35" * 50"	
2 cc. "	1' 6"	1' 6"	*	1' 22"	1' 26"	*	1' 50"	1' 11" * 1' 39"	
3 cc. "	1' 40"	1' 40"	*	2' 2"	2' 9"	*	2' 46"	1' 45" * 2' 31"	
4 cc. "	2' 13"	2' 12"	*	2' 46"	2' 54"	*	3' 44"	2' 21" * 3' 22"	
5 cc. "	2' 46"	2' 46"	*	3' 30"	3' 35"	*	4' 42"	2' 57" * 4' 15"	
								3' 33" * 5' 8"	
								4' 9" * 6' 2"	
								4' 45" * 6' 57"	
								5' 19" * 7' 51"	
								5' 56" * 8' 47"	
Inhibitions=(i) 22 %; (ii) 40 %; (iii) 53 %; (iv) 70 %.									

In the above two experiments the amount of ethyl butyrate at the commencement was 1.5 cc., and 0.1 cc. acetophenone was added at each *.

(The value of n is of course slowly changing throughout the experiment, as the concentration of substrate slowly diminishes while that of the inhibitor remains unaltered. The amount of substrate at any time is estimated from a knowledge of the rate of hydrolysis, *i.e.* of CO₂ output, and the time since the start of the experiment.)

Fig. 3 is drawn up from the following 22 sets of measurements, which in turn cover 8 separate experiments:

c_1	c_2	n (g. mol)	h	K	c_1	c_2	n (g. mol)	h	K
0.00674	0.00085	0.127	0.33	0.257	0.00674	0.00085	0.122	0.25	0.365
0.00674	0.00085	0.127	0.32	0.268	0.00673	0.00171	0.254	0.52	0.233
0.01030	0.00085	0.083	0.22	0.296	0.00673	0.00256	0.362	0.69	0.162
0.00701	0.00085	0.122	0.41	0.174	0.00986	0.00256	0.260	0.49	0.270
0.01080	0.00085	0.079	0.33	0.182	0.01030	0.00085	0.083	0.25	0.250
0.00674	0.00085	0.127	0.32	0.268	0.00994	0.00171	0.172	0.43	0.228
0.00606	0.00171	0.282	0.61	0.181	0.00986	0.00256	0.260	0.55	0.213
0.00945	0.00171	0.181	0.415	0.254	0.01030	0.00085	0.083	0.22	0.283
0.00701	0.00085	0.122	0.39	0.190	0.00994	0.00171	0.172	0.40	0.258
0.00631	0.00171	0.271	0.65	0.144	0.00986	0.00256	0.260	0.53	0.232
0.01012	0.00171	0.169	0.44	0.215	0.00945	0.00342	0.362	0.70	0.156

When the difficulty of accurately adding 0.1 cc. of a substance (which cannot be added in dissolved form) through the long side-tube projecting

above the water level in the bath and possessing a bend, is borne in mind, it will be appreciated that the extent of deviation observed is not more than the limit of experimental error.

The amounts of both acetophenone and ethyl butyrate used in the experiments exceed the solubility limits of the substances in water, and they are present in the system as an emulsion. The high speed of rotation of the stirrer however ensures that the emulsion is very fine and has the same composition all through except perhaps in the constriction leading to the exit tap. Furthermore the two substances are soluble in one another, so that the enzyme is presented with a uniform mixture of the two. The high speed

Fig. 3. Competitive adsorption between ethyl butyrate and acetophenone.
[The curve is the theoretical for $K=0.23$].

of stirring ensures that mechanical access to the enzyme should not be dependent in any way on diffusion or any other factor of that nature. In conditions where that was the case these phenomena might well fail to be observed. The effect does not appear to be dependent on the amounts of the substances dissolved in the aqueous phase, for if that were so, increase of the concentration of either component above the aqueous solubility level would have no further effect on the velocity of hydrolysis, which is not the case. This must be especially emphasised, since a possible criticism of these results might be that effects attributed to chemical structure of substances were in reality merely due to greater or less solubility.

Comparison of competitive adsorption with effect of fluoride.

It is interesting at once to contrast this type of inhibition, which we have termed "competitive adsorption," with a quite different type, namely that brought about by fluoride. On chemical grounds it would not be expected that fluoride would be likely to compete with the substrate, and quantitative experiments show that the behaviour of this substance is in fact quite different.

Thus the experiment cited in the accompanying table shows that after addition of fluoride with resulting fall of the velocity to a new constant level, a further addition of substrate will not raise the level again as with competitive adsorption types. The inhibition is thus not dependent on the relative concentration of inhibitor to substrate at all. But it is dependent on the relative concentration of inhibitor to enzyme. Whereas in cases of competitive adsorption the enzyme concentration does not affect the value of the percentage inhibition, in this case, the less enzyme the more is the percentage inhibition caused by a given quantity of fluoride. Fluoride in fact behaves as if it formed a compound with the enzyme itself, which no amount of substrate will reverse. Yet this compound is not irreversible since Loevenhart and Peirce [1906] showed that after dialysis a completely inactivated sample regains its activity, and quantitative relationships confirm this. No exact quantitative experiments were included in this series, but it was shown that if x , say, was the amount of fluoride required to give 50 % inhibition, *i.e.* to inactivate half of the enzyme, then $2x$ would not suffice to inactivate the whole, as it would were the combination irreversible.

Pancreatic lipase is very sensitive to $M/40$ fluoride (1 in 1000), this concentration giving an inhibition of nearly 90 % with the amount of enzyme employed in these experiments, while $M/40$ chloride would have no appreciable effect. One part of fluoride in 7000 gives an inhibition of 25 % in these conditions.

Showing non-competitive inhibition of fluoride.

1 cc. CO ₂ in	46"	45"	*	57"	59"	†	1' 1"	1' 1"
2 cc. "	1' 30"	1' 31"	*	1' 57"	1' 58"	†	2' 7"	2' 1"
3 cc. "	2' 16"	2' 13"	*	2' 58"	2' 59"	†	3' 10"	3' 2"
4 cc. "	3' 2"	2' 57"	*	4' 0"	4' 3"	†	4' 11"	4' 2"
5 cc. "	3' 50"	3' 42"	*	5' 1"	5' 6"	†	5' 14"	5' 0"

* 1 cc. 1 % sodium fluoride solution added.

† 0.5 cc. ethyl butyrate added.

Effect of other molecular structures.

The experiments with acetophenone, and worked out to a less extent with other ketones, *e.g.* methyl propyl ketone, lead to the conclusion that ketones are adsorbed by the active centres of lipase, and compete with the normal substrates for the surface of the centres. It was next necessary to investigate to what extent other compounds exerted an influence on the velocity.

Effect of ethers.

As stated before, the possibility exists that the esters could be adsorbed partially by the ethereal oxygen atom, and ethers would then act as inhibitors. Anisole (methyl phenyl ether), was particularly tried since the groups flanking the oxygen are the same as those present in acetophenone. It was found, as the table shows, that in comparison with the effect of the latter, the inhibition given by anisole is very feeble:

Showing the effect of anisole on hydrolysis of ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO ₂ in	32"	33"	30"	*	40"	35"	41"
2 cc. "	1' 5"	1' 7"	1' 2"	*	1' 19"	1' 10"	1' 20"
3 cc. "	1' 37"	1' 40"	1' 35"	*	1' 57"	1' 46"	1' 57"
4 cc. "	2' 8"	2' 12"	2' 7"	*	2' 36"	2' 21"	2' 33"
5 cc. "	2' 39"	2' 48"	2' 41"	*	3' 13"	3' 1"	3' 9"

* 0.1 cc. anisole added here.

$$\text{Inhibition } h = \frac{V_0 - V}{V_0} = 1 - \frac{V}{V_0} = 1 - \frac{t_0}{t} = 13 \%$$

A similar volume of acetophenone gives an inhibition of 33 %.

Some controls. Hydrocarbons.

As experiments showed that a wide variety of compounds had some small amount of inhibitory power, as indeed is not unexpected, it was thought desirable to make a fairly wide survey, particularly including aromatic derivatives, since these in general gave greater inhibitions. It is to be expected that hydrocarbons, having very inert molecules and no polar groups (besides being insoluble) would exert no action at all. Pentane and hexane among aliphatic, benzene, toluene, ethylbenzene and cyclohexane among cyclic hydrocarbons, were investigated. Pentane, hexane and benzene had no final effect whatever, an immediate rise in the velocity of gas evolution lasting a few minutes being followed by a permanent return to the original level. As much as 1 cc. of benzene in 70 cc. reacting fluid has been used with no effect. Toluene and ethylbenzene act as very weak inhibitors: no reason is known for this phenomenon. Very frequently, but not always, if more than 0.1 cc. were used, no steady state set in, but a gradual diminution of velocity was observed. Cyclohexane was unique in producing a permanent activation. The cause was not discovered, but presumably must be physical rather than chemical in nature. Presence of the substance did not affect the power of other substances to inhibit, *e.g.* acetophenone was able to bring about its usual inhibition substituting the new value for V_0 . The activation was equally marked in the case of completely soluble substrates such as triacetin, so that the effect, whatever it may be, appears to be on the enzyme itself.

Some soluble aromatic derivatives.

More soluble benzene derivatives were tried, *e.g.* phenol and aniline. 0.1 g. phenol in the total content gives an inhibition of 15 %, whereas 0.1 g. acetophenone gives over 30 % inhibition, in spite of the fact that phenol is considerably more soluble. This again points against the criticism that the effect is merely one of relative solubility. Aniline, which is fairly soluble, gives a very weak inhibition. Two experiments, each using 0.1 g. of this substance, have given inhibitions of 5.5 % and 6.5 % respectively. This highly polar substance therefore does not seem to be very actively adsorbed by the enzyme.

The following two tables give the experiments the results of which have

been quoted. The substance was suspended in bicarbonate solution, and added at the points marked *:

Substrate: ethyl butyrate.

A. Effect of 0.1 g. phenol.

1 cc. CO ₂ in	40"	36"	35"	*	48"	49"	41"
2 cc. "	1' 20"	1' 12"	1' 10"	*	1' 27"	1' 33"	1' 25"
3 cc. "	1' 58"	1' 49"	1' 47"	*	2' 5"	2' 20"	2' 8"
4 cc. "	2' 35"	2' 26"	2' 23"	*	2' 47"	3' 2"	2' 52"
5 cc. "	3' 11"	3' 3"	3' 3"	*	3' 33"	3' 46"	3' 36"

Inhibition = 15 %.

B. Effect of 0.1 g. aniline.

1 cc. CO ₂ in	35"	32"	30"	*	34"	32"	34"	33"
2 cc. "	1' 7"	1' 3"	1' 0"	*	1' 5"	1' 4"	1' 6"	1' 5"
3 cc. "	1' 36"	1' 33"	1' 29"	*	1' 37"	1' 36"	1' 40"	1' 39"
4 cc. "	2' 5"	2' 5"	1' 59"	*	2' 8"	2' 9"	2' 13"	2' 11"
5 cc. "	2' 36"	2' 36"	2' 30"	*	2' 44"	2' 40"	2' 48"	2' 43"

Inhibition = 5.5 %.

Effect of phenylmethylcarbinol.

Further experiments designed to show that the very powerful inhibitory action of acetophenone is due to its carbonyl group consisted in investigating derivatives in which this group was no longer present. The first compound investigated was phenylmethylcarbinol. Since alcohols are reactants in the general lipase system, this substance might be expected to be adsorbable by the lipase, and the results are in agreement with this supposition. 0.1 g. of this substance gives an inhibition of 25 % when the ethyl butyrate present is 1 cc. (0.88 g.), or about three-fourths of the inhibition given by acetophenone. When quantitatively worked out, the inhibition given by this substance was found to conform to the competitive adsorption type, the gram-molecular affinity relative to ethyl butyrate being about 2.5–3 for this compound as against about 4–5 for acetophenone. (These figures represent $1/K$ for the substances.) This shows that the alcohol does not give rise to any appreciable back action although being powerfully adsorbed. The case is analogous to the effect of glucose and fructose on the hydrolysis of cane-sugar by invertase. They occupy the surface of the enzyme.

The following table represents a typical result with this substance:

Effect of phenylmethylcarbinol on hydrolysis of ethyl butyrate, 1 cc. Substrate and inhibitor present as emulsion.

1 cc. CO ₂ in	42"	41"	*	52"	53"	54"	*	1' 12"	1' 11"
2 cc. "	1' 22"	1' 22"	*	1' 48"	1' 48"	1' 50"	*	2' 31"	2' 28"
3 cc. "	2' 5"	2' 4"	*	2' 44"	2' 44"	2' 46"	*	3' 48"	3' 49"
4 cc. "	2' 46"	2' 45"	*	3' 39"	3' 41"	3' 41"	*	5' 8"	5' 7"
5 cc. "	3' 27"	3' 29"	*	4' 34"	4' 38"	4' 37"	*	6' 24"	6' 28"

Inhibitions = (i) 25 %; (ii) 46.5 %.

* 0.1 g. phenylmethylcarbinol added.

Whether it is the same active centre for the adsorption of the ester and of the alcohol is still not clear. In the dehydrogenases the groupings —CO— and —CH.OH— seem interchangeable, *i.e.* either is equally well adsorbed at the same centre. It is not clear whether this is the same effect or whether phenylmethylcarbinol acts merely as an alcohol. In this case the ketone

exerts a more powerful action than the corresponding alcohol, and a keto-acid, as will presently be described, had more effect than a hydroxy-acid, but with the pair of substances, cyclohexanone and cyclohexanol, the reverse was true. Both, in comparison with the pair, acetophenone and phenylmethylcarbinol, are very weakly adsorbed, but cyclohexanol is very definitely more adsorbed than cyclohexanone, as the following two experiments show:

Substrate: ethyl butyrate 1 cc. Substrate and inhibitor present as emulsion.

A. Effect of cyclohexanone.

1 cc. CO ₂ in	37"	37"	*	43"	42"	41"	†	49"	49"	†	47"	46"
2 cc. "	1' 13"	1' 13"	*	1' 25"	1' 24"	1' 21"	†	1' 38"	1' 39"	†	1' 32"	1' 32"
3 cc. "	1' 51"	1' 52"	*	2' 7"	2' 6"	2' 3"	†	2' 28"	2' 31"	†	2' 17"	2' 15"
4 cc. "	2' 29"	2' 32"	*	2' 49"	2' 48"	2' 45"	†	3' 19"	3' 20"	†	3' 5"	3' 0"
5 cc. "	3' 8"	3' 10"	*	3' 31"	3' 29"	3' 27"	†	4' 10"	4' 10"	†	3' 53"	3' 43"

* 0.5 cc. added. † Another 0.5 cc. added. ‡ Another 0.5 cc. ethyl butyrate added.

B. Effect of cyclohexanol.

1 cc. CO ₂ in	38"	38"	*	50"	54"	50"	†	46"	45"	†	1' 5"	1' 0"
2 cc. "	1' 14"	1' 15"	*	1' 37"	1' 50"	1' 42"	†	1' 32"	1' 30"	†	2' 8"	2' 0"
3 cc. "	1' 52"	1' 52"	*	2' 27"	2' 45"	2' 34"	†	2' 17"	2' 15"	†	3' 8"	3' 0"
4 cc. "	2' 30"	2' 31"	*	3' 17"	3' 43"	3' 27"	†	3' 3"	3' 1"	†	4' 10"	4' 0"
5 cc. "	3' 8"	3' 8"	*	4' 8"	4' 38"	4' 22"	†	3' 49"	3' 48"	†	5' 12"	5' 1"

* 0.5 cc. added. † Another 0.5 cc. ethyl butyrate added. ‡ Another 0.5 cc. inhibitor added.

The gram-molecular constants ($1/K$) for these substances are approximately 0.17 for cyclohexanone and 0.6 for cyclohexanol.

Effect of acetophenoneoxime.

A more satisfactory attempt to affix the properties of acetophenone to its carbonyl group was in the use of the oxime. A difficulty admittedly arises in that this substance is a solid, but it is fairly soluble, and other solids have been investigated with which to compare it. The observed effect with solids is probably less than the full, as the solid tends to sink to the bottom, but if very finely powdered, nearly all the material is kept evenly distributed throughout the fluid by the stirrer.

Among solids used have been benzophenone, benzoin, and benzil. Two experiments with the former have yielded, for 0.1 g. of the substance, inhibitions of 15 % and 17 %, corresponding to a gram-molecular constant of 2.5, less than the observed value for acetophenone, but this may be accounted for by steric hindrance, the position of the carbonyl group between two rings quite possibly presenting an obstacle to adsorption. Benzoin has given figures of about the same order, but the error here is probably greater as the substance appears more prone to aggregate and fall to the bottom. Benzil, which, being a diketo-compound, is of especial interest, is particularly prone to this objection, the finely powdered material aggregating to large lumps as soon as it enters the fluid, nevertheless a considerable inhibition has been observed.

With these, then, it was considered fair to compare acetophenoneoxime. Whilst at first it was suspected of having some residuary inhibiting power, repeated recrystallisation finally gave a product giving practically no inhibition at all.

The following table compares acetophenoneoxime with benzophenone.

Substrate: ethyl butyrate, 1 cc. Inhibitors present as suspensions.

A. Effect of 0.1 g. benzophenone, added at *.

1 cc. CO ₂ in	35"	36"	36"	*	42"	40"	39"
2 cc. "	1' 9"	1' 12"	1' 12"	*	1' 22"	1' 20"	1' 18"
3 cc. "	1' 44"	1' 49"	1' 48"	*	2' 4"	2' 1"	2' 0"
4 cc. "	2' 20"	2' 25"	2' 24"	*	2' 45"	2' 43"	2' 42"
5 cc. "	2' 55"	3' 0"	3' 0"	*	3' 27"	3' 24"	3' 25"

Inhibition = 15 %.

B. Effect of 0.1 g. acetophenoneoxime, added at *.

1 cc. CO ₂ in	34"	34"	*	36"	35"	36"
2 cc. "	1' 5"	1' 8"	*	1' 12"	1' 9"	1' 7"
3 cc. "	1' 37"	1' 43"	*	1' 47"	1' 44"	1' 42"
4 cc. "	2' 14"	2' 17"	*	2' 23"	2' 19"	2' 17"
5 cc. "	2' 53"	2' 53"	*	2' 59"	2' 55"	2' 53"

No inhibition.

Miscellaneous examples.

Benzaldehyde also gives an inhibition of an order approximating to that given by acetophenone, the inhibition by 0.1 cc. in the usual volume being 26.5 %, corresponding to a gram-molecular constant (1/K) of 2.75. The effect of the carbonyl group is thus again evident. The oxime of this substance has not been investigated.

The behaviour of ethyl benzoate should not pass unnoticed. Preliminary experiments had shown that this ester was quite unattacked by the lipase preparation used. Further experiments showed that the substance acted as a simple inhibitor, not a very powerful one, but displaying competitive inhibition when added to a system actively hydrolysing ethyl butyrate or other substrate.

In connection with the experiments using methyl propyl ketone, acetylacetone was investigated. This substance, containing as it does two keto-groups, might be expected to have high inhibitive properties. Actually it was not so active as methyl propyl ketone itself. This is probably due to the fact that it is actively tautomeric, with a labile hydrogen atom, and does not therefore function as a ketone.

Effect of pyruvate and lactate.

Lastly remain to be described some experiments in which the grouping to be investigated was part of the anion of a salt. The sodium salt of a keto-acid, pyruvic, was compared with the similar salt of the corresponding hydroxy-acid, lactic acid, while the unsubstituted propionic acid was used as a control. The necessary concentrations of these entirely soluble substances were much larger than those of the compounds previously considered, amounts of the order of 1 g. being used. In the experiments quoted 1 g. each of pyruvic and lactic acids (whose molecular weights differ only by 2) and the corresponding equimolecular weight of propionic acid, all neutralised with sodium hydroxide, were used. With pyruvate it was very difficult to obtain the value

¹ The results with solids other than benzophenone have not been included in the general table, as for reasons stated they are not capable of strict comparison with the other results. This does not affect the view that the comparison just drawn represents a true qualitative difference of behaviour between the substances.

of the final velocity, as there tended to be a gradual decline in this quantity, but the inhibition was in the zone from 30 to 40 %. With lactate the inhibitions observed varied from 18 to 22 %, while propionate gave 11 %; equimolecular sodium chloride gave 8 %, while the dilution error (for these substances had to be introduced in a volume of at least 5 cc.) was up to 5 %. The relative effects of these substances are shown graphically in Fig. 4.

Fig. 4.

Incidentally the approximation between the values given by sodium propionate and sodium chloride shows that the fatty acids, present as they are as ions at this p_H , are very weakly adsorbed. This agrees with the statement made at the beginning of this paper that the continuance of the linear curve of hydrolysis depended on the alcohol produced, and scarcely at all on the acid.

Finally, Fig. 5 represents graphically the relative extent of the inhibition produced by the different substances studied. It shows the diminution in velocity brought about by a concentration of one-tenth of a gram-molecule of the inhibitor per gram-molecule of ethyl butyrate.

Fig. 5.

The values of $1/K$ relative to ethyl butyrate ($= 1$) for a number of chemically related substances are appended:

	$1/K$		$1/K$
Acetophenone	4.35	Phenol	1.25
Benzophenone	2.5	Aniline	0.45
Benzaldehyde	2.75	Cyclohexanol	0.60
Phenylmethylcarbinol	2.75	Cyclohexanone	0.17
Anisole	1.25		

CONCLUSION.

Whilst the nature of the active centres of lipase is still quite unknown, except in so far as we know from the work of Willstätter and his colleagues that they are not protein in nature, the work described throws some light on the manner of the attachment of the substrate to those active centres. Thus if substances containing the carbonyl group compete with the substrate for the enzyme, it may be claimed to show that it is by this group that the substrate is normally attached, which makes the relative non-specificity of the enzyme more understandable. The conversion of —CO— into —CHOH still yields a substance with inhibitive power, but in no case has it been found that replacement of —CO— by —CH_2 results in an active inhibitor. It is clear however that the non-polar moieties attached to the —CO— group also play an important part in rendering the substance accessible to the enzyme, as is indicated for instance by the greater inhibition by methyl propyl ketone than by acetone. The actual affinity of the enzyme for the substrate (the Michaelis constant), and the readiness of decomposition of the resulting complex are determined by factors in which the nature and configuration of the rest of the molecule play the chief part, and with regard to which lipases of different origin differ among themselves. The varying sensitivity of the different lipases to poisons such as alkaloids, arsenicals, etc., which has been much studied and which is held to be independent of the state of purity, is again a different phenomenon. The work described is meant to bear on the general affinities between substrates and the active centres of the lipase, the general rather than the particular having been the object of study. The results observed support the conclusions concerning the nature of enzyme action which have been obtained from the study of the dehydrogenating enzymes of bacteria.

SUMMARY.

1. A method is described by which the actual velocity of hydrolysis of esters by lipase can be followed immediately.
2. The effects of various compounds, studied from the point of view of their molecular structure, on this velocity is described.
3. Ketones (including aldehydes) produce an inhibition of the velocity of lipase hydrolysis out of proportion to that produced by the chemically related compounds investigated. Secondary alcohols also produce an inhibition, which may however be due to reversal of hydrolysis.
4. This power is completely lost by converting the ketone into its oxime.
5. It is claimed that this is due to a specific affinity between the carbonyl group and the active centre of the enzyme, which affinity is also responsible for the normal adsorption of esters preparatory to their hydrolysis by the enzyme.

In conclusion I wish to express my thanks to Sir F. G. Hopkins for his constant interest in this work, and to Mr J. B. S. Haldane and Dr J. H. Quastel for continued help and advice. I am also indebted to the Department of Scientific and Industrial Research for a supplementary grant.

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