CCXIII. THE INHIBITION OF ESTERASES BY EXCESS SUBSTRATE.

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It is now widely though not universally accepted that the first stage in enzymic hydrolysis consists of a reversible one-to-one union of enzyme and substrate, this complex later breaking down to give free enzyme once more and the products of the reaction. On this view Michaelis and Menten [1913] built up their equation giving the relation between velocity of reaction and substrate concentration, a relation which is similar to that between the degree of dissociation of a weak acid and the hydrogen ion concentration. That is, the relation is expressed by a rectangular hyperbola, or, if the concentration be plotted on a logarithmic scale, the familiar S-shaped curve is obtained. On either system of plotting, at high substrate concentration the curve should run parallel to the x-axis, the velocity being unaffected once the enzyme is saturated. Actually, Michaelis and Menten found in their experiments with invertase that the velocity began to diminish again when a concentration of about 6 % was exceeded, but they concluded that this might be due to failure of the laws of dilute solutions, the change in the concentration of water becoming an appreciable factor. The work of Nelson and Schubert [1928] subsequently showed that this assumption was both justified and, to a first approximation, quantitatively sufficient.

With other enzymes, however, the velocity passes a maximum and falls again at much smaller concentrations. Particularly with the liver esterases is this the case. Furthermore, the concentration at which this occurs varies very markedly with the species from which the enzyme is obtained [Bamann and Schmeller, 1929; Murray and King, 1930]. Thus with sheep-liver esterase hydrolysing ethyl butyrate the maximum velocity is observed when the substrate is at about $0{\cdot}005\,M$ concentration, with rabbit-liver esterase when it is at more than $0{\cdot}01\,M$. Obviously another explanation must be sought for this phenomenon.

Haldane [1930] has suggested that the explanation might lie in the formation of a complex of the enzyme with two molecules of the substrate, this complex being incapable of the breakdown leading to liberation of acid and alcohol. This idea leads to the following extension of the original Michaelis-Menten equation. Three reactions are to be considered, (1) $E + S \rightleftharpoons ES$,

(2) $ES + S \rightleftharpoons ES_2$, and (3) $ES \rightarrow E + P$. Let s, θ, p, q, be the concentrations of substrate, free enzyme, ES, and ES_2, σ the total enzyme concentration, K_1, K_2, the equilibrium constants of equations (1) and (2) and k the velocity constant of equation (3). Then $\theta s = K_1 p$ and $ps = K_2 q$, so that

$$\sigma = \theta + p + q = \theta\left(1 + \frac{s}{K_1} + \frac{s^2}{K_1 K_2}\right).$$

But $$V = kp = k \cdot \frac{s}{K_1} \cdot \theta = k\sigma \frac{s}{K_1 + s + \frac{s^2}{K_2}} \text{ or } k\sigma \frac{1}{1 + \frac{K_1}{s} + \frac{s}{K_2}}.$$

It will be seen that this expression is similar in form to that obtained by Michaelis [1911] for the dissociation residue of an ampholyte, *i.e.*

$$\rho = \frac{1}{1 + \frac{K_a}{[H^\cdot]} + \frac{K_b}{[OH']}},$$

if for [OH'] is written $\frac{K_w}{[H^\cdot]}$. Haldane's suggestion thus calls for an activity-p_s curve symmetrical about the highest point and of the familiar bell-shaped type.

Some of the curves given by Bamann and Schmeller approximate to this type, but the number of observations is too small to form the basis of a theory. In the present work therefore the curves have been extended into more concentrated solutions, and the effect of competitive inhibitors has been studied and compared with that predicted on theoretical grounds on the basis of the assumption described.

The activity-p_s *curve and the determination of* K_1 *and* K_2.

The procedure followed is that of Murray and King [1930], with certain additions as described in the experimental section. Sheep-liver esterase was the enzyme chosen, since for this the optimum concentration falls about the middle of the logarithmic scale of practicable values. Ethyl butyrate was the substrate. The general symmetry of the activity-p_s curve was quickly apparent, so that the next step was to calculate the best representative values of K_1 and K_2 and plot the theoretical curve with a view to observing how the experimental points lay with respect to it.

The product $K_1 K_2$ was determined as follows. By virtue of the symmetry of the curve, for all pairs of points with equal y co-ordinates (velocity) the sum of the x co-ordinates (log. concentration) must be equal. But $s = K_1$, $s = K_2$, are such a pair of points, so that this sum equals $\log K_1 + \log K_2$ or $\log K_1 K_2$. Hence a mean value of $K_1 K_2$ can be obtained directly from the experimental points.

The following method was devised to calculate the quotient K_1/K_2: transfer the y-axis to the axis of symmetry of the curve, that is, to the point $s = \sqrt{K_1 K_2}$, so that $x = \log s - \log \sqrt{K_1 K_2}$. Therefore

$$s = e^{(x + \log \sqrt{K_1 K_2})} = \sqrt{K_1 K_2}\, e^x.$$

Therefore

$$y = \frac{\text{const.}}{1+\frac{K_1}{s}+\frac{s}{K_2}} = \frac{\text{const.}}{1+\frac{K_1}{\sqrt{K_1K_2}}e^{-x}+\frac{\sqrt{K_1K_2}}{K_2}e^{x}} = \frac{\text{const.}}{1+\sqrt{\frac{K_1}{K_2}}(e^{x}+e^{-x})} = \frac{\text{const.}}{1+2\sqrt{\frac{K_1}{K_2}}\cosh x}.$$

Before using this equation, the values of x, if given in ordinary logarithms, must be multiplied by $\log_e 10$ (2·303), since the values required for the equation are Napierian logarithms.

A mean value of K_1/K_2 was found by taking a number of pairs of points and eliminating the arbitrary constant of the numerator.

The best representative values of the product and of the quotient were as follows:

$$K_1K_2 = \text{antilog}\,\bar{5}\cdot03 = 0\cdot0000107, \quad K_1/K_2 = 0\cdot1137,$$

whence

$$K_1 = 0\cdot00114 \text{ and } K_2 = 0\cdot00971.$$

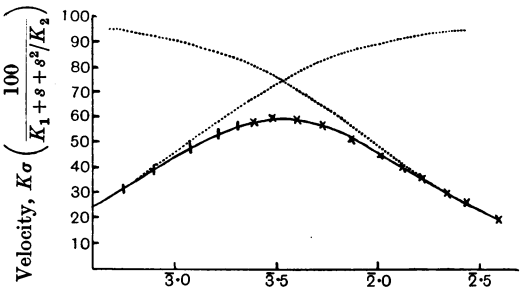

Fig. 1.

The continuous curve in Fig. 1 is the curve obtained by plotting the equation

$$y = \frac{100}{1+\frac{0\cdot00114}{s}+\frac{s}{0\cdot00971}}.$$

The dotted curves are the simple Michaelis curves

$$y = \frac{100}{1+\frac{0\cdot00114}{s}} \text{ and } y = \frac{100}{1+\frac{s}{0\cdot00971}}.$$

The points are the experimentally observed velocities, the scale being chosen so that one standard point (0·00517M or 0·48 mg./cc.) falls on the curve as drawn.

The agreement of the points on the right-hand side of the curve is as good as is to be expected, while on the left-hand side, where the velocities required (the initial velocities) have to be obtained by extrapolating backwards, with a consequent increased margin of error, the "theoretical" velocity is always within the limits of the experimental values.

The ordinate of the graph represents the percentage of the limiting velocity, $k\sigma$, which would be reached if the whole of the enzyme could be "saturated" in the ordinary Michaelis sense, that is, if the combination with the second substrate molecule did not occur. It will be seen that even in the optimal conditions only 60 % of that activity is reached. This point will be more fully considered in the next Section.

The effect of competitive inhibitors.

The satisfactory agreement between the observed velocities and those calculated on Haldane's assumption led next to an extension of the equations to a system containing a competitive inhibitor. The additional equilibrium $E + I \rightleftharpoons EI$ has to be considered[1]. If h = concentration of inhibitor, r = concentration of EI, the extra relationship is $\theta h = K_h r$. Then

$$\sigma = \theta + p + q + r, \quad \sigma = \theta\left(1+\frac{s}{K_1}+\frac{s^2}{K_1K_2}+\frac{h}{K_h}\right),$$

$$V = k\,.\,\frac{s}{K_1}\,.\,\theta = k\sigma\,\frac{s}{K_1+s+\frac{s^2}{K_2}+\frac{hK_1}{K_h}} = k\sigma\,\frac{1}{1+\frac{K_1}{s}\left(1+\frac{h}{K_h}\right)+\frac{s}{K_2}}.$$

This is equivalent to a change in the first affinity constant but not in the second. A curve of the same family is obtained, but the left-hand side is pushed in. In such a family the maximum value of the ordinate depends on the quotient K_1/K_2, just as in the case of the ampholyte [Michaelis, 1911] the percentage capable of existing as a dissociation residue depends on the product $K_a K_b$.

Certain quite definite differences from the case of an enzyme such as invertase are thus to be expected if this theory is correct. In the invertase system the same ultimate maximum velocity is obtained with or without inhibitor, the presence of inhibitor merely necessitating a greater substrate concentration before the saturating concentration is reached. In this case the addition of inhibitor affects the actual maximum velocity obtainable, diminishing it as well as pushing it into the direction of higher substrate concentration. If sufficient of the inhibitor be present, the enzyme can be completely inactivated whatever the concentration of substrate.

From already published results of Murray and King [1930] it is evident that this is what actually occurs in the case of the inhibitor methyl-*n*-hexylcarbinol. Fig. 1 of that paper is expressed with the concentration on the ordinary scale, which brings it out less clearly than the logarithmic, but shows clearly enough that the optimum substrate concentration is raised while the maximum velocity reached is lowered. Fig. 2 of this paper shows the effect (freshly determined with certain corrections as described in the experimental part) with the concentration plotted logarithmically.

[1] Haldane has worked out the case, considering also the reactions $EI + I \rightleftharpoons EI_2$ and $EI + S \rightleftharpoons ESI \rightleftharpoons ES + I$. The coefficient of the $1/s$ term is thereby altered by a different extent, the coefficient of s still remains unaltered, *viz.* $1/K_2$. Only the quantitative, not the qualitative, effect is thus altered by these extra considerations.

An attempt was made to substitute simpler substances for the rather complex secondary alcohols employed in the previous work. With the four primary alcohols, methyl to *n*-butyl, however, it was found that the simple relation

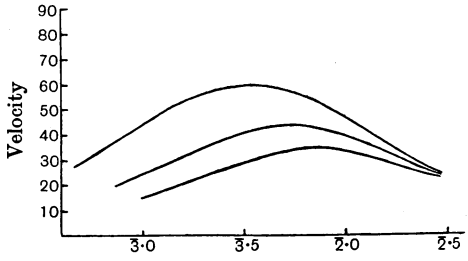

Fig. 2. Velocity-p_s curve in presence of (i) 0·00016 M, (ii) 0·00032 M *d*-methyl-*n*-hexyl carbinol.

deduced did not hold. Superimposed on the competitive inhibition which produced a shift of the position of the maximum velocity point towards higher concentration was a non-competitive inhibition which resulted in a depression of the curve over its whole length. In other words, a part of the inhibition produced was independent of the substrate concentration, though a part apparently was competitive. Fig. 3 shows the effect of *n*-butyl alcohol. The

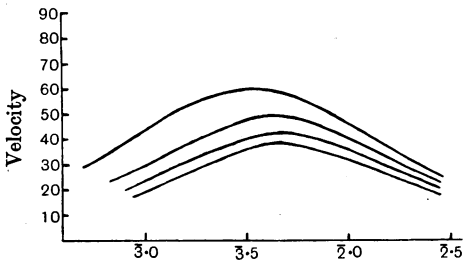

Fig. 3. Velocity-p_s curve in presence of (i) 0·0034 M, (ii) 0·0068 M, (iii) 0·0102 M *n*-butyl alcohol.

secondary alcohols, on the other hand, were true competitive inhibitors, and in high substrate concentrations the inhibition was extremely small and tending to zero.

The results with those inhibitors which are strictly competitive therefore further confirms the suggestion of Haldane. It is not necessary here to discuss the exact significance or nature of the ES_2 complex. Haldane himself has suggested that the most likely interpretation is that for hydrolysis to take place the substrate has to be united to the enzyme by two points, one characteristic of the acid part of the molecule, one of the alcohol. Then, if the acid end of one molecule and the alcohol end of another become attached to the same enzyme

molecule, the strain on the molecules leading to decomposition would not be produced. Diagrammatically the idea is expressed:

Capable of breakdown Incapable of breakdown

At the present time there is not sufficient evidence to go further than merely to suggest this view.

Experimental.

As the technique used was described in the previous paper [Murray and King, 1930], attention will be drawn to only a few points, the importance of which has been realised later, and in particular to two sources of error which affect the quantitative though not the qualitative findings.

The first of these is connected with the use of alcoholic solutions of indicators. Although only three drops of indicator were added to the 8 cc. of experimental fluid, controls showed that this produced an inhibition. As explained before, ethyl alcohol inhibits both competitively and non-competitively, so that the whole length of the curve was depressed, but especially so the low substrate concentration end. At 0·005 M concentration of substrate (near to the optimum) the inhibition was about 15 % and was greater at higher dilution. Further controls showed that the indicator itself, if made up as an aqueous solution of its sodium salt, had no inhibitory properties, and subsequently it was always used in this form.

The second error was due to dissolved carbon dioxide. The amount dissolved in the reagents could be roughly gauged by the number of drops of alkali required to adjust the p_H before the experiment began. Whenever this was more than about 3 drops or 0·075 cc. of $N/100$, the left-hand side of the curve was found to be depressed, although the high concentration figures were hardly affected. Thus the sodium bicarbonate formed appeared to act as a competitive inhibitor. Whether this could be due to its carbonyl group is an interesting question. The competitive nature of the inhibition was further demonstrated by repeatedly shaking the contents of the tube with air to dissolve more CO_2 and then neutralising with $N/100$ NaOH; in this way increasing inhibitions were produced. It was not possible to add any considerable quantity of $NaHCO_3$, since its buffering properties prevent its use in this essentially buffer-free technique.

Previous to this finding the stock solution of the sparingly soluble ethyl butyrate had been made by shaking violently with water in the presence of air, the resulting fluid being then neutralised, so that it contained some bicarbonate. Subsequently the shaking was done in the absence of air, the drops being broken up by small pieces of glass rod. Owing to ethyl butyrate being lighter than water the bottle had to be filled and inverted under cold boiled water and the ethyl butyrate introduced by pipette, the pieces of glass being pushed

in from beneath before the stopper was inserted. This procedure, though complicated, gave the most satisfactory CO_2-free solution of the substrate.

The effect of these two discoveries was to show that the maximum of the curve was considerably more to the left than had been at first supposed. Thus, whereas this was given as $0{\cdot}005\,M$ before, when these sources of inhibition were removed $0{\cdot}003\,M$ was nearer to the mark. Bamann and Schmeller [1929] found the maximum for methyl butyrate at still higher concentration, *viz.*, nearly $0{\cdot}01\,M$. Their system was very possibly already partially inhibited in this or another way. As these workers state, the value found for the affinity should always be qualified by the epithet "apparent," since the effect of a competitive inhibitor is to alter the value of the constant which is taken to be the measure of the affinity.

Summary.

The suggestion of Haldane, that the inhibition of certain enzymes by excess of substrate is due to the formation of a non-hydrolysable complex of the enzyme with two molecules of the substrate, is tested for the esterase of sheep-liver and found to explain the facts satisfactorily and to predict correctly the effect of competitive inhibitors.

The work described was performed during the tenure of the Benn W. Levy Studentship, to the managers of which I wish to express my thanks. I am particularly grateful to Prof. J. B. S. Haldane for informing me of his views before they appeared in print, and for suggesting simplifications in the mathematical treatment. To Sir F. G. Hopkins I express my thanks for his continued interest and encouragement.

REFERENCES.

Bamann and Schmeller (1929). *Z. physiol. Chem.* **183**, 149.
Haldane (1930). Enzymes (London), p. 84.
Michaelis (1911). *Biochem. Z.* **33**, 182.
—— and Menten (1913). *Biochem. Z.* **49**, 333.
Murray and King (1930). *Biochem. J.* **24**, 190.
Nelson and Schubert (1928). *J. Amer. Chem. Soc.* **50**, 2188.