ENZYMES

BY

J. B. S. HALDANE, M.A.

SIR WILLIAM DUNN READER IN BIOCHEMISTRY, CAMBRIDGE UNIVERSITY
FULLERIAN PROFESSOR OF PHYSIOLOGY, ROYAL INSTITUTION

WITH DIAGRAMS

LONGMANS, GREEN AND CO.
LONDON • NEW YORK • TORONTO
1930

CHAPTER III.

THE UNION OF ENZYME WITH ITS SUBSTRATE AND RELATED COMPOUNDS.

With the exception of Barendrecht [1924], who supported a radiation theory of enzyme action, almost all authors have postulated a union of enzyme and substrate. But while some have favoured a chemical union, others have postulated adsorption on the surface of the enzyme particles or molecules. While recent work in physical chemistry, notably that of Langmuir [1916], has tended to obliterate the sharpness of such a distinction, two points may at once be raised with regard to adsorption. In the first place, since enzyme molecules are probably of the same order of magnitude as those of proteins and starch, only a small number at most of substrate molecules can be united with one of protease or amylase. And secondly, the number is not very large even where the substrate is simpler.

Thus an enzyme molecule of molecular weight 60,000, and density 1·1, would have a radius of 28 Ångstrom units if spherical, and an area of 9856 square Ångstroms. The area occupied by a triglyceride in a condensed film at a water-air interface is 63·0 square Ångstroms. Such an enzyme molecule could therefore accommodate 156 fat molecules, if they were packed as closely as possible. This figure would be increased four times if the enzyme molecule were eight times as large, and so on. It would also be increased if the enzyme molecules were not spherical. Thus, if the molecule were a sheet 10 Å. thick, its flat surfaces would have an area of 18,000 Å., and it could accommodate about twice as many fat molecules. The number of molecules of a biose or hexoside, since there is reason to think that the whole ring of a hexose molecule is in contact with the enzyme, would probably be about half this. But it is certain that the whole enzyme surface is not catalytically active, for Michaelis [1908] and Nelson and Griffin [1916] found that yeast saccharase loses none of its activity when adsorbed by a variety of solids, and several, though not all, other enzymes behave in the same way. If the catalytic activity is confined to a few spots on the surface, as seems likely, we may

legitimately, at least as a first approximation, assume that the events at each go on independently. This assumption has worked fairly well in the case of hæmoglobin, of which, according to Adair [1928] each molecule can take up four molecules of O_2. If as Willstätter [1922] believes, an enzyme consists of a small chemically active group and a large colloidal carrier, the active centres must be very few in number. In many cases, as we shall see, the assumption that an enzyme molecule activates only one substrate molecule at a time has worked remarkably well. But provided the different active points are far enough away to be independent, the same equations are reached in the case of a large active surface, for example, a platinum surface catalysing gaseous oxidations [Langmuir, 1916].

The Adsorption of Enzyme by its Substrate.

Where an enzyme acts on a solid substrate it is generally adsorbed by it. This proves little, as enzymes are adsorbed by a variety of solids. Nevertheless, in some cases the adsorption is specific. Thus Willstätter and Waldschmidt-Leitz [1922] found tristearin a very effective adsorbent for pancreatic lipase. Moreover, lipase is partially active while adsorbed on kaolin or alumina, but quite inactive when adsorbed on fat or cholesterol. This suggests that it is adsorbed to the latter by its catalytically active group. Similarly casein and fibrin adsorb activated but not inactive trypsinogen [Waldschmidt-Leitz, Schäffner and Grassmann, 1926]. Fibrin also adsorbs enterokinase. On the other hand, this specificity is often absent. Thus Nishikawa [1927] found that fibrin adsorbed amylase, lipase, and rennin, as well as trypsin, from " takadiastase." Northrop [1919] found a maximum removal of pepsin from solution by boiled egg albumin at pH 2 (Fig. 11) which is also the optimum for digestion over short periods, according to Sörensen [1909]. At a later stage in digestion the optimum changes towards pH 1. This agreement suggests that the main effect of pH on peptic digestion is to favour the union of enzyme and substrate. Equally suggestive are the experiments where the enzyme is removed from an adsorbent by a solution of its substrate. In some cases this removal is specific. Thus Michaelis [1921] found that yeast invertase is not removed from colloidal ferric hydroxide by water, or solutions of fructose, lactose, and several other sugars, but is so by its substrates sucrose and raffinose, and also by maltose. Willstätter and Kuhn [1921], however, found that sucrose was the only sugar capable of removing it from alumina. These effects might have been due to the sugar

competing for the adsorbent with the enzyme. Hedin [1907] found that casein removed trypsin from adsorption by charcoal, and that the amounts removed increased with the total amount of casein in the system, but were independent of the volume of water in which it was dissolved. It is therefore clear that casein and charcoal were competing for the trypsin, not casein and trypsin for the charcoal. In fact trypsin must unite with dissolved casein.

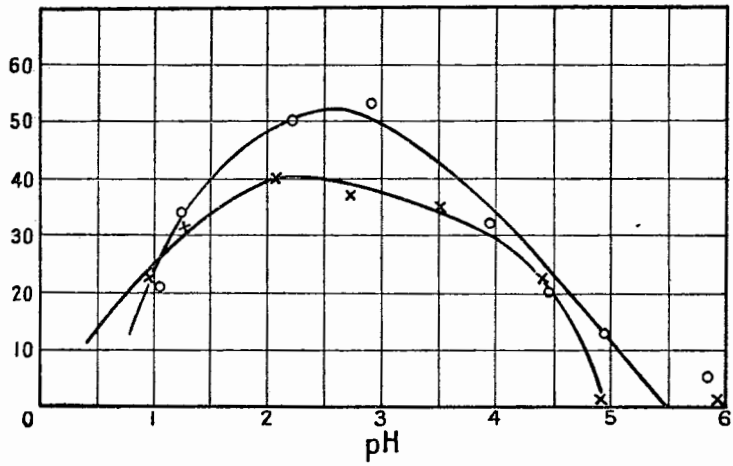

FIG. 11.—Percentages of pepsin removed from solution by coagulated egg albumin in two experiments, as functions of pH. [Northrop, 1919.]

The Effect of Varying Substrate Concentration on Reaction Velocity.

If the concentration of substrate be varied, the initial velocity of reaction at first increases, then reaches a maximum, and finally may diminish. The velocity is at first generally nearly proportional to the substrate concentration (Fig. 12). In many cases there is a fairly wide range of substrate concentrations over which the velocity is constant (Figs. 13, 14), and the falling off in strong solutions is only serious at concentrations where deviations from the mass-action law may be expected to be serious.

The best data for concentrated solutions are those for yeast saccharase. Nelson and Schubert [1928] found a maximum velocity of sucrose hydrolysis at about 5 per cent., and after this a falling off, the relation between sucrose content and velocity being nearly linear between 10 per cent. and 70 per cent. sucrose. In order to determine whether this was due to a falling off in water content they compared the results when the water content was lowered by the

addition of sucrose and of alcohol. The differences found were small (Fig. 15), and may be explained on the assumption that the

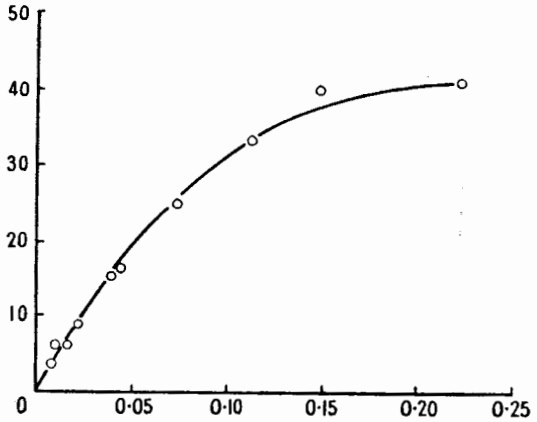

FIG. 12.—Rate of raffinose hydrolysis by yeast invertase as a function of substrate concentration. Abscissa, molar concentration of raffinose. Ordinate, initial velocity of hydrolysis. [After Kuhn, 1923, I.]

sucrose and alcohol were partly hydrated, and hence the amount of free water was somewhat different to that calculated. Water is, of course, a reactant in the reaction catalysed, and it is clear that

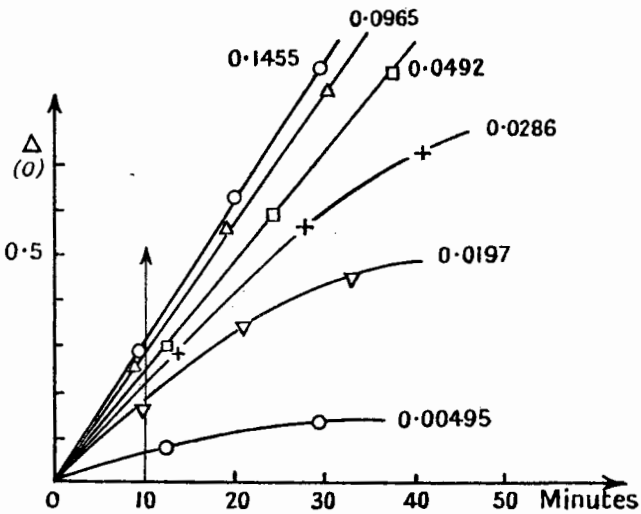

FIG. 13.—Course of hydrolysis of saccharose by yeast saccharase at different initial substrate concentrations. [Kuhn, 1923, I.]

over a wide range the velocity is proportional to the water concentration. The affinity of the enzyme for water, if any, must be very low, K_m exceeding 100. The inhibitory effects of methyl and ethyl alcohols

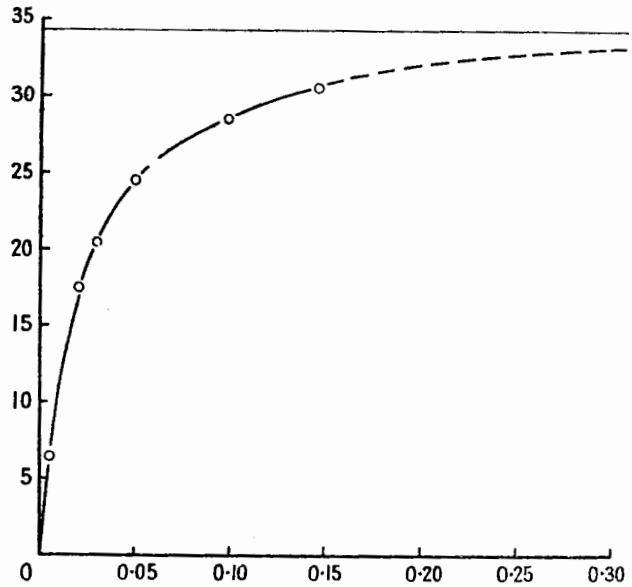

FIG. 14.—Rate of sucrose hydrolysis by yeast saccharase as a function of substrate concentration (from data of Fig. 13). Abscissa, molar concentration of sucrose. Ordinate, initial velocity of hydrolysis. [After Kuhn, 1923, 1.]

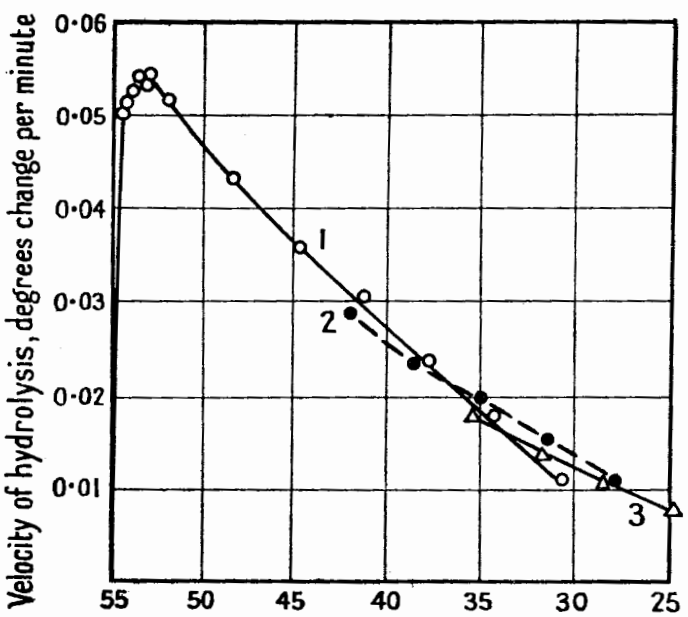

FIG. 15.—Concentration of water, moles per litre. Curve 1, sucrose and no alcohol; 2, sucrose and 10 per cent. alcohol; 3, sucrose and 20 per cent. alcohol.
[Nelson and Schubert, 1928.]

on the initial rate of β-glucoside hydrolysis by prunase [Josephson, 1925, I] are mainly, but not wholly, explicable as due to dilution of the water.

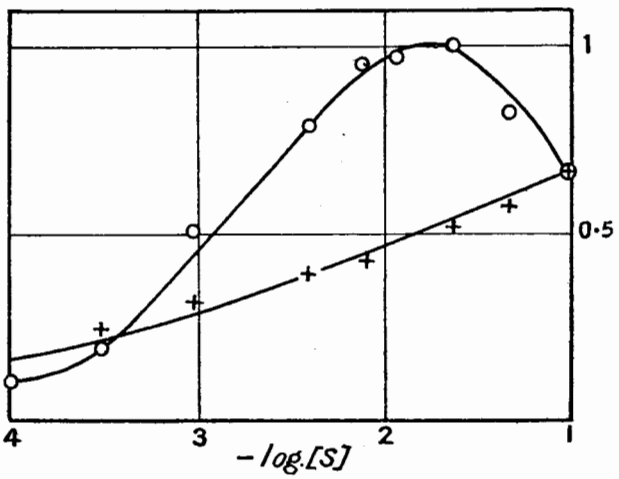

FIG. 16.—Velocities of hydrolysis of ethyl *d*-mandelate (+) and ethyl *l*-mandelate (O) by dog's liver lipase as functions of the logarithm of substrate concentration. [Bamann, 1929.]

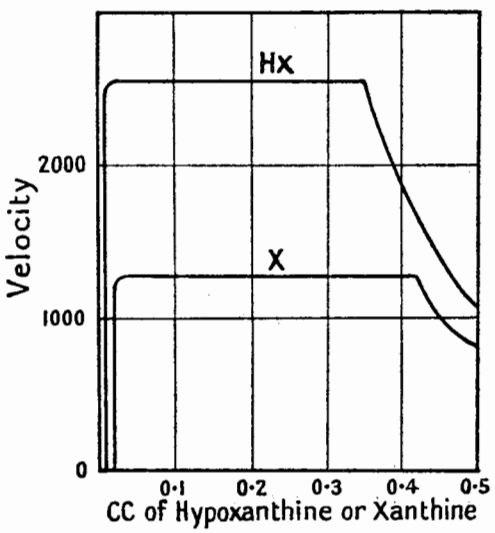

FIG. 17.—Milk xanthine-oxidase. Initial part of hypoxanthine and xanthine concentration curves. Each tube contained: 5·0 c.c. strong enzyme solution (in buffer); 0·5 c.c. methylene blue; x c.c. hypoxanthine or xanthine (2 mg. per c.c.); $(0{\cdot}5 - x)$ c.c. water. [Dixon and Thurlow, 1924.]

No explanation of this type is possible in the case of urease (Fig. 31), where the inhibitory effect of strong substrate concentrations is combated by glycine, or liver lipase (Fig. 16); in those of xanthine-oxidase

(Figs. 17, 18) peroxidase, and citric oxidase [Bernheim, 1928] where the effect is very marked in quite weak solutions, or in that of salivary amylase (ptyalin) where Lovatt Evans [1912] found a maximum hydrolysis in 1·5-2·5 per cent. solution, falling off to one-sixth in 5 per cent. (though this latter effect is conceivably due to changes of pH, owing to traces of acid present in his soluble starch preparation). Inhibition by strong substrate concentrations is further discussed in Chapter V.

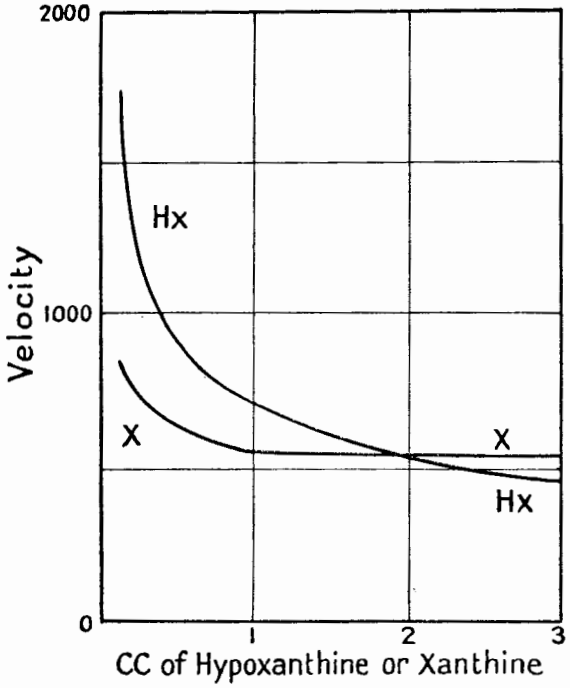

FIG. 18.—Milk xanthine oxidase. Hypoxanthine and xanthine concentration curves compared. Each tube contained: 4·0 c.c. enzyme solution (in buffer); 0·5 c.c. methylene blue; x c.c. hypoxanthine or xanthine (2 mg. per c.c); $(3 - x)$ c.c. water. [Dixon and Thurlow, 1924.]

Generally the curve relating initial velocity and substrate concentration is straight or concave downwards in weak solutions. This is, however, not the case with malt amylase, according to Sjöberg and Ericcson [1924], confirmed by Eadie [1926], where no action whatever occurs in sufficiently weak concentrations of amylose, amylopectin, or glycogen (see Fig. 20). It is also quite possible (see p. 52) that the initial position of the curve for insect saccharase is concave upwards.

Table V. gives a list of the substrate concentrations at which half

TABLE V.

SUBSTRATE CONCENTRATIONS GIVING HALF MAXIMUM VELOCITY (IN MANY CASES IDENTICAL WITH MICHAELIS' CONSTANT).

Enzyme.	Source.	Substrate.	Concentration.	Reference.
Phosphatase	Bone	Glycerophosphate	<·003 M	Martland and Robison (1927).
,,	*Aspergillus*	,,	·09 M	Kobayashi (1926).
Lipase	Pig pancreas	Ethyl butyrate	> ·03 M	Willstätter and Memmen (1924, 1).
,,	,, ,,	Methyl ,,	> ·09 M	,, ,, ,, ,,
,,	,, ,,	Methyl *d*-mandelate	·016 M	Weber and Ammon (1929).
,,	,, ,,	,, *l*-mandelate	·016 M	,, ,, ,,
,,	,, liver	,, *d*-mandelate	·00018 M	,, ,, ,,
,,	,, ,,	,, *l*-mandelate	·0013 M	,, ,, ,,
,,	,, ,,	Ethyl *d*-mandelate	·0005 M	Willstätter, Kuhn and Bamann (1928).
,,	,, ,,	,, *l*-mandelate	·0016 M	,, ,, ,, ,,
,,	Sheep liver	,, *d*-mandelate	·0007 M	Bamann (1929).
,,	,, ,,	,, *l*-mandelate	·0017 M	,, ,,
,,	Rabbit liver	,, *d*-mandelate	·0021 M	,, ,,
,,	,, ,,	,, *l*-mandelate	> ·01 M	,, ,,
,,	Dog liver	,, *d*-mandelate	·0011 M	,, ,,
,,	,, ,,	,, *l*-mandelate	> ·002 M	,, ,,
,,	Man's pancreas	,, *d*-mandelate	·0017 M	,, ,,
,,	,, ,,	,, *l*-mandelate	> ·01 M	,, ,,
,,	Ox pancreas	,, *d*-mandelate	> ·02 M	,, ,,
,,	,, ,,	,, *l*-mandelate	> ·05 M	,, ,,
,,	Horse pancreas	,, *d*-mandelate	> ·02 M	,, ,,
,,	,, ,,	,, *l*-mandelate	> ·05 M	,, ,,
,,	Sheep liver	Methyl butyrate	·0013 M	Bamann and Schmeller (1929).
,,	Rabbit liver	,, ,,	·0010 M	,, ,, ,,
,,	Dog liver	,, ,,	·0028 M	,, ,, ,,
,,	Man's liver	,, ,,	·008-·009 M	,, ,, ,,
,,	Ox liver	,, ,,	> ·02 M	,, ,, ,,
,,	Horse liver	,, ,,	> ·03 M	,, ,, ,,
Nucleosidase	Liver	Adenosine	> ·02 M	Levene, Weber and Yamagawa (1924).
Maltase	Yeast	Maltose	·12-·30 M	Willstätter, Kuhn, and Sobotka (1924).

TABLE V.—*continued.*

Enzyme.	Source.	Substrate.	Concentration.	Reference.
Maltase	Yeast	α-methyl glucoside	·037-·075 M	Willstätter, Kuhn, and Sobotka (1924).
,,	,,	α-phenyl glucoside	·021-·050 M	,, ,, ,, ,,
Prunase (=β-glucosidase)	Almond	β-glucose	·185 M	Josephson (1925).
,, ,,	,,	Helicin	·016 M	,, ,,
,, ,,	,,	Arbutin	·042 M	,, ,,
,, ,,	,,	Salicin	·017-·035 M	Willstätter, Kuhn, and Sobotka (1923).
,, ,,	,,	β-methyl glucoside	·60-1·12 M	,, ,, ,, ,,
,, ,,	,,	β-phenyl glucoside	·040-·065 M	,, ,, ,, ,,
Saccharase	Yeast	Saccharose	·016-·04 M	Kuhn (1923, 1).
,,	,,	Raffinose	·24-·66 M	,, ,,
,,	Honey	Saccharose	·02 M	Nelson and Cohn (1924).
,,	Gut	,,	·02 M	Cajori (1930).
Lactase	Almond	Lactose	abt. ·1 M	Armstrong (1924).
"Emulsin"	,,	Amygdalin	·003 M	Auld (1908).
Amylase	Saliva	Starch	0·4 per cent.	Lovatt Evans (1912).
,,	,,	,,	0·08 ,,	Myrbäck (1926, 2).
,,	Pancreas	,,	0·25 ,,	Kendall and Sherman (1910).
,,	Liver	,,	·095 ,,	Eadie (1927).
,,	Muscle	Glycogen	> 0·5 ,,	Lohmann (1926).
,,	Malt	Amylose	0·5 ,,	Sjöberg and Ericsson (1924).
,,	,,	Amylopectin	0·4 ,,	,, ,, ,,
,,	,,	Glycogen	abt. 1 ,,	Eadie (1926).
Urease	Soya bean	Urea	abt. ·025 M [1]	Van Slyke and Cullen (1914), etc.
Asparaginase	*Aspergillus*	Asparagine	< ·05 M	Bach (1928).
Erepsin	Gut	Glycyl-glycine	< ·05 M	Euler and Josephson (1926).
,,	,,	Glycyl-leucine	·02-·07 M [1]	Northrop and Simms (1928).
Trypsin	Pancreas	Casein (conductivity)	2 per cent.	Bayliss (1904).
,,	,,	Casein (amino N)	·75 ,,	Northrop (1922, 4).
,,	,,	,, (first stage)	< 5 ,,	,, ,,
,,	,,	Gelatin (amino N)	·5 ,,	,, ,,
Pepsin	Stomach	Ovalbumin	4·5 ,,	,, (1920, 3).
Glyoxalase	Liver	Methyl-glyoxal	<·005 M	Kuhn and Heckscher (1926).

[1] Varies with pH.

Enzyme.	Source.	Substrate.	Concentration.	Reference.
Glyoxalase	Liver	Phenyl-glyoxal	> ·01 M	Kuhn and Heckscher (1926).
Fumarase [1]	*B. coli*	Fumaric acid	< ·04 M	Woolf (1929).
Carboxylase	Yeast	Pyruvic acid	·01 M	Hägglund and Rosenquist (1927).
Zymase [1]	,,	Glucose	·006 M	Euler and Myrbäck (1923, 2).
Myozymase	Muscle	Amylose	0·1 per cent.	Meyerhof (1926).
Catalase	Liver	H_2O_2	·025 M	Euler and Josephson (1927, 1).
,,	Yeast	,,	> ·02 M	Issajew (1904).
Hæmin (as catalase)	Blood	,,	·008 M	Euler and Josephson (1927, 3).
Peroxidase	Root	H_2O_2, leucomalachite green	6×10^{-6} M	Willstätter and Weber (1926, 1).
Mean tissue oxygenase [1]	*Tenebrio*	O_2	$< 1{\cdot}5 \times 10^{-6}$ M	Thunberg (1905).
Mean tissue oxygenase [1]	Yeast	,,	$< {\cdot}5 \times 10^{-7}$ M	Warburg (1927).
Mean tissue oxygenase [1]	*Micrococcus*	,,	$< 10^{-8}$ M	Warburg and Kubowitz (1929).
Lactic oxidase [1]	Muscle	Lactate	·007 M	Ahlgren (1925).
,, ,,	Yeast	,,	abt. ·005 M	Bernheim (1928, 2).
,, ,,	,,	α-Hydroxybutyrate	abt. ·015 M	,, ,,
Succinoxidase [1]	Muscle	Succinate	·001 M	Widmark (1922).
Malic oxidase	Cucumber	Malate	·007 M	Thunberg (1929).
Citric oxidase	Liver	Citrate		Bernheim (1928).
,, ,, [1]	Muscle	,,	·0005 M	Ahlgren (1925).
,, ,,	Cucumber	,,	2×10^{-5} M	Thunberg (1929).
Glutaminic oxidase [1]	Muscle	Glutaminate	·0005 M	Ahlgren (1925).
Xanthine oxidase	Milk	Adenine Xanthine Hypoxanthine	$< 3 \times 10^{-5}$ M	Dixon and Thurlow (1924).
,, ,,	,,	Acetaldehyde	> 1 M	,, ,, ,,

[1] Not in solution.

the maximum initial velocity is reached for a variety of enzymes. These values are on the whole much less certain than the pH optima. They may, moreover, differ for samples of the same enzyme from different sources or differently treated, when the pH effects do not. In the case of the proteases they differ according to what is measured. Thus Northrop [1922, 4] found that the rate of casein digestion, as measured by increase of amino N, was unaltered when the concentration of casein was increased from 3 to 5 per cent., whereas the amount rendered incapable of precipitation by trichloracetic acid was almost exactly proportional to the concentration between 1·5 per cent. and 5 per cent. Presumably casein has a less affinity for trypsin than have some of the products of its digestion.

It will be noticed that a number of the oxidizing-reducing enzymes are saturated by their substrates at considerably lower concentrations than any of the hydrolytic enzymes acting on crystalloidal substrates. Aldehydes with xanthine-oxidase form an exception, but xanthine-oxidase has a very high affinity for its specific substrates. The reducing substrates of peroxidase appear to act, not by combining with it as a preliminary to oxidation, but by combating the inhibition of its activity by the relatively high H_2O_2 concentrations used (see p. 59). The figures obtained for them are therefore not included in the table. Catalase occupies an intermediate position as regards affinity. This may well prove to be a fundamental distinction between hydrolytic and oxidizing-reducing enzymes. Many of the latter also agree in giving a falling off in activity when the substrate concentration is increased beyond the optimum. This is still true even when full allowance has been made for the destructive effect on peroxidase of H_2O_2. It may be that this phenomenon is quite a general one, which, however, in the case of many hydrolytic enzymes cannot be distinguished from the effects of mere diminution of the amount of water in solution.

Michaelis' Theory.

Michaelis and Menten [1913] developed a theory adumbrated by Brown [1902], Henri [1903] and others, and applied it to the initial velocities of hydrolysis of sucrose by yeast invertase in varying sucrose concentrations. If E represent an enzyme molecule, S, G, and F molecules of sucrose, glucose, and fructose, they assume the following reactions to occur :—

1. $E + S \rightleftharpoons ES$.
2. $ES + H_2O \rightarrow E + G + F$.

Now let e be the total molar concentration of enzyme, x of sucrose (supposed to be very much greater than e), and p the concentration of ES molecules, so that the concentration of E molecules is $e - p$.

Then if K_m be the dissociation constant of the compound ES,

$$\therefore K_m p = x(e - p), \ \therefore p = \frac{ex}{K_m + x},$$

and if k be the velocity constant of the break-up of ES, and v the velocity $\frac{-dx}{dt}$ of hydrolysis, then the amount of H_2O is nearly constant,

$$\therefore v = kp$$
$$= \frac{kex}{K_m + x},$$

or if V be the velocity when x is large compared with K_m,

$$v = \frac{Vx}{K_m + x}, \text{ or } K_m = x\left(\frac{V}{v} - 1\right).$$

Plotted graphically the above equation gives a rectangular hyperbola (Fig. 14). K_m is the substrate concentration at which half the limiting velocity is reached.

We have thus two constants available for fitting a series of results, but V should be (and is found to be) proportional to the enzyme concentration, while K_m, which is generally called the Michaelis constant, is a characteristic of the enzyme. The above equation for the velocity is followed, probably within the limits of experimental error, by saccharase, provided the sucrose concentration is not too high, in which case H_2O concentration becomes a limiting factor. Thus in Michaelis and Menten's experiments the velocity, in degrees of polarimetric reading per minute, agrees well with the values calculated from V = ·0786, K_m = ·0167.

TABLE VI.

Molar concn. sucrose	·7700	·3850	·1920	·0960	·0480	·0308	·0154	·077
v. obs	·063	·075	·075	·0682	·0583	·0500	·0350	·0267
v. calc. . . .	·077	·075	·072	·0670	·0583	·0507	·0382	·0244

On the alkaline side of the optimum, K_m does not vary appreciably down to pH 8, but on the acid side it increases, being about doubled at pH 2·7, according to Josephson [1924]. Where K_m does not vary with the pH, it follows that the pH-activity curve is independent of the substrate concentration. Hence saccharase and its sucrose compound have the same acid dissociation constant, i.e. lose $\overset{+}{H}$ equally

readily. But on the acid side the free enzyme is a stronger base (or weaker acid) than the compound, as if the sucrose combined with an amino group of the enzyme. Nelson and Anderson [1926] found K_m independent of temperature, whilst Euler and Laurin [1920] with a different saccharase preparation found a diminution of 34 per cent. on raising the temperature from 1° to 39°, giving a heat of reaction of only 2000 calories per gram molecule. This has a considerable bearing on the nature of the union.

A Possible Criticism of the Above Theory.

It has been assumed above that the combination of enzyme and substrate is always in equilibrium, i.e. that the velocities of formation and dissociation of their compound are infinite in comparison with that of its decomposition to form the products of the reaction. This is rather an improbable assumption, for it seems likely that an invertase molecule can invert about 2000 sucrose molecules per second at 15° C., so that the half-period of the reaction $ES \rightarrow E + G + F$ is less than ·0005 second. It seems a little rash to postulate half-periods of 10^{-5} second or less for the other reactions. For only 10^7 cane-sugar molecules in a 0·1 N solution collide with a given point on the enzyme surface per second, and we cannot suppose that the orientation would always permit of union with the enzyme.

Hence Briggs and Haldane [1925] were led to consider the situation when the velocities of the three reactions are comparable.

Let the velocity constants of the reactions

$$E + S \rightarrow ES,\ ES \rightarrow E + S,\ \text{and}\ ES \rightarrow E + G + F$$

be k_1, k_2, k_3 respectively, e and p having their previous significance.

Then $$\frac{dp}{dt} = k_1x(e - p) - k_2p - k_3p.$$

But so long as the velocity of the reaction is constant, p is constant, and even when it is altering, the rate of change of p must be infinitesimal compared with that of x. Hence $\frac{dp}{dt}$ may be taken as zero,

$$\therefore k_1x(e - p) = (k_2 + k_3)p$$

$$\therefore p = \frac{k_1ex}{k_1x + k_2 + k_3}$$

$$= \frac{ex}{x + \frac{k_2 + k_3}{k_1}}.$$

Hence provided we take $\frac{k_2 + k_3}{k_1} = K_m$, the result is the same as that of Michaelis and Menten. Van Slyke and Cullen [1914] arrived at the same equation for urease, on the assumption that both reactions are irreversible, i.e. $k_2 = 0$. Actually then we cannot, with methods at present available, estimate the magnitude of k_2, i.e. the velocity of the reaction $ES \rightarrow E + S$. But the importance of the Michaelis constant is undiminished, though it need not be a dissociation constant. It has, however, the dimensions of such a constant, and should be expressed as a molar or percentage concentration.

In spite of this criticism it would seem that Michaelis and Menten's theory must be substantially correct for yeast invertase between pH 4 and 8. Over this range K_m does not alter with pH, whilst k_3

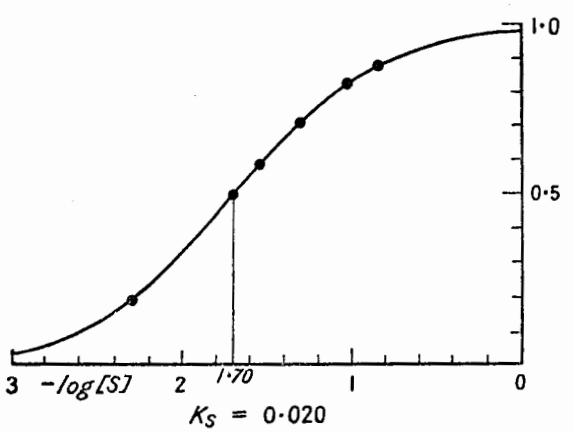

FIG. 19.—Velocities of initial saccharose hydrolysis as a function of the logarithm of substrate concentration, from data of Fig. 13. $K_m = \cdot 020$. [Kuhn, 1923, 1.]

varies twenty-fold. Unless then we make the quite improbable assumption that both k_1 and k_2 vary proportionally with it, the constancy of K_m shows that k_2 is large compared with k_3. On the other hand, in the case of such enzymes as urease, where K_m varies considerably with pH, either the theory of Van Slyke and Cullen, or that of Briggs and Haldane may turn out to be applicable. The question is further considered in Chapter V., pages 80-83.

In order to calculate K_m from a given set of data, it is convenient to plot the velocity v against the logarithm of the concentration x. Since $\log x = \log K_m - \log \left(\frac{V}{v} - 1\right)$, the curve obtained (Fig. 19) is of the same form as when the amount of dissociation of a weak electrolyte is plotted against pH. The logarithm of the substrate

concentration is usually called pS. The advantage of this method is that the points corresponding to low concentrations, which are important in determining K_m, are not crowded together, as would otherwise be the case. The affinity, which is the reciprocal of K_m, is often denoted by the symbol K_M.

Exceptions to the Michaelis Law.

In the case of the oxidases K_m (if the expression has a meaning) is often so small that the relation between v and x in sub-optimal substrate concentrations is still unknown. Liver catalase, and hæmin acting as a catalase, obey the Michaelis law within the limits of experimental error [Euler and Josephson, 1927, 2, 3], K_m being three times as great for the enzyme, although it is at least 10,000 times as active a catalyst as hæmin per unit weight. Among the hydrolytic enzymes several causes may contribute to apparent exceptions. The

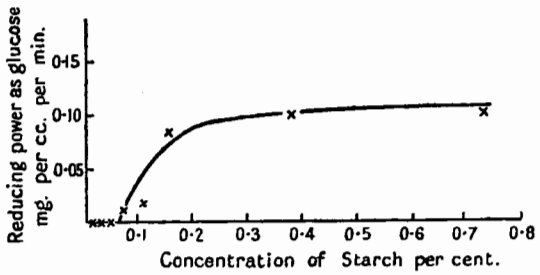

FIG. 20.—Initial velocities of starch hydrolysis (measured by maltose production by malt amylase, as a function of starch concentration in per cent.
[Eadie, 1926.]

proteases present great difficulties in the measurement of reaction velocity as they catalyse a number of different reactions. In other cases the enzyme may be inactivated by its substrate or impurities in it. Or the "enzyme" may consist of several components, and the velocity-substrate relation depend on their ratio, as is probably the case with urease.

Yet in many cases it is hard to escape the conclusion that the Michaelis law does not hold. This is conspicuously so where no action takes place below a certain limiting velocity (Fig. 20), but in a few cases the deviation consists largely in a failure of the v, x curve to reach an asymptote, and it is more nearly represented by a "parabola" of equation $v = ax^b$ (where b is less than 1) than by a rectangular hyperbola. Just as the hyperbola is the isotherm of a monomolecular reaction, so is such a curve that of an adsorption isotherm according to Freundlich's [1922] equation. Nevertheless,

such a curve has not yet been found to fit a v, x curve as accurately as the rectangular hyperbola of Michaelis fits it in favourable cases. In such cases defenders of the Michaelis equation might perhaps suggest that the enzyme consists of a mixture of different chemical individuals, each with its own K_m, or that the several reactive centres on a single enzyme molecule have somewhat different values of K_m. This is plausible, because K_m is much less specific than optimum pH. It varies considerably with invertase preparations from different yeasts, and even with different preparations from the same yeast, whilst their pH behaviour is always the same. The velocity equation would then be of the form

$$v = \frac{V_1 x}{x + K_1} + \frac{V_2 x}{x + K_2} + \ldots$$

which would allow of a relatively slow approach to the asymptote.

In a few cases the asymptote is approached too rapidly for the Michaelis equation, as in the case of taka-invertase where the relation between velocity and substrate concentration remains almost linear until saturation is reached, according to Hattori [1924]. This is equally hard to explain on the combination or adsorption theories. In Eadie's [1926] case a very good fit of the observed relation was obtained with the equation $v = a \log x - b$, which Quastel and Whetham [1925, 1] had successfully applied to surface catalyses where no action occurs below the critical concentration $10^{\frac{b}{a}}$, but which embodies no definite theory as to the union.

Variations in K_m for the Same Enzyme.

All preparations of yeast invertase not only hydrolyse sucrose to glucose and fructose, but raffinose to melibiose and fructose, and the two processes are identically affected by pH, except on the acid side of the optimum, where K_m for sucrose at least varies with pH. Nevertheless in ·138 M solutions the ratio of the velocity of hydrolysis of sucrose to that of raffinose varied, in the case of different yeasts, from 5·1 to 12·3, which led to the view that the enzymes concerned were present in different proportions in them. The true explanation was found by Kuhn [1923, 1], who measured the values of the Michaelis constants and limiting velocities for the two sugars. If we represent the v, x relation by $v_s = \frac{V_s x}{K_s + x}$, and $v_r = \frac{V_r x}{K_r + x}$, for the two sugars, we have the following typical results for different yeasts :—

TABLE VII.

v_s/v_r (·138 M).	K_s.	K_r.	$\frac{K_r}{K_s}$.	V_s/V_r.
4·8	·016	0·24	15	2·0
5·0	·016	0·27	16	1·9
8·3	·040	0·66	17	1·9

Hence for all yeast invertases so far investigated, sucrose has about sixteen times the affinity of raffinose, and the compound, when formed, is hydrolysed about twice as fast. This is as near as the errors of observation allow to the ratio of 1·6 found for acid hydrolysis, and suggests that the mechanism may be similar. Now at a concentration of ·14 M the enzyme is always saturated with sucrose, whilst its degree of saturation with raffinose depends on the value of K_r, and thus the ratio of the velocities of hydrolysis is different. Also the inadequacy of invertase to raffinose is determined more by low affinity than by low hydrolysing power. When the same enzyme attacks two substrates the quantitative differences will, in general, depend both on differences in affinity and in velocity of hydrolysis when saturated.

The proportionality of the affinities of sucrose and raffinose may be compared with the proportionality, for the same hæmoglobin, of the affinities for O_2 and CO when both are altered by varying pH or otherwise, found by Douglas, Haldane, and Haldane [1912]. On the other hand, the ratio of these affinities is the same for all yeast saccharases, though different for different hæmoglobins. In this respect, and with regard to the relative values of V, β-glucosidases differ more among themselves than invertases. Thus Willstätter, Kuhn, and Sobotka [1923] found the values of Table VIII.

TABLE VIII.

	Salicin.		β-phenyl-glucoside.		β-methyl-glucoside.	
	K_M.	V.	K_M.	V.	K_M.	V.
Bitter almonds *a* .	·035	8·5	·065	1·06	·60	1
,, ,, *b* .	·035	7·3	·040	·84	·40	1
Apricot . .	·041	9·3	—	—	·65	1
Sweet almonds .	·017	6·8	—	—	1·12	1
Strong acid . .	—	4·3	—	10·0	—	1

The maximal velocities for β-methyl glucoside are arbitrarily taken as unity. It will be seen that the sweet almond emulsin has

a greater affinity for salicin and a less for β-methyl glucoside than has that of bitter almonds. It is just possible that the different ratios of V for the different enzyme preparations are due to experimental error. Similar results were found for the hydrolysis of maltose, α-methyl and α-phenyl glucosides by Willstätter, Kuhn, and Sobotka [1924]. Here the ratios of the values of V for α-methyl glucoside, α-phenyl glucoside and maltose were as 1 : 28 : 140, the corresponding values for $\overset{+}{H}$ catalysis being 1 : 25 : 180, that is to say, the same within the limits of experimental error.

In general, purification of invertase does not affect its K_M. In one case [Kuhn, 1923, 1] the affinity was diminished by the addition of boiled invertase solution. This has been variously ascribed to a real alteration in the nature of the enzyme particles, and to the addition of an inhibitory substance.

The Union of the Enzyme with Compounds Related to its Substrate.

It was early found that, even when an enzyme reaction was not reversible, it might be slowed down by the addition of its products. This cannot, in most cases, be due to a back reaction, for the velocity of this is negligible in such cases as that of sucrose hydrolysis. It is sometimes due to pH changes, as in the case of unbuffered urease solution, but it is usually due to different causes. As a matter of fact the classical case of invertase still remains to be fully investigated. The reaction catalysed appears [1] to be—

$$\alpha\ (1, 5)\ \text{glucosido-}\beta\ (2, 5)\ \text{fructoside} + H_2O \rightarrow \alpha\ (1, 5)\ \text{glucose} + \beta\ (2, 5)\ \text{fructose}.$$

Subsequently the β (2, 5) fructose changes into an equilibrium mixture of α and β (2, 6) fructose, and part of the α (1, 5) glucose mutarotates more slowly into β (1, 5) glucose. The changes have been followed polarimetrically by Hudson [1909] after rapid enzyme hydrolysis at 0°. Now the old experiments on the influence of reaction products refer entirely to equilibrium mixtures of normal (6-membered ring) glucose and fructose. Later the effects of α- and β-glucose, and of $\alpha\beta$- and β-fructose were separately determined. But the unstable (2, 5) or γ-fructose, which is the immediate product of reaction, has an inhibitory effect no greater than normal fructose according to Nelson and Bodansky [1925].

[1] Cf. Appendix to Chap. VI.

An added compound may affect the velocity in two different ways. It may reduce V, without altering K_m. In this case the velocity is equally reduced at all substrate concentrations. Thus Michaelis and Pechstein [1914, 2] found that 1·0 M glycerol reduced the velocity of inversion of ·05 M and ·20 M sucrose solutions to 40 per cent. and 45 per cent. respectively, while α-methyl glucoside behaved similarly. Presumably such inhibitors, apart from slight effects due to dilution of the water, must unite with the enzyme in such a way as to reduce or abolish its catalytic activity, but are not displaced from it by the substrate. They must therefore be attached to a different part of its surface from the substrate. If the reaction be reversible, and the compound quite devoid of catalytic power, as appears to be the case with that of invertase and α-methyl glucoside, we have

$$\frac{v'}{v} = \frac{1}{1 + \frac{h}{K_h}}$$

where v is the velocity without inhibitor, v' the velocity with it, h the concentration of inhibitor, and K_h the dissociation constant of the combination. This formula is followed provided h does not rise too high, when strong solution effects may also intervene. Fructose, on the other hand, produces a far greater percentage inhibition in weak than in strong sucrose solutions. If we assume, then, that the sucrose and fructose molecules compete for the same spot in the enzyme molecule, and if f be the concentration of fructose, and q that of enzyme-fructose compound, we have the three reactions

$$E + S \rightleftharpoons ES\,; \quad E + F \rightleftharpoons EF\,; \quad \text{and } ES + H_2O \rightarrow E + F + G.$$

If K_f be the dissociation constant of EF,

$$\therefore \text{ (as on p. 39)}$$

$$K_m p = x(e - p - q), \text{ and } K_f q = f(e - p - q),$$

$$\therefore p = \frac{ex}{x + K_m\left(1 + \frac{f}{K_f}\right)}$$

$$\therefore v' = \frac{Vx}{x + K_m\left(1 + \frac{f}{K_f}\right)} = \frac{v}{1 + \frac{fK_m}{K_f(x + K_m)}},$$

where v is the velocity in the absence of fructose, v' in its presence.

Hence the net effect of a competing substance is to increase K_m,

and the amount needed to double it is equal to the dissociation constant K_f. This may also be calculated from the equation

$$K_f = \frac{fK_m}{\left(\frac{V}{v'} - 1\right)x - K_m} = \frac{fK_m}{\left(\frac{v}{v'} - 1\right)\left(x + K_m\right)}.$$

The agreement between values so obtained is satisfactory, considering the errors involved [Kuhn and Münch, 1927]. In Nelson and

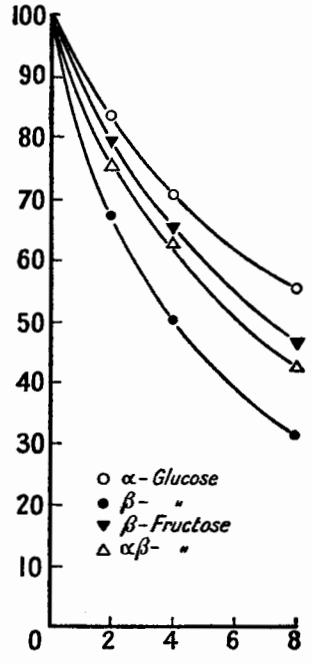

FIG. 21.—Effect of varying glucose and fructose concentrations on initial velocity of hydrolysis of 2 per cent. sucrose by yeast saccharase. Abscissa, hexose concentration per cent. Ordinates, velocities of hydrolysis of sucrose.
[After Nelson and Anderson, 1926.]

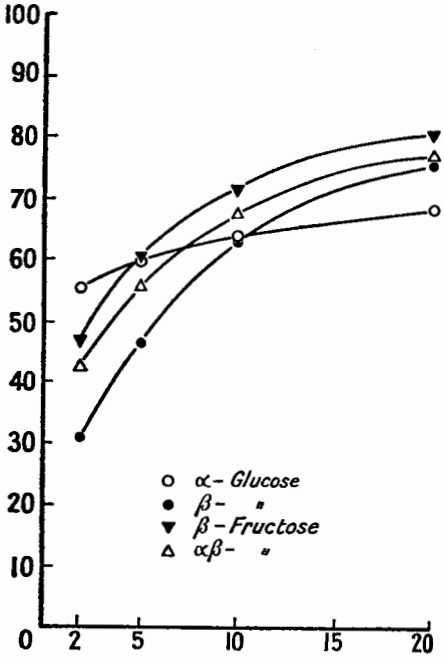

FIG. 22.—Effect of varying sucrose concentration on initial rate of its hydrolysis by yeast saccharase in presence of 8 per cent. glucose or fructose. Abscissa, sucrose concentration per cent. Ordinates, velocities of sucrose hydrolysis as percentages of velocity in absence of hexose.
[After Nelson and Anderson, 1926.]

Anderson's [1926] experiments (see Figs. 21 and 22) β-glucose had about half the affinity of sucrose for the enzyme, fructose a somewhat less value. Older data give similar results, though it is not absolutely clear whether all the competing affinities vary proportionally from one enzyme to another. However, Kuhn and Münch's [1927] results make this rather unlikely.

A few substances, e.g. α-glucose in the case of some invertases, and methyl and ethyl alcohols in the case of emulsin, according to Josephson [1925], act in both ways. With sufficient data, it is, however, quite possible to disentangle the two effects.

The literature regarding the combination of inhibitory substances with invertase is voluminous, but it is exceedingly misleading owing to the following facts :—

1. The earlier authors ignore the difference between the action of α and β sugars, first discovered by Kuhn in 1923 ; and almost all ignore the probable difference between the effects of (2, 5) and (2, 6) fructose.

2. Yeast invertases differ greatly with regard to their behaviour with α-glucose, some being inhibited by it, while others are not.

3. There is a general confusion, long after Michaelis and Pechstein's paper, between competitive and non-competitive inhibition. Hence in the case of many of the substances considered, we do not know in which way they inhibit.

Among the most satisfactory data are those of Nelson and Anderson [1926], from whose results Figs. 21 and 22 are constructed. For their enzyme α-glucose inhibited in an almost non-competitive manner, while β-glucose, α-fructose, and αβ-fructose were competitive inhibitors. Since the effect of αβ-fructose is slightly greater than that of β-fructose, α-fructose would be still more active, probably about as active as β-glucose.

The following substances [1] do not inhibit yeast saccharase except perhaps to a small extent in high concentrations :—

Maltose, gentiobiose, cellobiose, lactose, melibiose, tetramethyl-β-methyl glucoside, gluconic acid, amygdalin, lactic acid.

The following inhibit non-competitively :—

α-glucose and trehalose (in some cases), α-methyl glucoside, salicin, glycerol, αβ-mannose (? both components), ethyl alcohol,[2] α-*l*-arabinose.

The following inhibit competitively :—

β-glucose, α-fructose, β-fructose, α-galactose, β-galactose, β-*l*-arabinose.

The following inhibit, according to which law is not certain :—

α-rhamnose, β-rhamnose, α-xylose, β-xylose, β-methyl glucoside, mannitol, hexose-diphosphoric acid.

The cases of other poisons, e.g. metals, halogens, and organic

[1] Euler and Josephson [1924, 1], Kuhn [1923, 2], Kuhn [1924], Kuhn and Münch [1927].

[2] Only as a diluent.

bases, will be dealt with in Chapter VIII. Of the sugars and hexosides the α-form is the more active in the following :—

Fructose, xylose, methyl glucoside;

and the β-form in the following :—

Galactose, arabinose, mannose, ? lactose, rhamnose.

It is noteworthy that galactose may inhibit more strongly than glucose or fructose.

Nelson and Bodansky [1925] compared the rates of hydrolysis of sucrose (*a*) alone, (*b*) in presence of " nascent " invert sugar, as formed during hydrolysis, and (*c*) in presence of equivalent amounts of mutarotated invert sugar. (*a*) was always greatest, but (*b*) exceeded (*c*) in the early stages of the reaction, (*c*) exceeding (*b*) later on. It follows that α- (2, 5) fructose is probably less inhibitory than αβ- (2, 6) fructose, and may be much less so. The question, however, requires further study.

It is noteworthy that α-methyl glucoside may inhibit in some cases when α-glucose does not. No clear laws can at present be laid down as to the influence of structure on affinity. It is possible that such may emerge when the type of inhibition is determined in each case. We do not know, moreover, whether, for example, α-glucose and α-methyl glucoside unite with the enzyme at the same spot, though this could be determined. The importance of structure is, however, obvious. Josephson [1924] found that the inhibitory effect of fructose increased slowly with the pH, rising by 31 per cent. between pH 2·75 and 6·75. That of glucose was maximal at the optimum, falling to 76 per cent. at pH 2·75, and 60 per cent. at 6·65. Kuhn and Münch [1927] showed that the inhibition by α-glucose and α-galactose was nearly independent of pH, that of β-glucose being maximal at the optimum.

The Affinity of a Synthesizing Enzyme for its two Substrates.

Although the hydrolyses catalysed by enzymes are really bimolecular, the concentration of H_2O cannot be substantially altered without bringing in strong solution effects, and there is so far no experimental ground for supposing that it unites with any enzyme. Where the reverse reaction is measurable, the concentrations of both substrates can be altered. This has been done by Josephson [1925] for prunase, the β-glucosidase component of almond emulsin. Using a large excess of alcohol he found that for glucose $K_m = 0{\cdot}18$ and

0·185 for β-methyl glucoside synthesis in 30 per cent. and 40 per cent. alcohol, and 0·19 for β-ethyl glucoside synthesis in 30 per cent. alcohol at the optimal pH. Owing to the slowness of the reaction the data are of course for equilibrium αβ-glucose, not for pure β-glucose. When the methyl alcohol concentration was varied, matters were complicated by strong solution effects. Half the maximum velocity was reached at a concentration of 3·4 M, but the true value of K must be somewhat higher. Hence the enzyme has a very small affinity (of the competitive type) for methyl alcohol.

Now, in the case of the enzymatic hydrolysis of β-glucosides, glucose exerts a retarding influence, mainly but not wholly due to the β component. The dissociation constants of the enzyme—αβ-glucose combination, deduced from the inhibition of the hydrolyses of salicin, helicin, and arbutin respectively, were 0·16, 0·15, and 0·20, in extremely satisfactory agreement with the result (0·185) found directly. This constitutes a very strong argument for the validity of the theory by which the figures were calculated.

At the optimum pH Josephson found the values of K_m given in Table IX.

TABLE IX.

Alcohol.	β-Glucoside.	K Alcohol.	K Glucoside.	Ratio.
Methanol	β-methyl glucoside	3·7	·71	5·2
Phenol	β-phenyl glucoside	·21	·050	4·2
Saligenin	Salicin . . .	·17	·030	5·7
Salicylaldehyde	Helicin . .	·10	·016	6·2
Quinol	Arbutin. . .	·09	·042	2·1

The figures for the glucosides are obtained by measuring the initial velocities at different concentrations, those for the alcohols by inhibition. It is clear that the two sets of figures are intimately connected. The affinity of the enzyme for quinol is 2 to 3 times that expected from the affinity of its glucoside. This is not due to the quinol having two alcoholic groups, for its monomethyl ether has a K of about ·10. Nevertheless, it is clear that the enzyme has a high affinity for phenols and a much smaller for aliphatic alcohols. The affinity for a glucoside is no doubt also a function of the affinity for its sugar residue, and it would be of very great interest to study quantitatively the behaviour of such sugars as maltose, *d*-epirhamnose, and the methylated glucoses, together with their β-compounds towards this enzyme. Josephson found that maltose (like cane-sugar, lactose,

raffinose, and fructose) caused no appreciable inhibition, although galactose, xylose, and arabinose had a small effect. The affinity of the β-maltosides should therefore be low. If K_s, K_a, K_b be the dissociation constants of the compounds of the enzyme with its substrate, and with the two products of reaction, and V_1, V_2 the velocities of hydrolysis and synthesis when the enzyme is saturated, while K is the dissociation constant of the equilibrium reached in the catalysed reaction, it is obvious from a consideration of the velocities in a dilute solution, that $\frac{K_a K_b}{K_s} = \frac{K V_2}{V_1}$ (see p. 82). If, then, V_1 and V_2 were proportional, for different sets of substrates, to the velocity constants in the absence of a specific catalyst (which is often roughly true), then $\frac{K_a K_b}{K_s}$ should be a constant for any given enzyme.

The inhibitory compounds showed both competitive and non-competitive inhibition. It was possible, however, to correct for the latter, and the figures given, which are not very accurate, relate to it only. Salicylate gave only non-competitive inhibition. Incidentally, Josephson's work proves as conclusively as is at present possible the identity of the enzyme hydrolysing the different β-glucosides.

pH had a marked effect on the affinity of this enzyme for its substrate. Thus the dissociation constant of the salicin-enzyme compound rose from ·031 at the optimal pH of 4·4 to ·046 at pH 6·5, and ·044 at pH 2·8. Hence in a dilute solution the velocity falls off more sharply on each side of the optimum than in a strong solution, and it is probably for the same reason that the velocity of hydrolysis of β-methyl glucoside, with which saturation of the enzyme has never been reached, falls off more sharply than that of the aromatic glucosides. It is clear that Michaelis' formula for the relation of velocity and pH can only be expected to hold either if the enzyme is saturated with substrate, or if K_m does not vary with pH.

Acceleration by Products of Hydrolysis.

This phenomenon is generally due to changes of pH, but one case has been observed of a more direct action. Nelson and Cohn [1924] found that in strong sucrose solutions the rate of hydrolysis by honey (bees' salivary) invertase at first increased with time. Nelson and Sottery [1924] found that this was due to the glucose and fructose liberated, mainly the former. Fig. 23 shows the effects on initial rate of hydrolysis of equilibrium mixtures of α, β-glucose and

α, β-fructose added to 10 per cent. sucrose at pH 5·76. It will be seen that the maximum acceleration is produced by about 0·6 per cent. glucose and 0·1 per cent. fructose. α-glucose is less efficient, either at acceleration or retardation, than β-glucose, while β-fructose and $\alpha\beta$-fructose have the same effect. In 3 per cent. sucrose the maximum acceleration by glucose occurs on adding between 0·1 and 0·2 per cent., though the acceleration is not so great. The optimum concentration of glucose is therefore roughly proportional to that of sucrose. A similar case occurs outside the field of enzyme chemistry, where Douglas, Haldane, and Haldane [1911] found that, while large amounts of O_2 displace CO from its combination with hæmoglobin, small amounts increase the amount combined. On the other hand, this only occurs with CO concentrations insufficient to saturate the hæmoglobin at all completely, while the enzyme, according to Michaelis' theory, should be very nearly saturated by a 5 per cent. solution of sucrose.

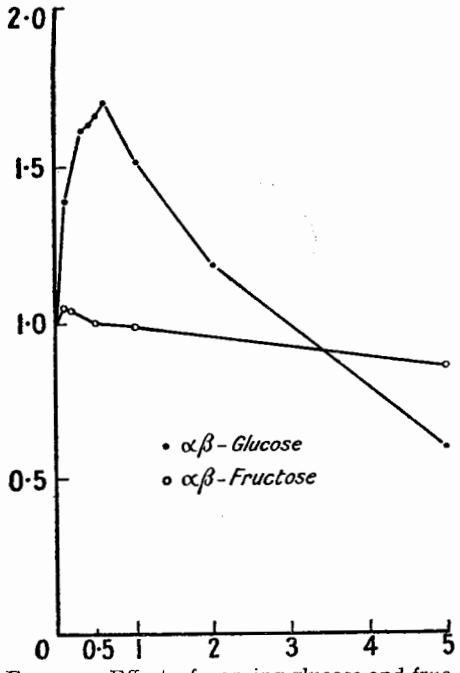

FIG. 23.—Effect of varying glucose and fructose concentration on velocity of sucrose hydrolysis by honey saccharase. Abscissa, hexose concentration per cent. Ordinates, initial rate of hydrolysis of 10 per cent. sucrose.
[After Nelson and Sottery, 1924.]

Lipase.

The substrate concentrations at which certain lipases reach half their maximum velocity of hydrolysis are given in Table V. It will be noticed that these vary very greatly. Moreover, they differ in the case of optical antipodes, showing that a purely physical interpretation is impossible. There are similar differences in the falling off of reaction velocity in high substrate concentrations. Where the enzyme acts on an emulsion, either in a non-aqueous phase or at the phase boundary, the concentration cannot be determined satisfactorily. Murray [1929] investigated the hydrolysis of mixtures

by pancreatic lipase. He points out that the affinity of this enzyme is greater for tributyrin than for dibutyrin and monobutyrin [Terroine, 1910] and confirms this for triacetin and monoacetin. The affinity for triacetin also appears to be greater than that for ethyl butyrate.

Murray also investigated inhibition. While fluorides inhibit non-competitively, a number of organic compounds inhibit in a competitive manner. The ratio of their affinities to that of ethyl butyrate is given in Table X.

TABLE X.

Acetophenone	4·35
Phenyl-methyl-carbinol, Benzaldehyde	2·75
Benzophenone	2·5
Phenol, Anisole	1·25
Cyclohexanol	0·60
Aniline	0·45

Other ketones had a moderate effect. Hydrocarbons had very little, and salts of propionic, lactic, and pyruvic acids had no more than sodium chloride. It is also known that glycerol has only a slight inhibitory effect, if any, since glycerol extracts of organs often have a powerful lipase action. It appears probable, from the powerful inhibitory effect of the carbonyl group, that the enzyme unites with the carbonyl group of the substrate. Since acetophenone oxime has no inhibitory effect, the carbonyl group of the inhibitor is clearly responsible. The effect of secondary alcohols is not so clear. Possibly the enzyme unites with an enolic tautomer of its substrate, or the secondary alcohol may unite with the enzyme in a different manner.[1] Willstätter, Kühn, Lind and Memmen [1927] found a latent period of the order of an hour in the hydrolysis of ethyl mandelate by liver lipase. This was traced to the presence of ethyl phenylglyoxylate, $C_6H_5—CO—CO—O—C_2H_5$, as an impurity. Until this substance was hydrolysed, no hydrolysis of the mandelate occurred. This is clearly due to the strong affinity of the enzyme for a carbonyl group in an uncharged molecule, the ionized keto-acid being ineffective, like pyruvic acid. The keto-ester is very slowly hydrolysed, in comparison with ethyl mandelate. The most plausible explanation of this fact is that it is largely united with the enzyme by the wrong carbonyl group, and therefore does not undergo hydrolysis.

[1] Murray and King (1929) have since shown that the affinities of the lævo forms of three secondary alcohols for sheep liver lipase are four to five times as great as those of their dextro isomers.