LI. ENZYME-ACTION, FACTS AND THEORY.

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The researches of the last few years on the kinetics of enzyme action have brought more confusion than clearness into this field. Even about the action of the best studied enzyme, invertase, there is contradiction and uncertainty; it seems as yet not quite established if, or under what conditions, the hydrolysis of cane-sugar by invertase follows the simple law of mass-action.

As to the nature of enzyme action there is a general inclination to suppose, that some chemical combination of enzyme and substrate plays a leading part in the process and that the general properties of the colloids, to which enzymes seem to belong, will supply sufficient explanation of their remarkable activities.

The aim of this paper is to clear up some of the contradictory statements in the literature about the kinetics of the most simple enzyme actions, and to show at the same time that there is another theory, which is more in accordance with the peculiarly characteristic behaviour of enzymes.

The hydrolysis of cane-sugar by invertase has been chosen by many investigators as the best process on which to study the velocity of enzyme action in relation with the different factors, which are here of importance. Both enzyme and hydrolyte are readily accessible, the action is simple, starts with the well-known substance saccharose and leads to the also fairly well-known substances glucose and levulose.

Chemically speaking, this hydrolysis is a monomolecular reaction. It was therefore a confirmation of what chemists expected, when O'Sullivan and Tompson [1890] found in an elaborate investigation, that the inversion of saccharose by invertase from yeast proceeded in accordance with the law of mass-action, i.e. if y represents the inverted fraction at a time t ,

$k = \frac{1}{t} \log \frac{1}{1-y}$ was nearly a constant.

Duclaux [1898] however, without doubting the correctness of the experimental results of O'Sullivan and Thompson, pointed out, that there was still a remarkable difference between the action of acids and invertase (and of enzymes generally).

In differently concentrated solutions of saccharose the same amount of acid gives the same velocity-constant k , thus for instance a 5, 10 or 15 % saccharose solution will be inverted by a 1 % acid solution in the same time to the same extent, e.g. half way. But for invertase Duclaux had found as early as 1883, that the same quantity of enzyme, acting in different concentrations of cane-sugar, inverted in the same time not the same fractions but the same absolute amounts.

Henri [1901] then took up again the experimental study of invertase and found, that not only was there the difference between the actions of enzymes and acids, established by Duclaux, but that k did not even remain constant during one and the same action of invertase on cane-sugar; on the contrary steadily increased.

Now it cannot be denied, that Henri's experiments were open to criticism owing to his not taking sufficiently into account the mutarotation of the freshly formed glucose in his polarimetric estimations.

It was therefore a valuable contribution on the experimental side of the subject, when A. Brown [1902] also investigated the kinetics of the invertase-action and found the same regular increase of the constant $k = \frac{1}{t} \log \frac{1}{1-y}$ for sufficiently concentrated saccharose solutions as Henri. On p. 375 of his article Brown says: "Conditions of experiment similar to those used by C. O'Sullivan and Thompson were employed, but the invertase used was prepared in a different manner from the enzyme with which these authors experimented."

To this last point we will return further on.

In order to remove all doubt, possibly also expressed by others as to the correctness of Brown's estimations, I wrote to him on this subject and was assured, that he "was well aware of O'Sullivan's experiences with regard to mutarotation and took exactly the same precautions to avoid errors in connection with it, which O'Sullivan adopted."

As I myself [1904] found the same deviation from the simple law of mass-action as Brown, without using a polariscope at all, but estimating the hydrolysis of cane-sugar by the accurate gravimetric method of Kjeldahl with Fehling's solution, Hudson's [1908] criticism of his predecessors (excepting Henri) must be rejected. How it was, that Hudson could again find validity

of the simple formula of mass-action in his experiments with invertase was explained by the work of Sørensen [1909].

In his elaborate article on: "The measurement and importance of the concentration of hydron in enzyme-action" this author shows, that low concentrations of hydron in the invertase-solution give the well-known increasing k . Going beyond this concentration one can work with an invertase-solution, where the reaction velocity follows the formula

$$k = \frac{1}{t} \log \frac{1}{1-y},$$

and in still more distinctly acid solutions k diminishes even more and more rapidly during the reaction. That this irregularity in the reaction-velocity is due to a gradually decaying of the enzyme by the action of the hydron was perhaps not sufficiently emphasised in this article, though it is clearly expressed on p. 146 for the series with the highest hydron concentration, where the reaction did not even come to a finish.

Indeed it follows from Sørensen's observations and deductions, that it is dangerous to work with enzymes at their optimum concentration of hydron as well as at the optimum temperature of their activity. Both factors in that condition give splendidly rapid enzyme action, but at the same time are gradually destroying the enzyme itself.

It is obvious, that, where the quantity of the active catalyst is diminishing during a reaction, the velocity of this reaction will diminish at the same rate and may thus seem to fulfil the simple formula of mass-action. With low hydron concentration, that is without adding acid or using extracts that have become acid by deterioration, one always gets for invertase (provided the saccharose solution is sufficiently concentrated) the same increase in the constant k , calculated as $k = \frac{1}{t} \log \frac{1}{1-y}$, or a real constant k , when it is calculated according to the empirical formula of Henri or to the theoretical formula, which I developed in 1904.

Sørensen drew attention to the fact, that O'Sullivan and Tompson brought their solutions by the addition of sulphuric acid to about the maximum of activity of the invertase, while Hudson worked with solutions containing acetic acid. Now we can add to this, that Brown "employed in (his) experiments an extract of invertase, prepared from dried yeast by digestion with water," and "without the complicating addition of sulphuric acid." In my own experiments I used an extract of fresh yeast, mixed with kieselguhr and dried rapidly at a low temperature. No acid at all was used.

When examining the results of O'Sullivan and Tompson as closely as these authors themselves did, one sees clearly, that the acidity they employed had not reduced the real velocities quite to an extent sufficient for the law of simple mass-action to be perfectly imitated. On p. 846 they say: "But if we look more closely at the diagram, we see that the deviations from the theoretical line, though small in themselves, are constant in the three experiments; in fact they correspond in a very striking manner." And a little further: "These figures all show a continuous increase in activity up to a certain point, about 80 per cent. of inversion, and after that a large and increasing decrease." That is exactly what the gradual destruction of the invertase must produce.

Sørensen goes however too far, when he generalises this influence of hydron concentration to suggest a sufficient explanation for the marked falling off of the constant $k = \frac{1}{t} \log \frac{1}{1-y}$ in the experiments of Armstrong with lactase. My own experiments as well as the theory of enzyme action, which I proposed some years ago, lead to a different explanation and to a real reaction-constant in this case also.

As the application of this theory may now be extended in some points beyond what was published before, it may be of interest to survey once more a part of this much entangled field of enzyme actions with the aid of this theory.

Any hypothesis of enzyme action must be founded on the principal characteristics of this kind of catalysis.

It seems more and more established that these are:

1. That the same quantities of enzyme, brought into differently, but sufficiently, concentrated solutions of substrate, produce in the beginning the same action in the same time.
2. That catalysis by enzymes has a marked specific character¹.

Starting from these general facts, I ventured the hypothesis [1904] that enzyme action spreads like radiation from an enzyme particle as centre, and that an enzyme particle contains the same molecule, which is liberated or acted upon by this enzyme, in some active state.

What kind of radiation this may be cannot yet be decided. It is however very remarkable, that an imitation of specific enzyme actions has been produced by Rosenthaler [1908] by electromagnetic oscillations. By placing

¹ This specificity is known to enzyme workers to be probably not so absolute as is often taken for granted. According to the following theory a limited specificity must also be looked for.

his substrate solutions inside a solenoid, through which a frequently interrupted direct current or an alternating current was passed, a diastase-like action on starch was observed for a frequency of 440–480 per second, and a hydrolysis of proteins for one of 320–360.

In order to explain the principal facts it is necessary to make the natural assumption, that this radiation may be absorbed by the substrate itself, by the substances liberated from it by the enzyme action, or by any other foreign substance added to the solution. The possible absorption by the water itself must be practically the same in every case and can therefore be left out of account.

It is evident that the sphere, within which this radiation-like effect around an enzyme particle may be expected to be active, must be very small; otherwise enzyme actions would be instantaneous.

In the beginning the spheres of action in differently, but sufficiently concentrated solutions of substrate will be inversely proportional to that concentration; therefore in the first time-unit the same quantity of enzyme will produce the same amount of effect in these solutions, for practically all radiation is absorbed by the substrate only.

In more dilute solutions not all the radiation will reach a substrate particle before it is too much weakened by spreading. This explains the fact that in moderately dilute solutions the constancy of the amount of initial action has disappeared. In sufficiently dilute solutions of substrate we shall practically obtain the condition, that on the average no two particles of substrate are on the same radius of the radiation sphere, so that all these particles will be acted upon at the same time and the amount of action will be directly proportional to the concentration of substrate; a fact observed by myself [1904] as well as by many other investigators.

The advantage of a reasonable working hypothesis proves here again to be its giving an indication, where to look for regularities and where these are not to be expected.

This hypothesis can also be quantitatively developed and leads, as I have shown, to a theoretical formula for the reaction-velocity in sufficiently concentrated solutions of substrate. We shall call the initial concentrations of the substrate a , the same after a time t of reaction x (all expressed for instance in grammes per 100 c.c.), and the amount of action in the first time-unit by a given quantity of enzyme in 100 c.c. of these sufficiently concentrated solutions m . According to theory as well as to experiment m is independent of a .

If further, n is the ratio between the absorption power for the enzyme

radiation of the reaction-products and that of the original substrate, the general differential equation for the rate of reaction is:

$$-dx = m \frac{x}{x+n(a-x)} dt.$$

As however catalysis by enzymes is generally recognised to be as a matter of fact a balanced action, this formula for the rate of reaction can only be applicable, where there is evidence that the reverse action, the synthesis, does not come into play. It will be shown further on, that this happens to be the case as a rule in the experiments with two of the best studied and relatively simple enzymes, invertase and lactase.

Bearing in mind this restriction, we obtain by substitution of $\frac{a-x}{a} = y$ and integration, the general formula for the rate of enzyme action in sufficiently concentrated solutions of substrate:

$$\log \frac{1}{1-y} + \frac{1-n}{n} 0.434y = \frac{m}{na} 0.434t$$

expressed in decimal logarithms.

The two constants in these experiments can be determined in different ways.

They may be calculated from the observed action at two different time-intervals, or m can be directly found by estimating the change during the first time-unit and n from the same estimation in similar conditions, but in a solution, in which had also been dissolved a known amount b per 100 c.c. of the reaction-products.

In the first estimation the initial velocity will be

$$\left(\frac{dy}{dt}\right)_{y=0} = \frac{m}{a},$$

in the second, from

$$-\frac{dx}{dt} = m \frac{x}{x+n(a-x)+nb} dt,$$

we get

$$\left(\frac{dy}{dt}\right)_{y=0} = \frac{m}{a} \frac{1}{1+n\frac{b}{a}}.$$

Evidently the value of n will depend only on the nature of enzyme and substrate, and must therefore be the same in the experiments of different observers.

For invertase from ordinary yeast the estimations of all the investigators, who avoided the errors mentioned in the first part of this article, agree with the value $n = \frac{1}{2}$, and I found the same also by direct estimation of initial velocities.

As $(a - x)$ grammes saccharose give by inversion $\frac{340}{112}$ grammes invert-sugar, the rate of inversion of cane-sugar in sufficiently concentrated solutions by unaltered invertase is thus generally represented by the equation :

$$\log \frac{1}{1-y} + 0.393y = kt.$$

The enzyme lactase is not as readily accessible as invertase, therefore its action is not so often studied. Ordinary brewers- or distillers-yeast is inactive in regard to milk-sugar. It is necessary to employ the special yeast, which is known to be the principal active agent of "kephir."

In order to extract the enzyme lactase as fresh and as unaltered as possible from the living cell, which produces it, I prepared a sufficient quantity of a pure culture of "*Saccharomyces kephir*" and worked only with the fresh extract from this yeast, which had been mixed with kieselguhr and dried in vacuum at a low temperature [1906].

In estimating the velocity of inversion of an 8% milk-sugar solution at 30° by this lactase, I found a series of figures, to which it will now be shown that the general equation :

$$\log \frac{1}{1-y} + \frac{1-n}{n} 0.434y = \frac{m}{na} 0.434t$$

can also be applied with the same success.

Only, as explained above, the value of n may be expected to be different from that for invertase.

Direct observations of the very large retarding influence of glucose and of galactose showed that n is here much greater than in the case of invertase.

Calculation from two estimations of the hydrolysis of lactose at different time-intervals gives about $n = 6$. Thus for lactose the velocity of inversion is represented by

$$\log \frac{1}{1-y} - 0.362y = kt.$$

For 8% milk-sugar at 30° I found, estimating the hydrolysis again by the gravimetric Fehling-Kjeldahl method :

t (minutes)	y	$k = \frac{1}{t} \left(\log \frac{1}{1-y} - 0.362y \right)$
5	0.23	0.0061
10	0.38	0.0070
15	0.47	0.0070
20	0.57	0.0080
25	0.61	0.0075
40	0.72	0.0073
66	0.82	0.0068
90	0.90	0.0075

Armstrong [1904], though also emphasising the importance of working rapidly and using only fresh extracts, did not it seems give care enough to these precautions. Kephir-grains, an extract of which he used, are a mixture of kephir-yeast cells with lactic acid bacteria and other residues, all together a mixture of somewhat unknown history and origin. The extract will contain some acid and in fact was not very active, compared with that obtainable from fresh pure lactose yeast. The experiments lasted several hours and even days, so that it was necessary to add toluene. The risk of decaying of the enzyme during these long experiments at 35°–37° was therefore not sufficiently excluded.

Still the first series of his investigations (p. 506) calculated with our lactose equation, gives

t (hours)	y	$k = \frac{1}{t} \log \frac{1}{1-y}$	$k = \frac{1}{t} \left(\log \frac{1}{1-y} - 0.362y \right)$
1	0.221	0.1085	0.0285
2	0.312	0.0812	0.0247
3	0.384	0.0713	0.0244
4	0.458	0.0665	0.0250
5	0.515	0.0629	0.0256
6	0.566	0.0604	0.0263
10	0.69	0.0509	0.0259
17	0.842	0.0471	0.0290
23	0.924	0.0461	0.033
29	0.953	0.0457	0.034
38	0.98	0.0447	0.035

The last of these estimations are less trustworthy, owing to exposure of enzyme and of milk-sugar to the combined action of the slight acidity and the temperature of 35°.

The other series of Armstrong's article lasted still longer, the inversions were far from finished at the end, and proceeded only very slowly during the last 24 hours. Evidently the enzyme was here to a large extent destroyed.

There are further regularities in the working of these relatively simple enzymes, invertase and lactase, which only become apparent when the investigator takes sufficient care to work only with enzymes, extracted with caution directly from the fresh living cells.

As I have shown before, the experiments not only agree with the conclusion, drawn from our hypothesis, that generally all neutral substances as well as the inversion products must retard the enzyme action, but striking quantitative regularities also exist.

For *unaltered*¹ invertase from ordinary yeast the facts are these :

¹ By drying the yeast at a high temperature or treatment with alcohol I got an invertase, the action of which was more retarded by levulose than by glucose.

If we represent the retarding power (or the absorption coefficient according to our conception) of glucose by $n = \frac{1}{2}$, then the retarding powers of levulose and of invert-sugar are found to be exactly $n = \frac{1}{2}$ also. For the retarding powers of galactose and mannose however the experiment gives the double value $n = 1$. Nearly all neutral substances give retardation of the same order as the sugars, e.g. dulcitol, mannitol, urea, salicin and other glucosides.

It is very remarkable, that milk-sugar alone does not appreciably retard the action of invertase. In connection with this may be mentioned the small absorption power that milk-sugar was also shown to have for lactase action, compared with that of all other sugars. The figure for the inversion couple from milk-sugar was found to be $n = 6$. Galactose retards twice as much as glucose, so for galactose the absorption power of lactase action is $n = 8$, that of glucose $n = 4$. For levulose, a hexose which is here not contained in the substrate, experiment gives also the sum of the absorption powers of the inversion products of milk-sugar, that is $n = 12$.

In connection with the recent work of Tanret [1905] and of Hudson [1908] I wish to draw attention here to another point about the action of invertase and maltase.

If enzymes, as seems now more and more probable, are able to decompose a substance as well as to actuate the reverse process, then invertase, maltase and lactase should produce an equilibrium between bihexose and hexoses. In the case of maltase Croft Hill [1898] seemed to have established some synthesis of maltose from glucose, but it was proved afterwards, that the bihexose formed with difficulty was, at least for the most part, another substance. As for invertase and lactase, the evidence of synthesis of cane-sugar and milk-sugar is even slighter.

The facts, that a fairly concentrated solution of cane-sugar is practically completely hydrolysable by means of invertase and that after all no appreciable enzymatic synthesis of maltose from glucose is observed, suggest the conclusion, that the hexoses, split off by these enzymes, undergo a secondary change, by which the reverse enzyme action is prohibited.

Through the investigations of the mutarotation of sugars, especially by Tanret and by Hudson, much clearness has been brought into our knowledge of the isomerism of the most important members of this group. It was proved by Tanret, that glucose, dissolved in water is at first totally in the α -form ($[\alpha]_D = 110^\circ$) and then gradually changes into a mixture in equilibrium ($[\alpha]_D = 52.5^\circ$) of about two-thirds α -glucose and one-third β -glucose ($[\alpha]_D = 19^\circ$).

As two-thirds of glucose in solution are finally in this α -modification, then, if the glucose, split off by enzyme action from cane-sugar or maltose should be originally in this same form, as is generally assumed, it is difficult to see why the reverse action of the enzyme should not produce a considerable amount of maltose.

Also the hydrolysis of cane-sugar by invertase should then not be expected to come to a finish.

The rate of mutarotation of this α -glucose, i.e. the velocity with which it comes to this equilibrium, was carefully measured by Hudson, who made a similar investigation of the mutarotation of levulose. Combining these investigations with his experimentally quite correct estimation of the velocity of inversion of cane-sugar by invertase, Hudson came to the conclusion, that it is really α -glucose, which is originally formed by enzyme action in this case.

As the mean value of 18 experiments he found however for the specific rotation of this "fresh" glucose 120.7° , while this figure for α -glucose is 110° . This difference is so considerable, that Hudson's work also seems to point to the non-identity of enzyme-made fresh glucose with α -glucose.

This result is in complete agreement with the experimental evidence I got in 1904 of an unstable form of glucose, which seems to be the primary product of enzyme action and which gradually is converted to ordinary glucose in solution, that is, as we know now, to a mixture of α -glucose and β -glucose, according to the new nomenclature of Tanret.

It was expected, if the inversion of maltose was accomplished by a very active maltase solution¹ rapidly enough for the velocity of inversion to be great, compared with the velocity of conversion of the freshly formed glucose, that after the real equilibrium point $\text{maltose} \rightleftharpoons \text{glucose}$ had been reached, the rate of inversion would appear to be approximately constant.

The curve, which must result from these two simultaneous reactions, may be demonstrated with the aid of Fig. 1.

After the few minutes that are needed for the principal reaction to come to the real equilibrium point P , the relatively slow secondary change of unstable to stable glucose has practically not yet started. Being a monomolecular reaction, this rate of change of glucose will be at any time proportional to the concentration of unchanged unstable glucose.

As soon however as the concentration of unstable glucose has been

¹ In my article of 1904 I omitted to mention, that these very active maltase extracts of the dried yeast-kieselguhr were obtained by using water with a small amount of sodium hydroxide. Extraction with water only gave a maltase solution of smaller activity.

diminished by this change, the equilibrium between maltose and glucose is disturbed and part of the maltose is split again. As we have arranged the conditions so that this inversion of maltose is much quicker than the transformation of glucose, the concentration of unstable glucose and therefore the rate of its change will be maintained constant (in first approximation).

Fig. 1.

Thus in Fig. 1 we can represent, also in first approximation, the formation of stable glucose by the dotted straight line PQ , and the amount of unstable glucose by the horizontal dotted line PR . At any time, e.g. after A minutes, the equilibrium between maltose and unstable glucose will be immediately restored by the quick enzyme action. As however the concentration of maltose at the time A is somewhat smaller than that at the time S , the concentration of unstable glucose, required to balance by synthesis the hydrolysis of maltose, is also somewhat smaller than SP or AB .

The unstable glucose which has disappeared therefore need not be totally replaced by newly inverted maltose and the result will be, that at the time A this inversion will not have proceeded exactly to D , but to C . The estimations of the inversion of maltose will thus give a curve PM , slightly bending downwards from the straight line PQ .

Fig. 2, which illustrates the type of result obtained in my experiments of 1904, may serve also here to demonstrate the concordance with the theoretical type of Fig. 1.

Fig. 2. A, 5 % maltose. B, 10 % maltose.

All the estimations of the hydrolysis of maltose were executed with Fehling's solution by the gravimetric method of Kjeldahl [1895] for mixtures of sugars.

These experiments with different concentrations of maltose showed further, that the positions of the equilibrium points, i.e. the points of flexure, where the curves become approximately straight lines, were connected together as these figures are in a balanced action between a monomolecular and a bimolecular reaction.

Following up the conception, that an enzyme particle extends its catalytic action in a sphere as regards both the reverse action and the hydrolysis of maltose, and that the size of this sphere in sufficiently concentrated solutions must be inversely proportional to the sum of the concentrations of maltose and glucose (the concentration of glucose multiplied by the absorption coefficient n), the complete formula for the velocity of action of maltase on maltose in conditions, where the secondary change of glucose is not yet perceptible, is

$$-dx = m \left\{ \frac{x - q(a-x)^2}{x + n(a-x)} \right\} dt.$$

After substituting again $\frac{a-x}{a} = y$, the equation, relating the equilibrium points (where $-\frac{dx}{dt} = 0$) with the initial maltose concentration, is:

$$1 - y - qay^2 = 0 \dots\dots\dots(E).$$

For a solution of 10 grammes maltose in 100 c.c. for instance, the experiment gave about $y = 0.15$, from which followed $q = 4$.

The other experiments with concentrations of 7, 5 (see Fig. 2), 3 and 1 g. for 100 c.c. agreed then with the figures, calculated from equation (E).

<i>a</i>	<i>y</i>
10	0.15
7	0.17
5	0.20
3	0.25
1	0.39

The synthesis of bihexoses in the living cell and the indications of synthesis *in vitro*, however slight they may be, suggest the conclusion, that the transformation of enzyme-made glucose to stable glucose is in fact also a balanced action, in which the equilibrium is far on the side of stable glucose.

In enzyme actions like this one, where the real equilibrium is so soon attained and the reverse action is thus vigorously hindering the hydrolysis, it is clear, that addition beforehand of the product of the reaction may retard more than that of any other compound of the same kind. The conversion of the unstable enzyme-made glucose is retarded considerably by the added glucose and with that the reverse action can assert itself.

For maltose I found a much larger retardation by glucose than by galactose and levulose.

Hence there is in fact no discrepancy between the peculiar retardation effects of foreign substances on the action of invertase and lactase on one side and the general rule, applied with success by ter Meulen [1905], on the other, that the enzymatic hydrolysis of a glucoside is most retarded by the same sugar, which is split off from the glucoside. The diagrams, representing the rates of hydrolysis of many of these glucosides, have the same character as that of maltose-inversion. Here also the reverse action would soon stop the further splitting of the glucoside, if the sugar, which is formed, were not liable to a secondary change, which change is effectively retarded by addition of a large amount of the stable form of the same sugar.

REFERENCES.

- Armstrong (1904), *Proc. Roy. Soc.* **73**, 500.
 Barendrecht (1904), *Zeitsch. physikal. Chem.* **49**, 456.
 — (1906), *Zeitsch. physikal. Chem.* **54**, 367.
 Brown (1902), *J. Chem. Soc.* **81**, 373.
 Croft Hill (1898), *J. Chem. Soc.* **73**, 634.
 Duclaux (1898), *Ann. Inst. Past.* **12**, 96.
 Henri (1901), *Compt. rend.* **133**, 891.
 Hudson (1908), *J. Amer. Chem. Soc.* **30**, 1564.
 Kjeldahl (1895), *Compt. rend. Carlsberg*, **4**, 329.
 O'Sullivan and Tompson (1890), *J. Chem. Soc.* **57**, 865.
 Rosenthaler (1908), *Sitzungsber. K. Preuss. Akad. Wiss.* **20**.
 Sørensen (1909), *Compt. rend. Carlsberg*, **8**, 120.
 Tanret (1905), *Bull. Soc. Chim.* (3), **33**, 337.
 ter Meulen (1905), *Rec. Trav. Chim.* **24**, 444.