[Contribution from the Chemical Laboratory of Johns Hopkins University.]

ON CATALYTIC REACTIONS INDUCED BY ENZYMES.[1]

(NINTH[2] COMMUNICATION ON CATALYSIS.)

By S. F. Acree.

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The very important work in the two recent articles by Hudson[3] on "The Inversion of Cane Sugar by Invertase" harmonizes completely with the theory of the action of enzymes advanced by Bredig,[4] Kastle,[5] Acree,[6] Euler[7] and others. This theory postulates that the addition of an enzyme to a reacting system brings about a catalytic reaction because the enzyme unites with some constituent (called a "substratum") of the reacting system, and forms a new compound which furnishes the same end products more (or less) readily than does the substratum. This hypothesis is practically identical with that advanced first by Kastle,[6] and especially by Bredig[8] and Lapworth[9] to account for the catalysis of esters, amides and sugars, the formation of acetoacetic ester and analogous condensations, the benzoin formation, etc., by hydrogen, hydroxyl, alcoholate, cyanide, and other ions, a theory which has been verified and broadened not only by the originators, but by the work of Acree,[10] Goldschmidt[11] and Stieglitz[12] in the field of organic chemistry;

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[1] We are being aided in our work on catalysis by grants from the Carnegie Institution of Washington.

[2] Acree and Hinkins, *Am. Chem. J.*, **28**, 370; *Dental Cosmos*, June, 1901. Acree and Johnson, *Am. Chem. J.*, **37**, 410; **38**, 258. Acree and Nirdlinger, *Ibid.*, **38**, 489. Acree, Johnson and Nirdlinger, *Ibid.*, **38**, 746; **39**, 544. Acree, *Ibid.*, **39**, 300.

[3] This Journal, **30**, 1160, and Dr. Hudson very kindly sent me the manuscript of his second article, which appears in this number of this Journal.

[4] See especially *Ergebnisse der Physiologie*, Vol. I, p. 134 (1903); *Biochemische Ztschr.*, **6**, 283, and *Ber.*, **41**, 740, 752, for references to Bredig's articles.

[5] See *Proc. Am. Assn. Adv. Sci.*, **47**, 238, for an abstract of a paper read before the Association in Boston, 1898; the original manuscript of this article was kindly loaned to me by Professor Kastle. See also Kastle and students, *Am. Chem. J.*, **24**, 491; **26**, 518; **29**, 397, 563; **31**, 521; **32**, 376.

[6] *Loc. cit.*

[7] *Z. physiol. Chem.*, **52**, 146; *Z. physik. Chem.*, **36**, 405, 663; **40**, 501; **47**, 356.

[8] *Loc. cit.* Especially, see *Z. Elektrochem.*, **9**, 118; **10**, 586; **11**, 528.

[9] Mellor's *Statics and Dynamics*, p. 289. See *J. Chem. Soc.*, **93**, 30, for a résumé of the literature by Lapworth and his students. *Ibid.*, **93**, 85; *Chem. News*, **97**, 248, 309. See also an article by Acree on the formation of ethylacetoacetate, and on its reactions, which will appear soon in the *American Chemical Journal.*

[10] *Loc. cit.* *Am. Chem. J.*, **37**, 71; Acree and Shadinger, *Ibid.*, **39**, 124. Acree, Johnson, Brunel, Shadinger and Nirdlinger, article on tautomeric compounds read before the Am. Chem. Soc., Jan. 1, 1908, and soon to appear in the *Berichte.*

[11] *Ber.*, **39**, 711; *Z. physik. Chem.*, **60**, 728. Goldschmidt and Brauer, *Ber.*, **39**, 97, 109. Goldschmidt and Reinders, *Ibid.*, **29**, 1369, 1899. For a case of negative catalysis through salt formation see Goldschmidt and Oslan, *Ibid.*, **32**, 3390.

[12] *Congress of Arts and Science, St. Louis*, 1904, **4**, 276. *Am. Chem. J.*, **31**, 458; **39**, 29, 166, 402, 437, 586, 719.

and similar work has been done by Miller[1] and his students, Bray,[2] Abel,[3] and others in the inorganic field.[4] This theory promises to be, with necessary modifications, a good working basis for further investigations.

The writer and Hinkins obtained results somewhat similar to Hudson's in the study of the action of enzymes on triacetyl glucose. Part of these results have been published and part are being held for further amplification; our evidence harmonizes in the main with that of Hudson. But Hudson's work is of very great importance chiefly because by making the first deep and exhaustive study of the underlying mechanism of the reaction, the mutarotation of the glucose and fructose formed in the inversion, he gives us further evidence for what will probably prove ultimately to be the correct conception of enzyme reaction, *viz.*, the pure chemical theory of enzyme catalysis *vs.* the vital-energy theory; that is, the theory that the enzyme has stored up in it a vital energy which is given up to the reacting system and, perhaps after transformation, causes an acceleration of the reaction just as do heat, light, etc.

This theory of catalysis through the formation of reactive intermediate compounds between the enzymes and the substratum of sugar, ester, etc., harmonizes with the facts and clears up the mystery of catalytic phenomena. It is thought by many that enzymes have the same amide groups, -CONH-, that are contained in large amounts in all proteid substances. If such is the case the enzymes must have both basic and acid properties or must be amphoteric compounds. The hydroxyl and carbonyl groups of the sugars and esters have been proved to have both basic and acid properties by the beautiful work of Baeyer and Villiger,[5] Collie and Tickle, Walker, Rosenheim, Euler, and others. If such is the case, all of the conditions necessary for the formation of such double compounds between the sugar or ester and the enzyme are present. These weak bases and acids form salts, as has been proved in so many cases. On the other hand, it may be that the enzyme also unites with the sugar because a carbonyl or other active group takes up a hydroxyl group of the sugar and forms an acetal compound which undergoes inversion

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[1] *J. Physic. Chem.*, **11**, 9, 54, 292; **12**, 1, and many other papers.

[2] *Ibid.*, **7**, 92. *Z. phys. Chem.*, **54**, 463, 569, 731. Dissertation, Leipzig, 1906.

[3] *Z. Elektrochem.*, **13**, 555. *Sitzungsber. kais. Akad. Wiss., Wien*, **116**, July, 1907.

[4] Professor Bredig has written me that he has never believed in the three so-called laws of catalysis. This is evident from his discussion in Spiro and Ascher's *Ergebnisse der Physiologie*, which Professor Bredig has kindly called to my attention, although the subject is not presented there with the detail given in the "Studies in Catalysis," by Acree and Johnson. It is a pleasure to me to make the above correction with the permission of Professor Bredig. Professor Lapworth has sent me extracts from his note-book for 1904, which were the basis of the articles giving him priority, with Bredig, in the present-day views of catalysis.

[5] For a general résumé of the literature see Acree and Johnson, *Am. Chem. J.*, **38**, 258, etc.

very easily. Whatever the nature of the *active molecules* it is easy to connect the facts with the theory by simply assuming the mass law to hold for the hydrolysis and ionization of the salt, or to hold for the formation of the active molecules.

If the sugar and enzyme in the concentrations C_{su} and C_e react as weak base and weak acid respectively and form a trace of salt, the concentration of the undissociated portion of which is $C_{saltund}$, then when equilibrium is established, the following equation holds *approximately* as has been developed in earlier papers:[1]

$$C_{su} \times C_e = (K\, C_{sucathion} \times C_{eanion} \times C_H \times C_{OH} =)\, K'C_{saltund.} \qquad (1)$$

If the equilibrium is established practically instantaneously, as is the rule in the neutralization of a base by an acid, and the *undissociated* salt is the substance inverted slowly according to the equation (2)

$$C_{saltund} + H_2O \rightarrow \text{enzyme} + \text{glucose} + \text{fructose}, \qquad (2)$$

then the reaction should follow the equation (3),

$$dx/dt = K_{trans}C_{saltund} \times C_{H_2O} = K'_{trans}C_{su} \times C_e \times C_{H_2O}, \qquad (3)$$

which is actually found to hold experimentally. On the other hand, *if* $C_{saltund}$ *is formed slowly and decomposes very rapidly* equation (3) still holds approximately, whether equation (1) be reversible or not.[2] The same reasoning will apply to the assumption of the inversion of *any kind of double compound* formed by one molecule each of cane sugar and enzyme according to the above equations: if n molecules of enzyme and m molecules of cane sugar form a trace of intermediate compound according to the above equations, then the reaction velocity will be proportional to the n and m powers, respectively, of the concentrations of the enzyme and cane sugar.[3] It is obvious, from the above equations and from the fact that invertase is a very weak acid and that the very weakly acid invertase used by Hudson is far more active than acids, that the sugar *cathion* $C_{12}H_{22}O_{10}.H$, which is the real substance hydrolyzed when cane sugar is inverted by acids, is probably not greatly concerned in the reaction. The significant fact that invertase seems to have a maximum activity in weakly acid solutions, makes it possible that on intermediate compounds formed by the acid, sugar and enzyme may be concerned to some extent. When the enzyme, enzyme-substratum, etc., are present in a second phase, the equations are more complicated, and this may account for the fact that other workers have found some

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[1] *Am. Chem. J.*, **37**, 71, 410; **38**, 258, *et seq.*; *Ber.*, **41**, heft 13.

[2] Acree and Shadinger, *Am. Chem. J.*, **39**, 237–248. Wegscheider, *Z. physik. Chem.*, **36**, 543; *Z. Elektrochem.*, **14**, 133.

[3] See Acree and Johnson, *Am. Chem. J.*, **38**, 349, *et seq.* Acree, *Ibid.*, **39**, 303, foot-note.

reactions of enzymes not to follow simple laws:[1] their solutions were in some cases heterogeneous.

It might be thought by some that a solution containing only a *trace* of the salt or double compound could not possibly yield the end products so rapidly. We have only to recall that in the hydrolysis of cane sugar and esters by acids only a *trace*[2] of the sugar cathion or ester cathion can be present in the solution at any moment. We have only to recall further that solutions of such double compounds as silver potassium cyanide, silver ammonium chloride, cadmium potassium cyanide, and a host of others yield only minimal concentrations, of the order 10^{-15}, of the metallic cathions, yet these compounds react with the only slightly dissociated hydrogen sulphide *practically instantaneously and completely.* Again, it was shown by Acree and Johnson[3] that acetylbromaminobenzene is catalytically changed practically instantaneously into *p*-bromacetanilide by hydrobromic acid because the hydrobromic acid and the weak base, acetylbromaminobenzene, form a *trace* of the corresponding hydrobromide, the *undissociated* form of which rearranges.

$$CH_3CONBrC_6H_5 + H^+ + Br^- \rightleftarrows CH_3CONHBr_2C_6H_5 \rightarrow CH_3CONHC_6H_4Br + H^+ + Br^-.$$

In fact all ionic reactions are due to a nearly instantaneous preliminary chemical change of undissociated or neutral components into ions. In the neutralization of a solution of ammonia with one of hydrochloric acid, for example, the neutral components NH_3 and NH_4OH, the chief substances present, must undergo nearly instantaneous change into NH_4^+ and OH^- ions. Such examples could be multiplied indefinitely.

On account of the great activity of the invertase and the precision with which the rate of its action on cane sugar can be measured, the methods used by Hudson should lead to a solution of the very important question as to *what are the correct units of concentration!*[4] Fortunately, most of our reactions are studied with solutions so dilute that the errors which may be involved are such as to make this question of no great importance in such cases. On the other hand, since thermodynamically the reaction velocity must be a simple function of the osmotic pressure, the greatest need is felt for the study of this problem with concentrated solutions. Caldwell,[5] working with concentrations not greater than normal, obtained fair proportionality between the reaction velocity and the concentration of the cane sugar in the inversion of cane sugar by acids when he used the weight-

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[1] See Arrhenius, *Immunochemie.* Acree, *Am. Chem. J.*, **39**, 308.

[2] Acree and Johnson, *Am. Chem. J.*, **38**, 297, 304, 334. See Bredig and Stieglitz, *loc. cit.*

[3] *Am. Chem. J.*, **37**, 410; **38**, 265, etc.

[4] See an article on tautomerism by Acree and collaborators, *Ber.*, **41**, 3199.

[5] *Proc. Royal Soc.*, A, **78**, 272 (1906).

normal system of Morse and Frazer. Lewis[1] has pointed out recently, however, that Raoult's formula for the osmotic pressure still remains the only one which holds in all of the concentrations measured directly and indirectly, from the lowest to the highest. If we examine Caldwell's data we find that his constants calculated on the basis of Morse and Frazer *increase* from 504 to 521 with increase in the concentration of the cane sugar from 0.25 N to 1.0 N. Since each successive increase in the concentration of the cane sugar produces a smaller increase in the volume of the solution it is probable that, if the proper data were applied, Caldwell's experiments would give as good, or better, constants on the basis of Raoult's formula as on that of Morse and Frazer. Since invertase is very active the solutions are comparatively dilute with respect to this, in most cases, and hence the chief factors to be considered are the changes in the concentrations of the water and cane sugar.

These facts are sufficient to show that in the future we shall probably find that all organic reactions, including the above catalytic reactions by enzymes, acids, bases, etc., are at bottom essentially like the inorganic reactions. That is, the velocity of the reaction depends simply upon the affinity and equilibrium constants and velocity constants governing the various substances present (often only in traces but at other times in large quantities) and their changes. Whether ions or neutral molecules, or both, yield the end products in a given reaction depends upon these same factors, which may vary enormously in magnitude in the different cases.[2]

These facts make unnecessary the assumption of a *vital energy* as a basis for the catalytic reactions of enzymes, an assumption made not only by some biologists but discussed also by chemists.[3] The chemical theory explains all the known facts sufficiently well; the vital theory is not only unnecessary but it also fails to harmonize with some known facts, and there is no corroborative evidence that these traces of enzymes have stored in them the energy necessary to bring about these reactions. O'Sullivan and Tompson showed that invertase can catalyze at least one hundred thousand times its own weight of cane sugar without losing its reactivity to a measurable extent.[4] If we assume a vital energy in the enzyme as the cause of the increase in the velocity of the reaction invertase would be compelled to give off as much energy perhaps as radium. Although the question could and should be tested further experimentally the discoveries of the last few years in the realm of catalytic reactions induced by enzymes, acids, bases, etc., have removed the doctrine of *vital energy* beyond the

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[1] THIS JOURNAL, 30, 668.

[2] *Am. Chem. J.*, 38, 348, *et seq.*; 39, 226; 39, 527.

[3] *Z. Electroch.*, 12, 605. Dietz, Dissertation, p. 44, Strassburg, 1907. O'Sullivan and Tompson, *J. Chem. Soc.*, 57, 835. Roberts, Lumlian Lectures, 1880.

[4] *J. Chem. Soc.*, 57, 927.

reasonable limits of possibility. He who would revive this creed must offer new facts.

[FROM THE CHICAGO LABORATORY OF THE REFEREE BOARD OF CONSULTING SCIENTIFIC EXPERTS TO THE U. S. DEPARTMENT OF AGRICULTURE,
NORTHWESTERN UNIVERSITY MEDICAL SCHOOL.]

THE ESTIMATION OF UREA IN URINE.

STANLEY R. BENEDICT AND FRANK GEPHART.

Bunsen[1] showed many years ago that if solutions of urea are heated to high temperatures the urea is converted entirely into ammonia and carbon dioxide. Many methods for the estimation of urea have been proposed which are based upon this fact. In the process for urea estimation most commonly employed at the present time, *viz.*, that of Folin,[2] the decomposition is secured through a high temperature obtained by boiling the solution after the addition to it of a considerable quantity of magnesium chloride. The digesting mixture is kept neutral or slightly acid through the frequent addition of small quantities of hydrochloric acid.

Where a number of decompositions are to be carried out simultaneously, the technique involved in Folin's method is laborious, and the results sometimes uncertain, owing to the difficulty of keeping conditions entirely uniform as to temperature and acidity. The employment of a magnesium salt in a method involving the distillation of ammonia from the mixture is unfortunate. Magnesium chloride is seldom obtained free from ammonia and is so hygroscopic that difficulty in uniform sampling introduces a distinct source of error. Kober[3] has recently called attention to the great difficulty of completely distilling ammonia from solutions containing magnesium salts. The distillation in Folin's process must be prolonged and carried almost to dryness, yet if continued too far, some of the excess of magnesium chloride is decomposed, with the liberation of acid, thus ruining the determination. When carried out with due regard to securing exactly similar conditions, Folin's process yields very uniform results.

The following method for urea estimation has been thoroughly tested for several weeks during the course of some nutrition experiments carried out in this laboratory. It permits of thirty or more decompositions being carried out simultaneously, as readily as one involves no special technique, shortens the time of subsequent distillation, and secures uniform conditions for the digestions. The procedure is as follows: Five

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[1] Bunsen, *Ann.*, **65**, 375.

[2] Folin, *Z. physiol. Chem.*, **32**, 504 (1901); **36**, 333 (1902); **37**, 548 (1903). Also *Am. J. Physiol.*, **13**, 45-47.

[3] Kober, THIS JOURNAL, **30**, 1279 (August, 1908).