LIV. SOME ENZYMES IN *B. COLI COMMUNIS* WHICH ACT ON FUMARIC ACID.

By BARNET WOOLF (*Beit Memorial Research Fellow*).

From the Biochemical Laboratory, Cambridge.

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It was shown by Quastel and Woolf [1926] that when fumaric acid and ammonia are incubated with a suspension of *B. coli communis* at p_H 7·4 in presence of an "inhibitor," such as 2 % propyl alcohol, there is a disappearance of free ammonia from the solution. Similarly, when *l*-aspartic acid is incubated under the same conditions, it is partially deaminated, and the time-course and final values of the ammonia uptake or output correspond with those required for the reversible reaction

$$COOH.CH=CH.COOH+NH_3 \rightleftharpoons COOH.CH_2.CH(NH_2).COOH$$

where the molecular equilibrium constant $K=\frac{[\text{Fumaric acid}]\,[\text{Ammonia}]}{[\text{Aspartic acid}]}$ has a value of about 0·04. In the absence of "inhibitors," there is also, under anaerobic conditions, an irreversible complete deamination of the aspartic acid, with production of succinic acid. It was shown by Cook and Woolf [1928] that the mechanism responsible for this reductive deamination is independent of the enzyme governing the aspartic-fumaric equilibrium, since it is present in strict aerobes and strict anaerobes, which do not bring about the latter reaction. When equilibrium has been attained, both aspartic acid and fumaric acid can be isolated, but, while the yields of aspartic acid agree satisfactorily with the theoretical, the yields of fumaric acid are invariably very low.

The presence in animal tissues of an enzyme capable of forming malic acid from fumaric acid was shown by Batelli and Stern [1911], who named it "fumarase." Einbeck [1919] reported that the reaction stopped when about three-quarters of the fumaric acid had been converted, and Dakin [1922] confirmed this and proved that the malic acid produced was exclusively the *laevo*-form. Clutterbuck [1927] followed polarimetrically the production of *l*-malic acid from fumaric acid in presence of muscle, and found that the reaction followed a linear course until the equilibrium was nearly reached, when it slowed down. Alwall [1929] completed the proof that a true chemical equilibrium was involved by showing that the same final state was reached when muscle tissue was allowed to act on *l*-malic acid.

The presence of fumarase in *B. coli* was reported by Quastel and Whetham [1924]. It is obvious that if this enzyme were present in the bacillus in any

considerable quantity it would interfere with the aspartic-fumaric equilibrium, giving a complex malic-fumaric-aspartic equilibrium as the final state. Quastel and Whetham, however, found that when fumaric acid was incubated anaerobically with relatively large concentrations of organisms, only minute quantities of malic acid could be detected. They therefore concluded that there was only a small quantity of fumarase in *B. coli*, and in the previous work on the aspartic-fumaric equilibrium this conclusion was accepted, and it was taken for granted that the production of malic acid was so slow as not to interfere appreciably with the main reaction. It will be shown in this paper that the interpretation given by Quastel and Whetham of their results was erroneous, though justified by the facts known at the time they did their work; that, in fact, *B. coli* shows a very high fumarase activity; and that the equilibrium constant previously reported for the aspartic-fumaric equilibrium is really that of the complex malic-fumaric-aspartic equilibrium. It will also be shown that malic acid itself does not take up ammonia, that the addition of water and of ammonia to fumaric acid is due to two distinct enzymes, and that it is possible to eliminate the fumaric-malic reaction and so obtain the true constant of the equilibrium between aspartic acid, fumaric acid and ammonia. The bearing of these results on the "active centre" hypothesis of Quastel and Wooldridge will be discussed.

Experimental.

All the work described was done with suspensions of "resting" *B. coli communis*. The organism was grown, either in Roux bottles containing 150 cc. of Cole and Onslow's tryptic broth, or on the surface of tryptic broth agar in Petri dishes. There was no apparent difference in the behaviour of the organisms obtained by the two methods. In each case, the nutrient medium was inoculated from an 18 hours old broth culture, and incubated at 37° for 2 days. When agar plates were used, the growth was washed off with normal saline and centrifuged; when broth was used, this was centrifuged from the organisms. The deposit of *B. coli* was then washed three times by centrifuging in normal saline, and finally suspended in saline and aerated for a few hours. It was stored at 0°, and, although generally used fresh, did not lose its activity after several months. The growth from one Roux bottle or one Petri dish generally corresponded to about 10 cc. of the suspension.

The following stock solutions were used: $M/2$ sodium fumarate, $M/2$ sodium *l*-aspartate, $M/2$ sodium *l*-malate, and M ammonium chloride. The organic acids were weighed out and neutralised with sodium hydroxide, and all solutions were brought to p_H 7·4. The buffer used was Clark and Lubs's phosphate buffer, p_H 7·4, containing $M/20$ phosphate. Ammonia was estimated on 0·5 cc. samples by the method of Woolf [1928]. Malic acid was estimated polarimetrically as the molybdate compound, by the method of Auerbach and Kruger [1923]. A 5 cc. sample was added to 10 cc. of 14·2 % ammonium molybdate, then 1 cc. of glacial acetic acid was added, and the mixture was

allowed to stand a few hours in the dark. It was then filtered through kieselguhr, exactly 5 cc. of water being used for wetting the filter-paper and washing, so that the total volume of fluid used was 21 cc. The presence of the molybdate helped to precipitate the bacteria, so that a crystal-clear filtrate was obtained. Another 5 cc. sample was added to 11 cc. of 6 % trichloroacetic acid, which was filtered in the same way with the addition of 5 cc. of water. The trichloroacetic acid acted as a protein precipitant and produced about the same degree of acidity as that due to the acetic acid in the molybdate mixture. The two solutions were examined polarimetrically in a 2 dm. tube, and the difference in rotation was proportional to the *l*-malic acid present. It was found that 10 mg. of malic acid, under these conditions, gave a rotation difference, with light from the mercury green line, of + 0·84°. This agrees with the value given by Needham [1927]. All the reaction mixtures were incubated at 37°, the mixtures without inhibitor being contained in filter-flasks evacuated at the water-pump, while those with inhibitor were placed in stoppered flasks, it having been previously ascertained that there was no difference in the course of the reaction anaerobically and aerobically.

Results.

The result of a typical experiment demonstrating the fumarase activity of the organism is shown in Fig. 1. The following reaction mixtures were made up, each containing in addition 50 cc. buffer solution and 2 cc. of *B. coli* suspension.

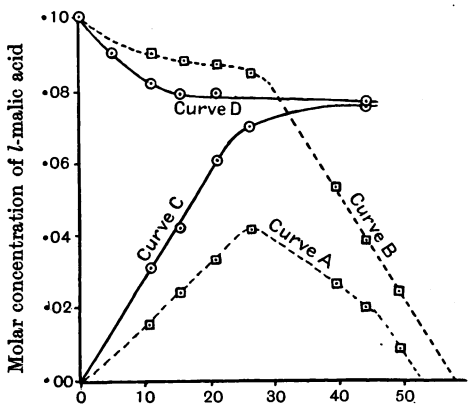

Fig. 1.

Mixture	*M*/2 fumaric cc.	*M*/2 *l*-malic cc.	Propyl alcohol cc.	Water cc.
A	20	—	—	28
B	—	20	—	28
C	20	—	2	26
D	—	20	2	26

Initial malic acid estimations were made, the mixtures were incubated, *A* and *B* being anaerobic, and at suitable intervals further samples were taken for estimation.

Curves *C* and *D* show that in presence of 2 % propyl alcohol an equilibrium is reached when about 76 % of the fumaric acid is converted into malic acid, or 24 % of the malic to fumaric. This equilibrium value agrees with that found by many workers for muscle. The initial portion of curve *C* is linear, as found by Clutterbuck [1927, 1928] for the fumarase of muscle and liver, which suggests that the enzyme is saturated with its substrate for nearly the whole course of the reaction. Curves *A* and *B* show that in the absence of an inhibitor the fumaric-malic equilibrium is masked by some other irreversible reaction which results in the destruction of the malic acid, and follows a linear course. The final portion of curve *A* is invariably parallel to the linear part of curve *B*, but sometimes the curves cross before this part is reached. The same phenomenon is observed in the absence of inhibitors for the aspartic-fumaric

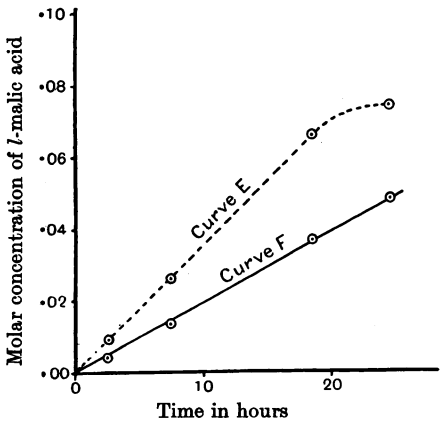

Fig. 2.

equilibrium [cf. Quastel and Woolf, 1926, figs. 1 and 2; Cook and Woolf, 1928, fig. 1], and, as in these cases, the chemical change involved is no doubt a reduction to succinic acid of at any rate part of the malic acid. The matter is under investigation. The figure also shows that the presence of propyl alcohol increases the velocity of the fumarase action. This activation is not always observed.

It was shown by Clutterbuck [1928] that phosphates had an activating action on animal fumarase. This has been confirmed for the enzyme of *B. coli*, as shown by Fig. 2. Curve *E* was obtained with a mixture similar in composition to mixture *C* of Fig. 1, and for curve *F* the buffer was replaced by water. The figure shows that the presence of *M*/40 phosphate at p_H 7·4 almost doubles the reaction velocity. All the other reaction mixtures used in this work contained half their volume of buffer solution.

It is now possible to explain the results of Quastel and Whetham [1924]. Their reaction mixture was similar to that used for curve *A*, save that they used a much higher concentration of *B. coli*. When they examined their solution, at the end of 24 hours' incubation *in vacuo*, it had probably reached the final part of curve *A*, and most of the malic acid previously formed had disappeared, thus leading them to believe that there was very little fumarase in the organism.

Having established the presence in *B. coli* of considerable quantities of fumarase, it became necessary to re-examine the supposed aspartic-fumaric equilibrium. A typical result is shown in Figs. 3 and 4. The reaction mixtures were:

Mixture	*M*/2 *l*-aspartic cc.	*M*/2 fumaric cc.	*M* NH_4Cl cc.	Propyl alcohol cc.	Water cc.
G	20	—	—	2	26
H	—	20	10	2	16
K	—	20	—	2	26

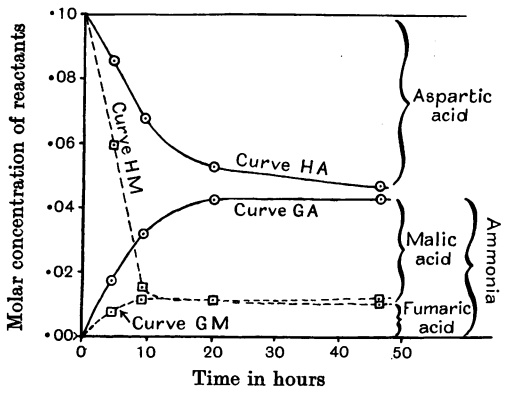

Fig. 3.

Ammonia and malic acid estimations were made on *G* and *H* and malic acid estimations on *K*; the solutions were then incubated and the estimations were repeated at suitable intervals. The results with *G* and *H* are shown in Fig. 3. Curves *GA* and *GM* represent the changes that take place when *M*/10 aspartic acid is incubated with the organism. The distance between *GA* and the base-line represents the molar concentration of free ammonia, found by estimation. This will of course be equal to the sum of the concentrations of fumaric and malic acids. The distance between curves *GA* and *GM* represents the concentration of malic acid, as found by estimation. Hence, by difference, the distance between *GM* and the base-line gives the amount of fumaric acid present, while the aspartic acid concentration is equal to the distance between *GA* and the horizontal line at the top of the figure. Similarly, for the other pair of curves, *HA* denoting free ammonia in *H* and the distance between *HA* and *HM* the malic acid. It will be seen that the ammonia curves *GA* and *HA* are similar

to those given by Quastel and Woolf [1926], but that, at equilibrium, the fumaric acid concentration is only about a quarter of the ammonia concentration, the remaining three-quarters having changed to malic acid, in accordance with the requirements of the fumaric-malic equilibrium.

Fig. 4 is strong evidence that the addition of water and of ammonia to fumaric acid is the work of separate enzymes. It is conceivable that the two reactions could be effected by a single enzyme, which activates fumaric acid, possibly on the lines suggested by Quastel [1926], the activated fumaric acid then "accepting" either water or ammonia, in the form of their ions, which do not need "activation" (Quastel). If this were the case, and one started with a system containing the enzyme and fumaric acid only, then the rate at which malic acid was formed would be governed by the speed with which the

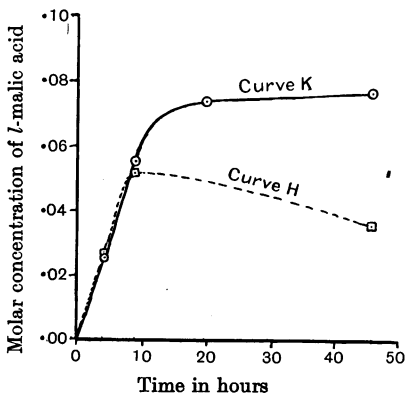

Fig. 4.

enzyme could activate fumaric acid. Now, if ammonia were added to the system, some of the fumaric acid which would have yielded malic acid would be diverted to form aspartic acid; there would be competition between water and ammonia for the activated fumaric acid. On the other hand, if two distinct enzymes were involved, then, so long as the fumaric acid was present in sufficient concentration to saturate the fumarase, the addition of ammonia would not affect the rate of formation of malic acid. Fig. 4 shows that this is in fact the case. Curve *H* shows the rate of malic acid formation from *M*/10 fumaric acid in presence of *M*/10 ammonia, *i.e.* the ordinate at any time equals the distance between curves *HA* and *HM* in Fig. 3. Curve *K* shows the production of malic acid in mixture *K*, which contains no ammonia, and it is clear that the initial rates of malic acid production in the two mixtures are identical.

When malic acid and ammonia are incubated with *B. coli* the final state is the same as that shown in Fig. 3, but the ammonia uptake is slower than with fumaric acid. It is, of course, possible that malic acid goes directly to aspartic acid without passing through the stage of fumaric acid. That this is unlikely is shown by Fig. 5. Mixture *L* contains *M*/10 fumaric acid and *M*/10

ammonia, together with a very small quantity of organism, while in mixture *M*, malic acid is substituted for the fumaric acid. The curves show the details of the beginnings of the ammonia uptakes. Fumaric acid immediately begins to react, but with malic acid there is a lag, indicating that fumaric acid must be formed before the ammonia uptake can begin.

The equilibrium constant K_1 for the reaction

$$COOH.CH = CH.COOH + H_2O \rightleftharpoons COOH.CH_2CH.OH.COOH$$

is given by the equation

$$K_1 = \frac{[\text{Fumaric acid}]\,[\text{Water}]}{[\text{Malic acid}]}.$$

The concentration of water can be taken as constant. Hence the ratio of fumaric acid to malic acid at equilibrium will be constant, whatever their initial concentrations may be. Now in the aspartic-fumaric equilibrium the constant K_2 is given by

$$K_2 = \frac{[\text{Fumaric acid}]\,[\text{Ammonia}]}{[\text{Aspartic acid}]}.$$

The value found for this constant by Quastel and Woolf [1926] was 0·04, but what they supposed was the fumaric acid concentration was really the sum of the fumaric and malic acid concentrations. The true concentration of fumaric acid is only a quarter of the value they used. It is this constancy of the

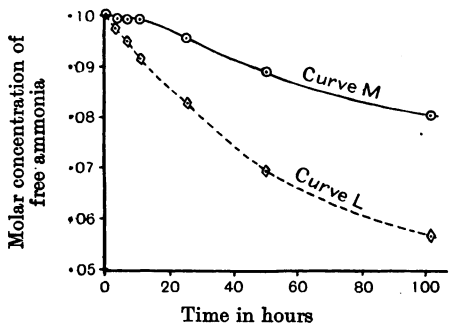

Fig. 5.

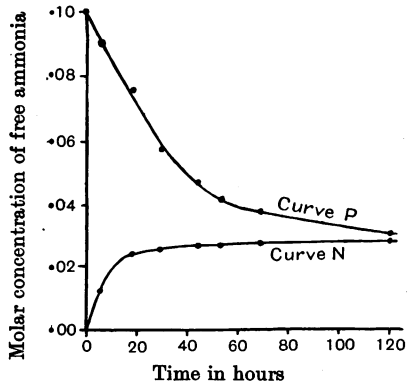

Fig. 6.

fumaric-malic ratio that enabled them to find the same value of K for the complex equilibrium when they varied the concentrations of their reactants. Substituting the true value of the fumaric acid concentration in the equation for K_2, one finds that K_2 is about 0·01. From this value it can easily be calculated that, if the fumaric-malic reaction could be entirely eliminated, the equilibrium mixture for the aspartic-fumaric equilibrium, when the reactants were present in the usual initial concentrations of *M*/10, would contain only about 28 % of free ammonia instead of the 47 % found for the mixed equilibrium.

Fig. 6 shows the result of an experiment in which these conditions are realised. The effect of cyclohexanol on the fumarase of *B. coli* is peculiar. If

the organism is incubated with 2 % cyclohexanol for an hour or two, the fumarase is markedly activated, but if the incubation is continued for 15 hours, the fumarase activity is sometimes found to have entirely disappeared. This is not always the case, and it is probable that the time required for the destruction of the enzyme varies for different strains and preparations of the organism. When the fumarase is destroyed in this way it is found that malic acid will show no ammonia uptake, but ammonia uptake by fumaric acid still occurs. In the experiment to be described, 20 cc. of stock *B. coli* suspension were added to 78 cc. of saline and 2 cc. of cyclohexanol, and the mixture was incubated for 15 hours, when the fumarase was found to be completely destroyed. The following reaction mixtures were then made up:

Mixture	*M*/2 *l*-aspartic cc.	*M*/2 fumaric cc.	*M* NH_4Cl cc.	Buffer cc.	Cyclo-hexanol cc.	Treated *B. coli* cc.	Water cc.
N	20	—	—	50	1·8	10	18·2
P	—	20	10	50	1·8	10	8·2

The mixtures were incubated, and ammonia and malic acid estimations made at intervals. The malic acid estimations were negative, and the ammonia results are shown in Fig. 6. It will be seen that the final value obtained is the true one for the aspartic-fumaric equilibrium.

This differential destruction of the mechanisms responsible for the two reactions is a further proof that two distinct enzymes are involved, and the absence of ammonia uptake by malic acid after the destruction of fumarase confirms the conclusion that malic acid itself is not converted into aspartic acid. It is convenient to have a name for the enzyme responsible for the aspartic acid reaction and it is proposed that it be provisionally termed *aspartase.*

It was found by Alwall [1928] that the fumarase in a succinoxidase preparation from muscle is destroyed by incubation at 50° for half an hour. Boiled *B. coli* shows no fumarase or aspartase activity, and preliminary experiments indicate that the inactivation temperature for each enzyme is in the neighbourhood of 50°.

Discussion.

B. coli communis is able to bring about several chemical reactions involving fumaric acid. One is the irreversible reaction shown in Fig. 1, which is inhibited by propyl alcohol. Possibly another is the oxidation of fumaric acid, though it seems probable that this may only take place *via* malic acid. Finally there are three reversible reactions: the fumaric-succinic-methylene blue equilibrium [Quastel and Whetham, 1924], and the equilibria governed by fumarase and aspartase. Each of these reactions seems to be catalysed by a distinct enzyme, which can effect one reaction only. Working with mammalian tissues, Alwall [1928] destroyed fumarase and left succinoxidase, and Clutterbuck [1928] destroyed succinoxidase and left fumarase. It has been shown in this paper that the fumarase of bacteria can be destroyed without elimination of aspartase. Quastel and Wooldridge [1927, 1] showed that the succinoxidase of

B. coli is destroyed by incubation with cyclohexanol for 5 minutes, whereas fumarase has been shown above to be far more resistant to this substance. They also showed that succinoxidase is little affected by one hour at 57°, a treatment that would destroy both fumarase and aspartase. Quastel and Wooldridge [1928] report that, after treatment with toluene, the affinity of the succinoxidase of *B. coli* for malonic acid is vastly increased, so that small concentrations have a large effect in retarding the reaction velocity. Experiments made on fumarase and aspartase show that they do not possess this property. Finally, repeated attempts have been made to demonstrate the presence of aspartase in muscle, with uniform lack of success, although the various preparations used showed marked fumarase activity.

It seems clear, therefore, that three reactions as similar as the addition across the double bond of fumaric acid of 2H, H and OH, and H and NH_2, require separate enzymes for their catalysis. Now according to the "activation" theory of Quastel [1926], as modified and extended by Quastel and Wooldridge [1927, 2] into the "active centre" theory, one would expect the essential happening in all these reactions to be the same, the activation of fumaric acid. The fumaric acid would be adsorbed or combined at an active centre, at which it would come under the influence of an intense electric field, becoming activated, so that its state could be diagrammatically represented as $COOH.CH_2.\check{C}.COOH$. The activated molecule would then react with ions from the solution, which do not need activation, and which would combine at the temporarily unsaturated or active carbon atom represented by the sign $\check{C}$. If this were the true mechanism, one would expect all three reactions to be effected by a single enzyme; or, if it were objected that the three reactions required fumaric acid activated to different extents, then it would be anticipated that the enzyme that could effect the most difficult of the three reactions —the one requiring the highest energy of activation or the most intense electric field—should also be able to bring about the other two reactions, while the enzyme catalysing the more difficult of these two reactions should also bring about the easier, and only the enzyme with the weakest field should be truly specific for one reaction only. But experiment shows that the contrary is the case; each enzyme is specific for one type of reaction. The differences in the enzymes seem not to be merely quantitative, as one would expect if the views of Quastel and Wooldridge were accepted as adequate, but there appear to be qualitative differences between them.

At the present stage it is only possible to give the merest indications of what the nature of these qualitative differences may prove to be. It will be noticed in curve *HA*, Fig. 3, that the initial portion is linear for a large portion of the reaction. This suggests that the enzyme is working at full saturation with its substrate. Now the reaction taking place involves two substances, fumaric acid and ammonia. If it were supposed that the ammonia were acting in solution, combining with activated molecules of fumaric acid at the enzyme when it came into contact with them by collision, then, by the law of mass

action, it would be expected that, as the ammonia concentration fell, the reaction velocity would fall proportionately, even although the enzyme was saturated the whole time with fumaric acid. One would only expect to get a linear reaction if the enzyme were saturated with both the reactants—fumaric acid and ammonia. It follows that one must suppose that ammonia is combined at the enzyme as well as fumaric acid. Similar considerations apply to the reduction of methylene blue by succinic acid in presence of bacteria. Quastel [1926] gives a figure (Fig. 1) showing that the rate of decoloration is linear during the greater part of the reaction. This implies that the enzyme is saturated with methylene blue, and therefore that there is combination between enzyme and dye. Further evidence leading to the same conclusion is furnished by the work of Dixon [1926] on xanthine oxidase and Quastel and Wooldridge [1927, 1] on several of the dehydrogenating enzymes of *B. coli*. These workers used various dyes of different reduction potentials, and found that the rate of reaction was not dependent merely upon the ease of reduction of the indicator. Dixon points out that the presence of sulphonic groups in the dye tends to slow its reduction rate. These facts suggest that the chemical nature of the dye affects its affinity for the enzyme, and hence its rate of reaction. Quastel and Wooldridge themselves state that "the velocity of reduction is governed by the concentration of indicator as well as by the concentration of activated donator at the surface," but they do not seem to mean that the combination between enzyme and indicator is of the Michaelis type, nor do they seem to the writer to bear this condition sufficiently in mind in the development of their theory.

These considerations, and similar ones applied to the facts known about some other enzymes, point to the rather attractive hypothesis that one of the conditions for a reaction to occur at these enzymes is that all the substrates shall be combined there together—succinic acid and methylene blue for succinoxidase action, fumaric acid and ammonia with aspartase, and fumaric acid and water with fumarase. Whether there is also activation by electric fields as Quastel postulates, or whether the mechanism by which reaction is effected is of a different nature, seems to the writer still a very open question. This hypothesis has at any rate the merit that it is capable of being tested on strictly quantitative lines, and it is hoped to carry out further studies on fumarase and aspartase with this object in view.

Summary.

1. The presence is demonstrated in *B. coli communis* of considerable quantities of fumarase, the enzyme governing the equilibrium between fumaric acid and *l*-malic acid. In the absence of inhibitors, such as 2 % propyl alcohol, the action of this enzyme is masked by an irreversible process resulting in the anaerobic destruction of malic acid. In presence of propyl alcohol the same equilibrium is attained as that given by animal fumarases.

2. The equilibrium previously reported between aspartic acid, fumaric acid and ammonia is really a complex malic-fumaric-aspartic equilibrium. The fumarase of *B. coli* can be destroyed by treatment of the organism with cyclohexanol, and then the true aspartic-fumaric equilibrium is obtained.

3. The three reversible changes involving fumaric acid—those to succinic, malic and aspartic acids—are catalysed by distinct enzymes. It is proposed to call the enzyme responsible for the third of these reactions *aspartase*.

4. The bearing of these results on enzyme theory is discussed, and a limited hypothesis on enzyme action is put forward.

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