LXXII. THE EQUILIBRIUM BETWEEN *l*-ASPARTIC ACID, FUMARIC ACID AND AMMONIA IN PRESENCE OF RESTING BACTERIA.

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It is an old observation that when fumaric acid, maleic acid or malic acid is heated in the presence of ammonia in a sealed tube to 200° a conversion to aspartic acid occurs. There has been no evidence, however, that such a conversion can take place under biological conditions. Experiments which will be described in this paper show that *l*-aspartic acid is formed in a solution containing sodium fumarate, ammonium chloride and resting bacteria, no amino-acid being synthesised in the absence of the bacteria. The yield of *l*-aspartic acid, obtained under certain conditions, has been as high as 60 % of the fumaric acid taken. A small amount of aspartic acid is synthesised from malic acid but none, under our experimental conditions, from maleic acid.

The results of experiments on oxidations, etc., in the animal body make it highly probable that the production of α -hydroxy-acids from α -amino-acids takes place *via* the α -ketonic acids. It will be shown in this paper, however, that aspartic acid in presence of resting bacteria rapidly liberates ammonia with the formation of fumaric acid. It is known that the latter slowly gives rise to malic acid (in presence of bacteria); hence it follows that aspartic acid presents an instance of an α -amino-acid proceeding to the corresponding hydroxy-acid *via* the unsaturated acid. Whether this is true in the animal body or with plant tissues still remains to be determined.

Raistrick [1917] showed that in the case of organisms of the *coli-typhosus* group histidine produces the corresponding unsaturated acid, urocanic acid. With aspartic acid, it will be shown, a similar reaction occurs reversibly, the following equilibrium being established:

and it is possible to obtain the equilibrium constant of this reaction. It is by no means a general phenomenon, however, that an α -amino-acid, in contact with *B. coli*, gives up ammonia to form the corresponding unsaturated acid. Under conditions such that aspartic acid readily loses ammonia to form fumaric acid, no liberation of ammonia has been observed to occur from

glutaminic acid or glycine. The latter amino-acids, doubtless, require oxidation before ammonia is set free.

It has been shown [Quastel and Whetham, 1924] that in presence of resting *B. coli* an equilibrium is established between succinic acid, fumaric acid, methylene blue and leucomethylene blue and also between fumaric acid and *l*-malic acid. Malic acid retards the reduction of methylene blue by succinic acid in presence of *B. coli* owing to the production of fumaric acid. Aspartic acid also retards the reduction of methylene blue [Quastel and Whetham, 1925] and it was this observation that made it seem possible that aspartic acid underwent a deamination to fumaric acid. That aspartic acid is a hydrogen acceptor has been shown, not only by experiments with methylene blue, but by Harden's observation [1901] that it is reduced completely to succinic acid in the presence of *B. coli* growing in a sugar solution and by the observation [Quastel and Stephenson, 1925] that it induces the anaerobic growth of *B. coli* upon glycerol, although neither aspartic acid nor glycerol alone can support the anaerobic growth of this organism.

Resting *B. coli* is prepared by growing the organism for two days on tryptic broth (in Roux bottles each containing 150 cc.), centrifuging and thoroughly washing with normal saline. The organism is made up to a thick emulsion and aerated for one hour. It must be used under such conditions that growth does not occur—otherwise the reactions it brings about may be due to changes consequent upon proliferation. Such conditions are usually easily secured if the experiments are carried out

- (a) anaerobically;
- (b) in a short time—usually under 30 minutes;
- (c) at a relatively high temperature—45°;
- (d) in the absence of a nutritive source of nitrogen among the substrates whose reactions are being investigated; and
- (e) by using, when possible, substrates which are known not to induce alone the anaerobic growth of the organism under investigation.

A mixture of sodium fumarate and ammonium chloride or one of sodium aspartate and ammonium chloride does not support the anaerobic growth of *B. coli* [Quastel and Stephenson, 1925], but it is quite possible in our experiments in the presence of these substances, even under strictly anaerobic conditions, where a relatively large amount of organism must be used, where the time of experiment is relatively long (two to seven days) and where the temperature of experiment is an optimum for growth, that a certain amount of growth will occur on the protein provided by the dead bacteria present in the emulsion of resting organism. Such a growth will mask any equilibrium owing to the fumaric or aspartic acid acting as a hydrogen acceptor and being converted to succinic acid. Hence the isolation of aspartic acid from fumaric acid and ammonia, or of fumaric acid from aspartic acid, is most satisfactorily accomplished in the presence of a suitable growth inhibitor, such as toluene or 2 % propyl alcohol. It will be shown later that these inhibitors do not

appreciably affect the velocities at which synthesis of aspartic acid or formation of fumaric acid occurs, whilst the formation of succinic acid is almost completely inhibited. In the absence of a growth inhibitor, but under anaerobic conditions, a synthesis of aspartic acid occurs within a short time and isolation may then be accomplished. If the mixture is left for a much longer period the aspartic acid disappears and gives rise to succinic acid. The most suitable time for isolation can be determined by ammonia estimations to be described later.

Preparation of the stock resting bacteria.

The crop of *B. coli* from twelve Roux bottles (*i.e.* 1800 cc. broth), after having been centrifuged, washed and aerated, is made up to 200 cc. with normal saline, and this suspension of bacteria kept as a stock preparation of the organism. It should be stored in the ice chest and exposed as little as possible to room temperature. Although the organism was generally used within a fortnight of its preparation, it will retain its activity apparently unimpaired for at least two months.

Isolation of aspartic acid in the absence of toluene.

One g. fumaric acid was neutralised with sodium hydroxide, mixed with 2 g. ammonium chloride and made up to 40 cc. with distilled water. To this were added 50 cc. phosphate buffer solution (Clark and Lubs) p_H 7.4, and 10 cc. of the stock emulsion of *B. coli*. The mixture was placed in a conical filtering flask, which was exhausted at the water-pump, filled with nitrogen and re-evacuated. The flask and its contents were then incubated at 37° for about 30 hours. After this period the contents of the flask were centrifuged and the supernatant liquid evaporated on the water-bath to low bulk. This solution was filtered from a little protein matter and the filtrate poured into 20 cc. of a hot saturated solution of copper sulphate. The mixture was boiled vigorously for a quarter to half an hour and filtered from the bulky precipitate. The clear deep blue filtrate was allowed to stand in the ice chest three days, when the crystallisation of the copper aspartate was complete. The latter was filtered off, washed well with cold distilled water, and dried in a desiccator over concentrated sulphuric acid. An average yield of 1 g. hydrated copper aspartate was obtained in this way from 1 g. fumaric acid. The copper salt was then suspended in boiling water and decomposed with H_2S . Aspartic acid was obtained from the filtrate in crystals with the characteristic pearly lustre.

Isolation of aspartic acid in presence of toluene.

The advantage of using an inhibitor such as toluene is that there is no fear of exceeding the time necessary for the optimum yield of aspartic acid, since no reduction to succinic acid occurs. Moreover, slightly better yields of the amino-acid are obtained.

Three g. fumaric acid were neutralised with sodium hydroxide, mixed with 6 g. ammonium chloride, and made up to 125 cc. with distilled water; 100 cc. phosphate buffer, p_H 7.4, 25 cc. of the stock suspension of *B. coli*, and 20 cc. toluene were added, and the mixture placed in a flask which was exhausted at the water-pump. Incubation was allowed to proceed at 37° for five days, after which time the isolation of aspartic acid through the copper salt was effected in the manner described above. The yield of hydrated copper aspartate was 3.5 g.

Identification of l-aspartic acid produced by synthesis.

For analysis of the copper salt, the hydrated copper aspartate obtained in the manner described was twice recrystallised from hot water and dried to constant weight over concentrated H_2SO_4 .

Found Cu = 25.8; N = 5.53 %.

Calculated for $C_4H_5O_4NCu \cdot 3H_2O$ Cu = 25.6; N = 5.63.

Aspartic acid produced from the copper salt by treatment with H_2S gave the following analytical figures:

Found C = 35.4; H = 5.20; N = 10.2 %.

Calculated for $C_4H_7O_4N$ C = 36.0; H = 5.29; N = 10.5.

The aspartic acid showed no definite melting point in an open tube, but in a closed capillary tube it commenced to melt at 272°.

0.0402 g. of the synthetic acid required 4.15 cc. of 0.0715 N NaOH for neutralisation, which is equivalent to 0.0395 g. aspartic acid.

0.1860 g. of the acid was dissolved in water, 0.5 cc. concentrated HCl added, the solution was made up to 25 cc. and examined polarimetrically in a 2 dm. tube. The rotation was + 0.41° (for the mercury green) giving $[\alpha]_{Hg\ green} = + 27^{\circ}.5$.

Isolation of fumaric acid from aspartic acid in presence of toluene.

Three g. l-aspartic acid were neutralised and made up to 50 cc. with distilled water; 175 cc. phosphate buffer solution, p_H 7.4, 25 cc. of the stock suspension of *B. coli* and 20 cc. toluene were added and the mixture was placed in a flask which was exhausted at the water-pump. Incubation was allowed to proceed at 37° for five days. The mixture was then made 10 % acid with sulphuric acid and thoroughly extracted with ether. The ether extract was poured through dry filter paper and evaporated. The residue was dissolved in water and to the solution a strong solution of silver nitrate was added. A heavy white precipitate of silver fumarate was formed which was filtered, washed and decomposed with H_2S . A yield of 0.55 g. fumaric acid was obtained in this way.

Identification of fumaric acid.

The acid decolorised potassium permanganate solution in the cold and was precipitated by silver nitrate from its aqueous solution.

In an open tube it did not melt at 200° but showed signs of sublimation; in a closed capillary tube it melted at 270°, its behaviour being unchanged by admixture with an authentic specimen of fumaric acid.

0.0330 g. of the acid required 7.9 cc. 0.0715 *N* NaOH for neutralisation, which is equivalent to 0.0327 g. fumaric acid.

Isolation of succinic acid.

1.3 g. *l*-aspartic acid was neutralised and made up to 40 cc. with distilled water; 50 cc. phosphate buffer solution, p_H 7.4, and 10 cc. of stock suspension of *B. coli* were added, and incubated *in vacuo* for a week at 37°, after which time the mixture was made 10 % acid with sulphuric acid and extracted with ether. The dried ether extracts gave a crystalline residue of succinic acid, no fumaric acid being detectable.

The succinic acid melted sharply at 187° (uncorr.), its behaviour being unaltered by admixture with an authentic specimen of succinic acid.

0.0185 g. of the acid required 4.30 cc. 0.0715 *N* NaOH for neutralisation, which is equivalent to 0.0181 g. succinic acid.

The acid had no decolorising effect on potassium permanganate solution.

THE EQUILIBRIUM BETWEEN *l*-ASPARTIC ACID, FUMARIC ACID
AND AMMONIA.

The most accurate method of determining the course of the reaction between fumaric acid and ammonia or of the breakdown of aspartic acid in presence of *B. coli* is to follow the absorption and elimination of ammonia. The technique adopted, therefore, consisted in estimating at frequent intervals the concentration of ammonia in a mixture which initially consisted of fumaric acid, ammonia and *B. coli* or of aspartic acid and *B. coli*. From the rates of variation of ammonia concentration in the two cases it will be possible to determine (1) whether an equilibrium exists between aspartic acid, fumaric acid, and ammonia and (2) the conditions affecting such an equilibrium.

The following stock solutions were prepared: *M*/2 sodium fumarate, *M*/2 sodium *l*-aspartate, *M* ammonium chloride.

The ammonia was estimated by a modification of the Van Slyke aeration method, the aeration being carried out for one hour in a boiling water-bath. The apparatus was arranged so that a steady stream of air (freed from ammonia by passing through sulphuric acid) could be drawn through the reaction tube, which contained the sample whose ammonia was to be estimated, into a known quantity of standard sulphuric acid contained in a 250 cc. flask. In the reaction tube were placed, in addition to the sample, 50 cc. distilled water, and 2 cc. liquid paraffin (to prevent frothing); 3 g.

potassium carbonate were then added, the apparatus was immediately connected up and a stream of air drawn through. After ten minutes the water-bath was brought to boiling and the aeration continued for an hour. The apparatus was then disconnected and the acid titrated back.

All control experiments (*i.e.* fumaric acid and ammonia in absence of organism, fumaric acid and organism in absence of ammonia, ammonia and organism in absence of fumaric acid, aspartic acid in absence of organism) gave negative results.

Fig. 1 shows typical curves of ammonia absorption by fumaric acid and of ammonia elimination by aspartic acid both in the absence and in the presence of inhibitors. The results are expressed for convenience in terms of molar concentrations. The initial composition of the mixtures used for the curves in Fig. 1 are expressed in Table I, the numbers representing cc.

Table I.

Curve	M/2 sodium aspartate	M/2 sodium fumarate	M/1 NH ₄ Cl	10 % NaNO ₂	Toluene	Propyl alcohol	<i>B. coli</i> suspension	Buffer solution pH 7.4
A	20	—	—	—	—	—	5	75
B	—	20	10	—	—	—	5	65
C*	20	—	—	—	2	—	5	75
D*	—	20	10	—	2	—	5	65
E	20	—	—	—	—	2	5	73
F	—	20	10	—	—	2	5	63
G	20	—	—	10	—	—	5	65
H	—	20	10	10	—	—	5	55

* The volume of aqueous phase, when toluene was present, was 100 cc. Samples were taken for analysis as free as possible from toluene.

Fig. 1.

The mixture was placed in a conical filtering flask, 10 cc. were withdrawn for an initial ammonia estimation, the flask exhausted and incubated at 37°. At suitable intervals further samples of 10 cc. were withdrawn for the estimation of free ammonia and the flask re-evacuated and re-incubated. About

seven or eight ammonia estimations were made for each curve over a period of from three to six days.

Absence of inhibitors.

The following features are observed with aspartic acid (curve A):

- (1) a relatively rapid liberation of ammonia at first;
- (2) a period during which there is a little more ammonia produced;
- (3) finally, a rapid linear liberation of all the remaining ammonia.

With fumaric acid (curve B) there are observed:

- (1) a relatively rapid uptake of ammonia at first, followed by
- (2) a long period of slow liberation of ammonia, and finally
- (3) a rapid linear liberation of all the remaining ammonia.

We have always found this last liberation of ammonia to follow a parallel course to the third portion of the aspartic acid curve. With different suspensions and different strains of organism the relative durations of the three periods of each curve vary, but the curves are always of the same general type.

These features of the curves are readily interpreted by the knowledge that in the case of each curve two processes are proceeding simultaneously, (1) in the case of curve B, absorption of ammonia to form aspartic acid, and a reducing process to form succinic acid and (2), in the case of curve A, elimination of ammonia to form fumaric acid and a reduction to succinic acid.

We suggest that the reduction is due to the growing organism, fumaric acid acting as a hydrogen acceptor and thus enabling anaerobic growth to occur, and that the aspartic acid-fumaric acid equilibrium is due to the resting organism and is independent of growth.

Effects of inhibitors.

With certain inhibitors, reduction to succinic acid appears to be practically entirely prevented and close approximations to true equilibrium curves are obtained.

The effect of toluene is shown in curves C and D. The two curves approach until they almost meet and then become parallel and rise very slowly, showing a very gradual elimination of ammonia. This rise is too slow to be shown in the figure. The equilibrium point can be taken to a first approximation as being half-way between the points at which the rise in the two curves first begins. Some of these values are given in Table II.

Table II. *Equilibrium concentration of ammonia obtained with various inhibitors and suspensions of B. coli with initial concentrations of M/10 sodium fumarate and M/10 ammonium chloride and of M/10 sodium l-aspartate (at 37° and p_H 7.4).*

Inhibitor	0.5 % NaNO ₂	0.7 % NaNO ₂	1.0 % NaNO ₂	Toluene	2 % C ₃ H ₇ OH	4 % C ₃ H ₇ OH	4 % C ₃ H ₇ OH aerobic	1 % NaNO ₂ aerobic
Molar conc. of NH ₃ at equi- librium	0.046	0.046	0.045 0.046	0.047	0.047 0.047	0.047	0.047	0.046

With a moderate concentration of propyl alcohol (2 % by volume) the curves are indistinguishable from those obtained with toluene as shown in Fig. 1, where curves E and F lie so close to curves C and D that they cannot be distinguished in the drawing. With 4 % propyl alcohol similar curves are obtained. If the concentration of propyl alcohol be increased, however, to 10 %, synthesis of aspartic acid from fumaric acid or breakdown of aspartic acid to fumaric acid is almost entirely inhibited. The output of ammonia from aspartic acid in the presence of 10 % propyl alcohol was equivalent to 6 % of the aspartic acid after 97 hours' incubation, and in the same period no detectable uptake of ammonia by fumaric acid was observed.

It was shown by Quastel, Stephenson and Whetham [1925] that resting *B. coli*, in presence of a concentration of sodium nitrite (0.4 %) which inhibited growth, was still capable of activating lactate and nitrate. It seemed possible therefore that resting *B. coli* in presence of a toxic concentration of nitrite might still bring about the synthesis of aspartic acid. This proved to be correct. A 0.5 % concentration of sodium nitrite in the initial mixture resulted in a good synthesis with little or no reduction, although the curves just crossed. The effect of a concentration of 1 % sodium nitrite is shown in Fig. 1, curves G and H. The curves are similar to those with toluene and propyl alcohol but there appears to be a slight retardation in the early period. Control experiments, in the absence of organism, showed that nitrite at p_H 7.4 and 37° had no effect on aspartic acid or on a mixture of fumaric acid and ammonia.

Aerobic experiments.

With 1 % nitrite and 4 % propyl alcohol the curves obtained under aerobic conditions are almost identical with those obtained anaerobically. Table III gives results with propyl alcohol.

Table III. *Free NH₃ in molar concentration.*

Time in hours	Initial mixture: <i>M</i> /10 aspartic acid; 4 % propyl alcohol		Initial mixture: <i>M</i> /10 fumaric acid + <i>M</i> /10 ammonia; 4 % propyl alcohol	
	Anaerobic	Aerobic	Anaerobic	Aerobic
0	0.001	0.001	0.098	0.101
9.25	0.021	0.020	0.082	0.083
24.5	0.032	0.032	0.061	0.060
33.25	0.035	0.035	0.051	0.053
52	0.041	0.040	0.049	0.049
80.5	0.045	0.041	0.050	0.048

The equilibrium constant.

If a true equilibrium exists between aspartic acid, fumaric acid and ammonia, then, from a knowledge of the constant *K* given by the equation

$$K = \frac{[\text{fumaric acid}][\text{NH}_3]}{[\text{aspartic acid}]},$$

it should be possible to determine the concentrations of ammonia at equilibrium with different initial mixtures. This proves to be the case, the calcu-

lated and observed readings agreeing satisfactorily (see Table IV). K was calculated from the values in Table II. It was found to be 0.04.

Table IV.

Initial concentration of sodium fumarate M	Initial concentration of ammonium chloride M	Inhibitor	NH_3 uptake at equilibrium observed M	NH_3 uptake at equilibrium calculated M
0.1	0.1	All inhibitors, see Table II	0.054	0.054
0.1	0.2	1 % NaNO_2	0.077 0.074	0.075
0.1	0.3	4 % $\text{C}_3\text{H}_7\text{OH}$	0.082	0.084
0.1	0.4	1 % NaNO_2	0.088	0.088

Effect of resting B. coli on sodium dl-malate and ammonium chloride.

Since malic acid gives rise to fumaric acid in presence of *B. coli*, it follows that some aspartic acid synthesis should be produced from sodium malate and ammonium chloride in presence of this organism. It was shown, however, that the velocity of fumaric acid formation from malic acid is slow, so that it is to be expected that the velocity of aspartic acid synthesis will also be slow. This is found to be the case (see Fig. 2 in which a fumaric acid and an aspartic acid curve obtained at the same time with the same organism and under the same conditions are included for comparison). The rate of ammonia absorption by *dl*-malic acid proves to be small compared with that by fumaric acid.

Fig. 2.

Thermolability of the mechanism controlling the aspartic acid-fumaric acid equilibrium. Resting *B. coli* which has been boiled cannot bring about a synthesis of aspartic acid from fumaric acid, or a breakdown of aspartic acid to fumaric acid.

Effect of B. coli on glutaconic acid, maleic acid and succinic acid. None of these acids absorbs ammonia in presence of *B. coli* (see Table V), the estimations being carried out over a long period.

Table V.

Initial mixture, p_H 7.4	NH ₃ uptake	NH ₃ output
	<i>M</i>	<i>M</i>
<i>M</i> /10 glutaconic acid + <i>M</i> /10 NH ₄ Cl	0	—
<i>M</i> /10 maleic acid + <i>M</i> /10 NH ₄ Cl	0	—
<i>M</i> /10 succinic acid + <i>M</i> /10 NH ₄ Cl	0	—
<i>M</i> /10 glutaminic acid (toluene)	—	0.002
<i>M</i> /10 glycine (toluene)	—	0.001

Maleic acid thus offers still another contrast to fumaric acid. It is interesting to summarise the differences between fumaric acid and maleic acid with regard to *B. coli*.

(1) *B. coli* grows luxuriantly aerobically on sodium fumarate, but slightly or not at all on sodium maleate.

(2) In presence of *B. coli*, fumaric acid oxidises leucomethylene blue; maleic acid has no such effect.

(3) In presence of *B. coli*, fumaric acid absorbs ammonia to form aspartic acid; maleic acid is inert.

Effect of B. coli on glutaminic acid and glycine. Anaerobically in the presence of toluene and the organism only a trace of ammonia is liberated from these amino-acids.

The mechanism controlling the aspartic acid-fumaric acid equilibrium.

We have carried out many experiments to elucidate the nature of the mechanism or enzyme controlling the aspartic acid-fumaric acid equilibrium. At present all we feel justified in stating definitely is that during the time of the most rapid synthesis of aspartic acid (*i.e.* within the first three days), no enzyme capable of accomplishing the synthesis can be detected in the cell-free medium obtained by filtration through a porcelain candle.

SUMMARY.

1. In presence of resting *B. coli* under anaerobic conditions at p_H 7.4 and 37°, *l*-aspartic acid is synthesised from fumaric acid and ammonia.

2. The equilibrium

is established and the constant of the equilibrium has been found.

3. The equilibrium is controlled by a thermolabile mechanism.

4. In the absence of growth inhibitors such as toluene, propyl alcohol and sodium nitrite, reduction to succinic acid occurs. In the presence of any of these inhibitors no reduction takes place, whilst the equilibrium remains unaffected.

5. In the presence of 1 % sodium nitrite or 4 % propyl alcohol, the same equilibrium is attained both aerobically and anaerobically; 10 % propyl alcohol almost entirely inhibits the reaction.

6. Under conditions such that aspartic acid liberates ammonia in the presence of *B. coli*, glutaminic acid and glycine are inert.

7. Similarly, under conditions such that fumaric acid absorbs ammonia in presence of *B. coli*, glutaconic acid, maleic acid and succinic acid are inert.

8. Malic acid gives a slight but definite ammonia uptake in presence of *B. coli*.

It is a pleasure to express our appreciation of the interest taken by Sir F. G. Hopkins in this work.

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