LXII. THE DEAMINATION AND SYNTHESIS OF l-ASPARTIC ACID IN THE PRESENCE OF BACTERIA.

By ROBERT PERCIVAL COOK
AND 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 *l*-aspartic acid is incubated anaerobically with resting *B. coli communis* at 37° and p_H 7·4, complete deamination occurs, fumaric acid appearing in the early stages of the reaction, to be replaced later by succinic acid. If fumaric acid and ammonium chloride are incubated under the same conditions, there is first an ammonia uptake and a synthesis of *l*-aspartic acid, followed by the reappearance of all the ammonia, at which point nothing but succinic acid can be found in the medium. In the presence, however, of certain inhibiting substances, such as 2 or 4 % propyl alcohol, *M*/20 or *M*/10 sodium nitrite, or toluene, the production of succinic acid is completely inhibited. Under these circumstances, only fumaric acid is formed from aspartic acid, and aspartic acid from fumaric acid and ammonia, the reaction being a true chemical equilibrium for which equilibrium curves were obtained and the constant calculated. Resting *B. coli* thus appears to be able to effect two distinct chemical reactions, one reversible

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

which persists in the presence of inhibitors which completely abolish the other, irreversible reaction

$$COOH . CH_2 . CH(NH_2) . COOH + 2H \rightarrow COOH . CH_2 . CH_2 . COOH + NH_3.$$

The object of the work to be described was to determine how far the power to effect these two reactions is distributed among other species of bacteria.

The following organisms were used:

Strict aerobes	Facultative anaerobes	Strict anaerobes
B. alkaligenes	*B. pyocyaneus*	*B. sporogenes*
B. subtilis	*B. prodigiosus*	*B. histolyticus*
B. phlei (Timothy Grass Bacillus)	*B. proteus*	*B. tertius*
B. megatherium	*B. fluorescens*	

The organisms were grown in Cole and Onslow's tryptic broth. The aerobes and facultative anaerobes were grown aerobically in Roux bottles containing 150 cc. of broth, which were inoculated from 12-hour old broth cultures. The

time of growth was usually 2 or 3 days, except in the case of *B. megatherium* and *B. phlei*, which were allowed to grow for 10 days. The strict anaerobes were grown in large flasks, either anaerobically, or aerobically in presence of 0·01 % cysteine [Quastel and Stephenson, 1926] for about a fortnight.

After growth, the organisms were centrifuged from the broth, and washed three times by centrifuging with 0·85 % saline. They were then emulsified in saline, the emulsion being put in the shaking machine for an hour or two; in this way it was possible to obtain a homogeneous emulsion, even with granular growths like *B. subtilis* and *B. phlei*, and slimy growths like *B. prodigiosus*. The emulsion was finally aerated for 4 hours, and was then ready for use. It was stored in the refrigerator. Although it was generally used fresh, there was no marked change in its activity for at least 2 months. The various emulsions were made very roughly comparable by diluting with saline until 1 cc., when diluted to 50 cc., showed a standard opacity. The dilution chosen was such that the growth from one Roux bottle of a prolific organism like *B. alkaligenes* generally corresponded to about 10 cc. of the emulsion. At least two strains of each organism, obtained from different sources, were used, and always gave concordant results.

All the experiments were done anaerobically at p_H 7·4 and 37°. Reaction mixtures, containing known concentrations of sodium aspartate, sodium fumarate, and ammonium chloride, with the organism to be tested, and the inhibitor (if any) were made up, and placed in filtering flasks. All the reaction mixtures contained at least half their volume of Clark and Lubs's phosphate buffer, p_H 7·4. The course of the reaction was followed by frequent estimations of the free ammonia, by the modified Van Slyke aeration method described by Quastel and Woolf [1926]. A sample was taken for an initial estimation, and the filtering flask was stoppered, exhausted at the water-pump, and incubated, and the flask was re-evacuated and incubated after each subsequent sample. Duplicate flasks were put up, which were used for the isolation of aspartic, fumaric or succinic acid, as described later, when the curve of ammonia uptake or output indicated that a suitable stage had been reached.

The organisms were found to fall into two distinct groups, one containing all the facultative anaerobes and the other all the strict aerobes and strict anaerobes.

Results with facultative anaerobes.

All the facultative anaerobes were found to behave in a similar manner to *B. coli communis*, giving both the equilibrium and the reduction in the absence of inhibitors, but only the equilibrium in the presence of inhibitors. The equilibrium point was the same with the various organisms and inhibitors, and the velocity was similar for equal concentrations of the various emulsions.

The concentrations of reactants generally employed were $M/10$ *l*-aspartic acid, and $M/10$ fumaric acid + $M/10$ ammonium chloride. The inhibitors used were 2 % and 4 % propyl alcohol, $M/10$ sodium nitrite, and toluene. The

bacterial emulsion was generally used at a strength of 1 cc. in 50 cc. of the reaction mixture, and the reaction was complete in from 3 to 5 days.

Fig. 1 shows a typical set of reaction curves, obtained with *B. fluorescens*, both without inhibitor (curves *A* and *F*) and in presence of 4 % propyl alcohol (curves *AP* and *FP*). The reaction mixtures were made up as follows.

	M/2 sodium aspartate cc.	*M*/2 sodium fumarate cc.	*M* NH_4Cl cc.	C_3H_7OH cc.	Buffer p_H 7·4 cc.	Bacterial emulsion cc.
A	10	—	—	—	39	1
F	—	10	5	—	34	1
AP	10	—	—	2	37	1
FP	—	10	5	2	32	1

The curves exhibit the following features.

Curve A. A rapid initial ammonia output which later slows down. During this period fumaric acid is being formed. There is then a rapid linear output of ammonia to completion, after which only succinic acid can be isolated.

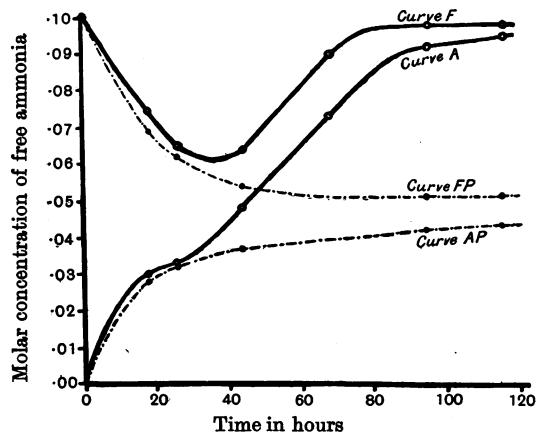

Fig. 1.

Curve F. There is an initial ammonia uptake by fumaric acid, with the production of aspartic acid. This is followed by a linear output of ammonia, parallel to that given by curve *A*, at the end of which only succinic acid is present.

Curve AP. An ammonia output, during which fumaric acid is produced, no succinic acid being detectable.

Curve FP. A synthesis of aspartic acid until the equilibrium concentration is reached.

Curves *A* and *F* differ from the corresponding curves obtained with *B. coli* by Quastel and Woolf in one respect. With *B. coli*, curve *F* invariably crosses curve *A* before the linear portion is reached. With the other facultative anaerobes this crossing is the exception. We have observed it in two cases only, once with *B. fluorescens* and once with *B. pyocyaneus*. Curves *AP* and *FP*, though close approximations to true equilibrium curves, are subject to

a small disturbing factor, which prevents the curves from meeting. They approach closely together, and then rise very slowly parallel to one another. The equilibrium value can be taken, to a first approximation, as being half-way between the curves at the point where the rise begins. The more closely the curves approach, *i.e.* the less prominent the disturbing factor, the lower the concentration of ammonia found at the equilibrium point. Some typical values of this equilibrium concentration are shown in Table I, for different organisms and inhibitors. Values already published for *B. coli* are given for comparison. The figures represent molar concentration of free ammonia at equilibrium, starting with reaction mixtures containing $M/10$ aspartate and $M/10$ fumarate + $M/10$ ammonia, and it will be seen that the equilibrium point is the same in all cases, allowing for the error due to the disturbing effect already mentioned.

Table I.

	Inhibitor			
Organism	2 % C_3H_7OH	4 % C_3H_7OH	Toluene	$M/10$ sodium nitrite
B. fluorescens	0·046	0·046	0·049	0·050
B. prodigiosus	0·049	0·049	0·048	0·051
B. proteus	0·045	0·047	0·047	0·047
B. pyocyaneus	0·048	0·049	0·048	0·049
B. coli	0·047	0·047	0·047	0·046

All controls, such as aspartic acid or fumaric acid and ammonia without organism, fumaric acid and organism without ammonia, ammonia and organism without fumaric acid, with and without the inhibitors used, were negative. Well-boiled emulsions of the organisms were unable to effect either the equilibrium or the reduction to succinic acid.

Results with strict aerobes and strict anaerobes.

All the strict aerobes and strict anaerobes tested show an entirely different type of behaviour. They give no sign of an equilibrium, but seem able to effect the reduction of aspartic acid to succinic acid as readily as the facultative anaerobes. When aspartic acid is incubated with one of these organisms in the presence of an inhibitor there is no ammonia output, even when 20 cc. of bacterial emulsion is used in 50 cc. of reaction mixture, and the experiment is extended for 2 weeks. Similarly, there is no ammonia uptake by fumaric acid, although the concentration of the organisms may be raised to half the volume of the reaction medium, and the fumaric acid be present in a concentration up to $M/2$, with ammonium chloride at molar concentration, over a period of a fortnight. Controls with a facultative anaerobe at these concentrations give an ammonia uptake to equilibrium in an hour or two.

In the absence of inhibitors, there is usually no ammonia uptake by fumaric acid. Aspartic acid, however, is completely deaminated, the only product being succinic acid. No fumaric acid is detectable at any stage of the reaction.

The course of the ammonia output from aspartic acid in two typical cases is shown in Fig. 2, one curve being obtained with an aerobe and the other with

an anaerobe. The aspartic acid was $M/10$, and the concentration of bacterial emulsion was 1 cc. in 50 cc. of the medium. It will be seen that the curves consist of two portions, a period of very slow ammonia output followed by a rapid linear deamination to completion. The relative durations of these two portions seem to be different for each emulsion, different preparations of the same organism giving sometimes a short latent period and a slow linear period and sometimes the reverse. There is no correlation between the relative durations and the bacterial species.

Although there is never any ammonia uptake by fumaric acid when inhibitors are present, there is sometimes a disappearance of ammonia in their absence. This phenomenon is especially noticed with *B. alkaligenes*. When this occurs, there is also a slow ammonia uptake with aspartic acid after deamination has proceeded to completion. This makes it very unlikely that the uptake is due to a synthesis of aspartic acid. It is reported by Quastel and Wooldridge [1925] that well-washed *B. alkaligenes* still shows great reducing activity in the Thunberg tube towards methylene blue, indicating that there are reactive

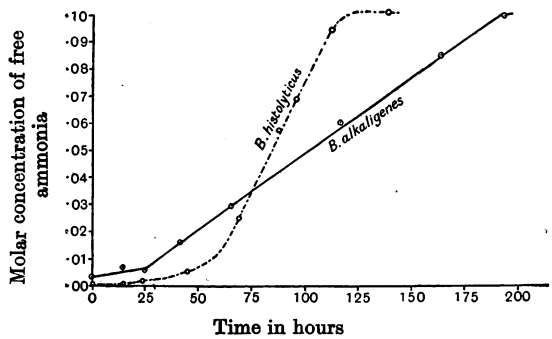

Fig. 2.

substances present which cannot be washed away. It seemed possible that these might be the cause of the disappearance of ammonia. An attempt was made to test this by washing a preparation of *B. alkaligenes* very thoroughly, in the hope that both reducing activity and ammonia uptake would diminish. It proved impossible, however, to increase the reduction time by repeated washing. 1 cc. of the original emulsion was placed in a Thunberg tube with 3 cc. of buffer and 1 cc. of 1/5000 methylene blue, and the reduction time at 37° was 9 minutes. A portion of the emulsion was washed four times by centrifuging with saline, made up to the original volume, and aerated for 6 hours. The reduction time was still 9 minutes. That the ammonia uptake was not due to a synthesis of aspartic acid was shown, however, by putting up a reaction mixture containing $M/10$ fumaric acid + $M/10$ ammonium chloride, with half its volume of this emulsion. In 5 days 15 % of the ammonia had disappeared, a much higher figure than usual. If aspartic acid had been produced in this quantity, it would have been easy to isolate. On attempting an isolation none was obtained, and the copper filtrate was pale blue, instead of

the dark blue obtained with even a trace of aspartic acid. It seems clear, therefore, that the uptake is due to some disturbing reaction that, unlike the aspartic-fumaric equilibrium, is stopped by inhibitors. With ammonium chloride alone, this emulsion showed no ammonia uptake.

Isolation of aspartic acid.

The reaction mixture was heated to boiling and saturated with solid copper sulphate. Boiling was continued for half an hour and the light blue precipitate was filtered off. The clear, deep blue filtrate was allowed to stand in the refrigerator for 3 days, when crystallisation of the copper aspartate was complete. The crystals were filtered off, well washed with cold water, and twice recrystallised from hot water. They were then dried to constant weight over sulphuric acid in the desiccator. Free aspartic acid was obtained by treating the copper salt with H_2S and decolorising if necessary with a little animal charcoal.

For identification, copper and nitrogen determinations were made on the copper salt, and melting-point and nitrogen determinations on the free acid. The rotation was also measured in presence of 3 mols. of HCl.

Copper salt. Found: Cu, 25·5–25·9 %; N, 5·37–5·49 %. Calculated for $C_4H_5O_4NCu$, $3H_2O$ Cu, 25·6 %; N, 5·63 %.

Free acid, M.P. in sealed tube, 269°–271° (uncorr.). Admixture with authentic aspartic acid did not alter the M.P., or raised it by not more than one degree.

N, found, 10·1–10·3 %. Calculated, 10·5 %.

Average for $[\alpha]_{Hg\ green}$ + 27°.

The yield of copper aspartate was between 60 and 80 % of that calculated from the reaction curve.

Isolation of succinic acid.

The organisms were removed by centrifuging and filtering through kieselguhr, 10 % phosphoric acid was added and the mixture thoroughly extracted with ether. The ether extract was evaporated to dryness, and the product taken up in water, decolorised with animal charcoal, and again evaporated to dryness. The crystalline residue of succinic acid had no decolorising effect on alkaline permanganate, and the different specimens melted between 184° and 186° (uncorr.). Admixture with authentic succinic acid either did not affect the melting-point or raised it by half a degree. The yield was from 40 to 60 % of the theoretical.

Isolation of fumaric acid.

The reaction mixture was freed from bacteria by centrifuging and filtering through kieselguhr, and toluene or propyl alcohol, if present, was removed by evaporation. 10 % of phosphoric acid was then added and the mixture thoroughly extracted with ether. The ether extract was evaporated to dryness and the residue was dissolved in water and precipitated with a strong solution of silver nitrate. The white precipitate of silver fumarate was filtered off,

washed, and decomposed with H_2S. The filtrate from the Ag_2S was decolorised with charcoal and evaporated to dryness. The residue of fumaric acid decolorised alkaline permanganate solution in the cold. It did not melt in an open capillary tube, but showed signs of sublimation just above 200°. In a closed capillary tube it melted between 278° and 279·5° (uncorr.), admixture with authentic fumaric acid having no effect on the melting-point. The yield was between 30 and 40 % of the theoretical.

Experiments with other amino-acids.

Preliminary experiments with glycine, alanine and glutamic acid indicate that all these substances are deaminated by all the organisms used when inhibitors are absent, but that no ammonia is given out when inhibitors are present.

Discussion.

It seems clear from the results described above that there are present in bacteria two distinct mechanisms capable of dealing with aspartic acid.

1. A mechanism which effects an irreversible reductive deamination to succinic acid, its time course being represented by a latent period followed by a straight line to completion. This mechanism is present in all the bacteria tested and is completely stopped by inhibitors.

2. A mechanism which effects a reversible equilibrium between aspartic acid and fumaric acid and ammonia. This mechanism is present only in facultative anaerobes, and is unaffected by inhibitors.

It is possible, therefore, by varying the conditions, to obtain curves showing the effect of mechanism 1 only (Fig. 2) or mechanism 2 only (curves *AP* and *FP*, Fig. 1) or a summation effect of the two mechanisms (curves *A* and *F*, Fig. 1).

Since the second mechanism acts in the presence of substances known to inhibit the growth of the bacteria concerned, there can be no question that it is due to the true "resting" or non-proliferating organism. Experiments with methylene blue in the Thunberg tube show that facultative anaerobes like *B. coli*, *B. proteus* and *B. prodigiosus* possess far more general activating powers than strict aerobes like *B. alkaligenes* [Quastel and Wooldridge, 1925] and strict anaerobes (unpublished results by these authors). It seems possible that the fumaric-aspartic mechanism is bound up with the activating mechanisms of the organism, and experiments are in progress to test this point.

Since the first mechanism is stopped by growth inhibitors, there is a possibility that it may be due to the growth of the organisms in the reaction mixture. This was the suggested explanation of Quastel and Woolf [1926]; they postulated that anaerobic growth might occur on the protein of dead bacteria in the emulsion, the aspartic acid acting as hydrogen acceptor. This explanation now seems unlikely for several reasons. Firstly, one would hardly expect strict aerobes to be able to grow anaerobically in this way. Secondly, slow-growing organisms like *B. megatherium* seem able to bring about the reaction as rapidly

as the more prolific organisms. Thirdly, the amount of bacterial substance present can hardly account for the complete reduction observed.

The only possible alternative explanation seems to be that the aspartic acid undergoes an oxidation-reduction process, a part being oxidised to provide for the reduction of the rest. The yields of succinic acid obtained leave an ample margin for this to be possible. If this explanation be correct, the peculiar nature of the curve might be explained as follows. During the latent period a very slow oxidation is going on, resulting in the appearance of some reactive product (*e.g.* pyruvic acid). When this substance has reached a certain concentration, it is oxidised, reducing several molecules of aspartic acid in the process. The straight line portion is then reached, being an expression of the steady production of the hypothetical reactive substance. Experimental tests of this supposition are in progress.

The teleological significance of this reaction may be that the organism needs a mechanism to disengage ammonia from amino-acids in order that it may enter into the synthetic processes of growth. The fact that other amino-acids are deaminated tends to support this view, which, if true, would fit in with the theory that when a bacillus grows on a medium containing amino-acids as the source of nitrogen, the nitrogen must be turned into ammonia before it can be utilised.

SUMMARY.

Eleven species of bacteria have been tested. At 37° and p_H 7·4 they all deaminate aspartic acid anaerobically to succinic acid. This reaction is completely prevented by the presence of inhibitors, such as 2 or 4 % propyl alcohol, $M/10$ sodium nitrite, or toluene. In addition, all the four facultative anaerobes can effect a reversible equilibrium

$$\text{aspartic acid} \rightleftharpoons \text{fumaric acid} + \text{ammonia},$$

giving the same equilibrium constant as that already observed with *B. coli communis*. The four strict aerobes and three strict anaerobes used show no trace of this reaction, which is unaffected by the presence of the inhibitors. Well-boiled bacteria have no deaminating effect on aspartic acid.

Preliminary work indicates that all the bacteria used will completely deaminate glycine, alanine and glutamic acid in the absence of inhibitors. When inhibitors are added there is no ammonia output.

It is a pleasure to express our appreciation of the interest taken by Sir F. G. Hopkins in this work. We wish to thank Mr E. G. D. Murray, of the Pathological Department, Cambridge, for kindly supplying us with some of the bacterial cultures used.

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