CCVI. FACTORS INFLUENCING BACTERIAL DEAMINATION

III. ASPARTASE II: ITS OCCURRENCE IN AND EXTRACTION FROM *BACTERIUM COLI* AND ITS ACTIVATION BY ADENOSINE AND RELATED COMPOUNDS

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THE bacterial deaminases of *Bact. coli*, previously studied, fall into two groups: the first consisting of the deaminases of glycine, *dl*-alanine and *l*-(+)-glutamic acid, and the second of the deaminases of *dl*-serine and *l*-(-)-aspartic acid. In these investigations deaminase activity has been measured in Q_N ($\mu\text{g. NH}_3\text{-N}$ liberated per mg. dry wt. bacteria per hr.). The deaminases of the first group [Stephenson & Gale, 1937] carry out only oxidative deamination and have an activity of the order $Q_N=30$ which remains constant throughout the growth period of the organism. Those of the second group [Gale & Stephenson, 1938] carry out both aerobic and anaerobic deaminations and have an activity of a much higher order—values of 1000 for *dl*-serine and 500 for *l*-(-)-aspartic acid have been recorded—and this activity varies greatly with the age of the culture.

In the study of *dl*-serine deaminase, it was shown that the washed suspension of the organism in water lost activity on standing. Investigation of this loss and its subsequent recovery led us to postulate the existence of a diffusible coenzyme for serine deaminase. The loss of activity could be prevented by incubation with a reducing agent in the presence of phosphate, or with a trace of adenylic acid. Also, if the activity were allowed to fall, then the lost activity could be recovered by incubation for 1–2 hr. with a reducing agent and phosphate—neither being effective alone. From this evidence, we deduced that the coenzyme could exist in a reduced or oxidized form and in a phosphorylated or non-phosphorylated form, the reduced and phosphorylated form being the active substance.

Since this work was published, it has been demonstrated that the alanine oxidase preparation from kidney requires a coenzyme [Das, 1936; Warburg & Christian, 1938, 1; Straub, 1938] and that this coenzyme contains adenine, phosphate and flavin [Warburg & Christian, 1938, 2].

Harden [1901] showed that a culture of *Bact. coli* grown in glucose broth would deaminate aspartic acid with the formation of succinic acid. Quastel & Woolf [1926] showed that this reaction took place anaerobically in the absence of inhibitors but that in the presence of an inhibitor such as toluene, an equilibrium was set up corresponding to

the action of the toluene being to stop the later reduction of fumaric acid to succinic acid. Woolf [1929] showed that the fumarase of the organism produced malic acid from the fumaric acid so that the final equilibrium was really an

aspartic-fumaric-malic- NH_3 system. He gave the name "aspartase" to the enzyme responsible for the formation of fumaric acid from aspartic acid. These workers used anaerobic conditions for all their experiments and showed that the effect of toluene was to inhibit the reduction of fumaric acid but, since the evolution of NH_3 from aspartic acid in the initial stage of the experiment was independent of the presence of toluene, this inhibitor can have no action on the aspartase enzyme itself.

Part of this paper will be devoted to showing that there is, in *Bact. coli*, an enzyme, other than the "aspartase" of Woolf, which is responsible for the deamination of aspartic acid but which requires for its action the presence of a coenzyme which is destroyed by the method used by previous workers for the preparation of the bacterial suspension.

Methods. The strains of *Bact. coli* used, methods of growth, preparation and measurement of bacterial suspensions and the determination of NH_3 liberated have already been described [Gale & Stephenson, 1938]. Since the results obtained with the two strains do not differ significantly, only those obtained with strain I are presented here. Fig. 1 shows the effect of pH on deamination of *l*-(–)aspartic acid by this strain.

The adenosine used in part of this work was obtained from Dr George Henning of Berlin-Tempelhof.

Fig. 1.

Fig. 2.

Fig. 1. Effect of pH on the rate of deamination of *l*-(–)aspartic acid by *Bact. coli* (I).

Fig. 2. Variation of deaminase activity with "age" of culture and conditions of growth: 1, in N_2 ; 2, in air; 3, in 2% glucose broth in N_2 (strain I).

Variation of deaminase activity with "age" of culture and conditions of growth.

Fig. 2 shows the Q_N variation due to culture time and conditions of aeration. In curve 1 the culture was grown anaerobically in N_2 for varying periods; in curve 2 the growth was carried out aerobically in liquid medium in Roux bottles; in curve 3 the organism was grown in 2% glucose broth with chalk and bubbled continuously with N_2 . In the last case samples were taken at intervals with sterile precautions, the chalk removed by centrifuging for 2 min. at 3000 r.p.m. and pouring off the supernatant liquid from which the organisms could then be removed by centrifuging in the usual way. The activities in each case were determined aerobically.

The presence of glucose in the growth medium inhibits deaminase activity, a result which has been shown with all the deaminases so far studied. The low activity obtained under such conditions remains constant with age. With curve 2,

however, there is a steadily increasing activity for the first 14 hr. of growth, after which the activity falls again, reaching the initial low value after about 24 hr. When the culture is grown anaerobically, there is a sharp rise in activity between the 8th and 10th hr. of growth and the high activity then attained does not decrease appreciably up to the 24th hr. of growth.

Fig. 3 shows how this variation of activity in an anaerobic culture is related to the growth curve and also to the E_h of the medium determined with the calomel electrode. The increase in activity reaches a maximum value at about the same time as active cell division ceases and the E_h reaches a steady value. It seemed likely that the increase in activity between the 8th and 10th hr. was due to a change in the medium consequent upon the metabolic activity of the cells. To investigate this point 900 ml. of liquid medium were inoculated with a 12 hr.

Fig. 3.

Fig. 3. 1, Variation of deaminase activity; 2, growth in dry wt.; 3, E_h of medium, with "age" of culture. Anaerobic culture (strain I).

Fig. 4.

Fig. 4. 1, Variation of deaminase activity; 2, growth curve for culture grown aerobically in inorganic medium + 2% lactate + 2% aspartate (strain I).

culture of the organism and incubated anaerobically for 10 hr., by which time the activity of the culture was approaching its maximum. The greater part of the organism was then centrifuged off and the medium sterilized by passage through a Seitz filter. This semi-exhausted medium was then re-inoculated with a 12 hr. culture, bubbled with N_2 and incubated. A turbidity was visible after 5 hr. and the organisms were spun off after 6 hr. The results of three determinations of the activity are set out in Table I. The earliest obtainable culture in the semi-

Table I. Activity of early cultures grown anaerobically in "semi-exhausted" medium

Exp.	Hr. of growth	Activity Q_N	Activity of control grown in normal medium (Q_N)
142	6	376	35
143	7	296	42
153	6	378	75

In exp. 153, 1% sodium aspartate was added to the semi-exhausted medium before the 2nd inoculation.

exhausted medium has an activity equal to the maximum activity attained by a culture grown normally for 14 hr. This indicates that variation in activity is dependent upon an alteration in the chemical constitution of the growth medium.

Fig. 4 shows that a culture grown in synthetic inorganic medium [Stephenson, 1930] with the addition of 2 % Na aspartate and 2 % Na lactate, shows an increase in activity before growth stops. If the increase in activity is due to the formation in the medium of some substance, then the organism is able to synthesize that substance from the constituents of this synthetic medium and, presumably, if that substance were added to the medium in the first place, then the earliest obtainable cultures would possess maximum activity. Accordingly 6 hr. cultures have been grown in liquid medium with the following additions and, in each case, the activity of the washed suspension determined:

1 % Na fumarate; 1 % Na succinate; 1 % Na pyruvate; 0.5 % Na formate; 0.5 % alanine; 0.05 % adenosine; excess CO_2 .

In no case has a significant increase of activity over the control been obtained.

Loss and recovery of activity. The greater part of the work to be presented was carried out with cultures grown anaerobically for 15 hr. and having a Q_N (aerobic) of approximately 300 after preparation of the washed suspension. As in the case of serine deaminase, it was found that the activity of the washed suspension "decayed" on standing whether at 0° or on incubation. Table II shows that if suspensions of different strengths are incubated *in vacuo* for 3 hr. at 37° there is a loss of activity which increases with dilution of the suspension, being 28 % for a suspension of 3.7 mg./ml. and 78 % for a 0.37 mg./ml. suspension.

Table II. *Effect of dilution with water during incubation for 3 hr. at 37°*

Suspension of bacteria incubated (mg./ml.)	Dilution	Suspension of bacteria for estimation (mg./ml.)	Q_N (initial = 338)	Loss of activity %
3.70	—	0.37	244	28
0.75	1/5	0.37	177	48
0.37	1/10	0.37	75	78

Fig. 5. Effect of "age" of (anaerobic) culture on: 1, activity (Q_N); 2, % loss of that activity during 3 hr. subsequent incubation at 37° .

With serine deaminase, it was found that the % loss of activity over any given period depended upon the "age" of the culture: an effect which was provisionally explained by differences in the permeability of the organism. Fig. 5 shows the relation between the "age" of the culture and its activity with aspartic acid (curve 1) and the % loss of activity over 3 hr. incubation *in vacuo* at 37° (curve 2). With serine deaminase, a maximum "permeability" was obtained between the 10th and 12th hr. of growth and the rate of loss of activity fell off rapidly on either side of this period: with aspartic deaminase, the rate of loss of activity remains approximately constant from the 12th to the 20th hr. of growth. The marked differences between the activity and the "permeability" curves for the two deaminases indicate that we are dealing with different systems in each case.

With serine deaminase, it was shown that the lost activity could be recovered again by incubating the bacterial suspension for 1-2 hr. with glutathione (GSH) or more negative reducing systems in the presence of phosphate. Similar experiments have been carried out with the aspartic deaminase and the results are set out in Fig. 6. A culture was grown anaerobically for 15 hr. and the aerobic activity of the washed suspension was determined immediately after its prepara-

Fig. 6. Recovery of activity (strain I).

tion ("initial Q_N " = 306). The suspension was then left at 0° for 3 hr. *in vacuo*, when its activity had fallen to 186. The activity of the "decayed" suspension was then redetermined aerobically in the presence of borate buffer at pH 7.5 to which the following systems had been added: (1) water; (2) boiled bacteria in water; (3) boiled bacteria in 0.01 *M* phosphate buffer at pH 7.5; (4) formate, 0.005 *M*; (5) formate, 0.005 *M*, and phosphate, 0.01 *M*; (6) glutathione, *M*/30,000 GSH; (7) *M*/30,000 GSH and 0.01 *M* phosphate; (8) lactate 0.005 *M*; (9) 0.005 *M* lactate and 0.01 *M* phosphate (final concentrations). The results show the following marked differences from those obtained with similar experiments carried out with serine deaminase.

1. With certain additions an immediate recovery of activity is obtained without incubation.
2. GSH, with or without phosphate, has no effect either before or after incubation.
3. Boiled bacteria or formate produce an immediate reactivation with or without phosphate and, in certain cases, this reactivation proceeds to a point above the "initial" value.

Fig. 7 shows the effect of adding certain of these substances to the washed suspension immediately after its preparation. Boiled bacteria and certain reducing systems again produce an activation of the suspension. The preparation of the bacterial suspension takes about $1\frac{1}{2}$ hr. and it can be shown that there is a loss of activity during this period and that it is this lost activity which is recovered in Fig. 7. The reactivation obtained with lactate is not satisfactorily reproducible while that obtained with formate is consistent so that it seems that a reducing system is required which is rather more negative than the lactate-lactic dehydrogenase system.

Fig. 7. Immediate activation produced by boiled organism or reducing systems.

The immediate reactivation effect of boiled bacteria suggests that they form a source of some essential factor which is lost on standing and a search for a coenzyme of *l*-(-)aspartic deaminase was next undertaken.

Nature of the coenzyme. A culture was grown anaerobically for 15 hr. and the initial aerobic activity of the washed suspension determined ($Q_N=251$). After 3 hr. at 0° *in vacuo* its activity had fallen to $Q_N=84$. The activity was then redetermined in the presence of (1) boiled cell-free liver extract; (2) boiled cell-free yeast extract; (3) a suspension of boiled bacteria; (4) a cozymase preparation in solution containing approximately 0.1 % coenzyme I; (5) a solution freshly prepared from a separate dry cozymase preparation containing 1 mg. of the dry material in 10 ml. The results are shown in Table III and a reactivation is obtained with the first four additions but not with the second of the cozymase preparations.

Table III. *Sources of coenzyme*

Initial activity of washed suspension	$Q_N=251$	
Activity after 3 hr. at 0° <i>in vacuo</i>	$Q_N=84$	
Addition to suspending medium	Resultant activity Q_N	Increase in Q_N
Boiled liver ext.	227	143
Boiled yeast ext.	161	77
Boiled bacteria	229	148
Cozymase prep. A	202	118
Cozymase prep. B	88	4

To confirm that the deaminase factor was not coenzyme I, the cozymase preparation which had proved active in the last experiment was treated in the various ways set out in Table IV. Coenzyme I is destroyed by boiling at pH 12 for 2 min. but is stable to boiling at pH 1 for 5–10 min. If, however, the

coenzyme is first reduced with $\text{Na}_2\text{S}_2\text{O}_4$ then these relations are reversed, the reduced form being stable to alkali and unstable to acid [Green & Brosteaux, 1936]. Excess $\text{Na}_2\text{S}_2\text{O}_4$ was oxidized by aeration. The form of the experiment was similar to those already described and it is obvious that the deaminase factor bears no relation to the activity of coenzyme I but would appear to increase slightly during alkaline hydrolysis and to decrease during acid hydrolysis. The factor is then, presumably, either an impurity in the cozymase preparation or a breakdown product of coenzyme I. The known impurities in the active cozymase preparation were then tested and are also listed in Table IV where it is seen that adenylic acid possesses a definite reactivation effect but of a rather low order.

Table IV. *Nature of active factor in cozymase prep. A*

Initial activity of washed suspension		$Q_N=292$			
Activity after 3 hr. at 0° in <i>vacuo</i>		$Q_N=206$			
Addition to suspending medium	Previous treatment.	Coenzyme I	Resultant activity Q_N	Q_N increase	Factor
	Boiled for 5 min. at pH				
Cozymase prep. A	—	Active	297	91	+
„	12	Inactive	324	118	+
„	1	Active	304	98	+
Cozymase reduced	12	Active	277	71	+
„	1	Inactive	266	60	+
Cozymase prep. B	—	Active	200	—	—
Flavine	—	—	196	—	—
Adenylic acid (0.1 mg./ml.)	—	—	246	40	+
Nicotinamide (0.1 mg./ml.)	—	—	205	—	—
Boiled bacteria	—	—	290	84	+

It has been shown that adenine compounds are readily attacked by *Bact. coli*, the probable course being as follows [Lutwak-Mann, 1936]:

Adenylic acid $\rightarrow$ adenosine $\rightarrow$ inosine $\rightarrow$ hypoxanthine + ribose

Inosinic acid $\rightarrow$ inosine $\rightarrow$ hypoxanthine + ribose

For this reason adenylic acid and its decomposition products were tested for their activating effect as follows: the activity of a washed suspension from a 15 hr. anaerobic culture was determined aerobically immediately after its preparation in the usual way with 0.03 *M* phosphate buffer and *M*/250 *l*-aspartate and then also in the presence of (1) adenylic acid; (2) inosinic acid; (3) adenosine; (4) inosine; (5) adenine; (6) hypoxanthine; (7) ribose; (8) boiled bacteria. The final concentration in all cases, with the exception of (8), was 1 mg./100 ml. The results are shown in the top portion of Fig. 8 as "initial recovery". The suspension was then allowed to stand in water at 0° for 3 hr. by which time its activity had fallen to $Q_N=100$ and the whole experiment then repeated with this "decayed" suspension: the results obtained are shown in the lower portion and overlapping the upper portion of Fig. 8. The most effective substance is adenosine, having an activation effect greater than that produced by the boiled bacteria. It is closely followed by inosine and inosinic acid. The further breakdown products of adenosine—adenine, hypoxanthine and ribose—produce a small reactivation in the second half of the experiment but their effect is generally rather less than in the example quoted.

These results suggest that adenosine is the activating substance replaceable by inosine or inosinic acid, possibly in virtue of their interchangeability under the action of the organism [Stephenson & Trim, unpublished]. The lower but still

marked activity of the other decomposition products of adenosine may be due to the synthesis from them of one of the three more active compounds. Until the mode of action of the adenosine etc. is explained, speculation on this point is useless. At first sight it might be thought that adenosine and inosine act as an

Fig. 8. Recovery in the presence of breakdown products of adenylic acid.

NH_3 -carrier system, the inosine reacting with aspartic acid to form adenosine and the latter then being deaminated to inosine again. That this cannot be the case is seen from a consideration of the rates of deamination of aspartic acid and adenosine respectively:

Rate of deamination of *l*-aspartic acid:

- (a) Aerobically in presence of adenosine $Q_N = 450$
- (b) Anaerobically in presence of adenosine $Q_N = 501$

Rate of deamination of adenosine:

- (a) Aerobically $Q_N = 122$
- (b) Anaerobically $Q_N = 120$

The effect of adenosine on the deamination of l-(−)aspartic acid. It was next necessary to determine whether this co-deaminase effect of adenosine would work anaerobically.

Fig. 9 shows the effect of adding an initial concentration of 0.67 mg. adenosine/ml. on the deamination and Fig. 10 the corresponding effects with one-quarter this quantity of adenosine. In the absence of adenosine there is a much slower rate of evolution of NH_3 anaerobically than aerobically. The effect of the addition of the adenosine is to increase the rate both aerobically and anaerobically but, whereas the aerobic rate is increased by a constant anaerobically the initial acceleration is much greater but rapidly falls off until the rate is back

to that of the control. That this is not due to the attainment of an equilibrium can be demonstrated by the addition of further adenosine, when the rate will again be temporarily accelerated.

Fig. 9.

Fig. 10.

Fig. 9. Effect of adenosine (1/15 mg./ml.) on evolution of NH_3 from *l*-(-)aspartic acid in presence of *Bact. coli* (0.1 mg. dry wt./ml.).

Fig. 10. Effect of smaller concentration of adenosine (1/60 mg./ml.) on evolution of NH_3 (cf. Fig. 9).

Fig. 11 shows the result of adding increasing concentrations of adenosine upon the Q_N . Since the accelerated rate is constant aerobically (Figs. 9 and 10) we can determine a true rate under such conditions but anaerobically this is not possible and the points on the anaerobic curve of Fig. 11 represent the rate for the first 30 min. after the addition of the adenosine. We have seen from Figs. 9 and 10 that a given amount of adenosine produces a more marked increase in the anaerobic than the aerobic rate and this may mean that the total activity obtained aerobically is due to an anaerobic process. In the absence of adenosine and with freshly prepared suspensions, the aerobic rate of deamination is greater than the anaerobic but this difference in the two rates disappears on incubation of the suspension for half-an-hour. Mesrobianu [1936] has shown that *Bact. coli* contains an appreciable amount of adenosine and this difference in anaerobic and aerobic activities of fresh cultures may be due either to a labile oxidative deaminase or to the natural adenosine of the bacterium having an effect which is obvious anaerobically but falls off so rapidly anaerobically (cf. Fig. 9) as not to be demonstrable.

Fig. 11.

Fig. 11. Effect of adenosine concentration on rate of deamination by strain I.

Fig. 12.

Fig. 12. Variation of critical concentration of adenosine with concentration of *l*-(-)aspartate.

From Fig. 11 it is also seen that, aerobically, the rate of deamination reaches a maximum in the presence of a final concentration of adenosine greater than the critical concentration of $M/7500$. The final concentration of aspartic acid in this case is $M/250$ so that a maximum rate of deamination is obtained when there is one mol. adenosine present to every 30 mol. aspartic acid. Fig. 12 shows that if the final concentration of *l*-(-)aspartic acid is doubled then the critical concentration of adenosine is also approximately doubled.

Deamination of l-(-)aspartic acid by cell-free coli-juice. At this point in the research the wet-crushing mill of Booth & Green [1938] became available and it was decided to test these findings with a cell-free juice obtained by crushing *Bact. coli*. 25 Roux bottles containing broth-agar with the addition of 0.5% Na lactate were sown with the organism and incubated for 24 hr., the culture then washed off and centrifuged, washed once in water and then made up into a thick cream (c. 35 ml.). This was poured into the mill and ground for 2 hr. by which time most of the cells had been disintegrated. The resulting juice was then centrifuged until microscopic examination showed it to be free from whole cells.

The juice was then tested for activity as aspartic deaminase and the results obtained are shown in Table V. 1 ml. each of the juice, $3M/250$ *l*-(-)aspartate and $M/10$ phosphate buffer at pH 7.5 were shaken aerobically in a bath at 40°. The figures given are corrected for the blank of the juice. The activity was then redetermined in the presence of adenosine and also after dialysis with and without adenosine. It appears that neither dialysis nor the addition of adenosine has any appreciable effect on the activity. This may be due to the fact that the juice contains the optimal activating amount of adenosine already. Also, since

Table V. *Deamination of l-aspartate by cell-free coli-juice*

	$\mu\text{g. NH}_3\text{-N liberated/}$ hr./ml. juice
(a) Juice + $M/250$ aspartate	97
(b) Juice + $M/250$ aspartate + 0.07 mg. adenosine	126
(c) Juice + 0.05 mg. adenosine	18
(b)-(c)	108
(d) Dialysed juice + $M/250$ aspartate	192
(e) Dialysed juice + $M/250$ aspartate + 0.07 mg. adenosine	107
(f) Dialysed juice + 0.07 mg. adenosine	18
(e)-(f)	89

Table VI. *Scheme of fractionation of coli-juice*

the bacteria had been grown in a manner different from that used for the organisms used in the first part of the work, it was possible that the juice contained other enzymes capable of deaminating aspartic acid.

Hence an attempt was made to fractionate the juice (Table VI).

Separation of aspartase I and aspartase II from cell-free coli-juice. The scheme provides three precipitates. Each of these was dissolved in water and then dialysed against distilled water until a blank of 10–20 $\mu\text{g. NH}_3\text{-N/ml.}$ of preparation, was obtained. Each of the preparations was then tested for deaminase activity with *l*-(–)aspartic acid, the activity redetermined in the presence of adenosine at a final concentration of 1/10 mg./ml. and, finally, the preparation was incubated with toluene for 2 hr. and its activity then determined in the presence of adenosine (1/10 mg./ml.). The results of one such set of experiments is given in Table VII where the activities are expressed as $\mu\text{g. NH}_3\text{-N}$ liberated per hr. per 100 mg. dry wt. of preparation. Fraction A has a small blank activity which is increased some 500% by the presence of adenosine and its activity is completely abolished by incubation with toluene; fraction B appears to have no activity towards *l*-aspartic acid under any of the circumstances tested. Fraction C has an activity which is unaffected by added adenosine and is completely unaltered by toluene treatment. These experiments were carried out anaerobically. The course of deamination by fraction A under various conditions is set out more fully in Fig. 13.

Table VII. *Analysis of fractions from coli-juice*

	$\mu\text{g. NH}_3\text{-N/hr./100 mg. dry wt.}$		
	Fraction A	Fraction B	Fraction C
(a) <i>M</i> /250 aspartate	110	0	90
(b) <i>M</i> /250 aspartate + 0.1 mg. adenosine	650	0	75
(c) 0.1 mg. adenosine	70	0	0
(b)–(c)	580	0	75
(d) Fraction (after 2 hr. with toluene at 37°) + <i>M</i> /250 aspartate + 0.1 mg. adenosine	0	0	85

Fig. 13. Deamination of *M*/250 aspartate by fraction A: 1, +adenosine 0.1 mg./ml.; 2, +adenosine 0.03 mg./ml.; 3, no addition; 4, adenosine alone 0.1 mg./ml.; 5, same as 1, with toluene-treated fraction.

This fractionation leads to the separation of two distinct enzyme systems which deaminate *l*-(–)aspartic acid anaerobically. The enzyme of fraction C is unaffected by toluene or adenosine and must therefore be the one studied by

previous workers and called "aspartase" by Woolf [1929]. It is proposed that, in future, this enzyme should be called "aspartase I" and the enzyme present in fraction A whose activity depends upon the presence of adenosine and is completely inhibited by toluene treatment, "aspartase II". The enzyme separated from *Bact. fluoresc. liquefaciens* [Virtanen & Tarnanen, 1932] was probably, since toluene was used in the extraction, aspartase I.

Distribution of aspartase I and II in Bact. coli. It has been possible to demonstrate the presence of aspartase II in cultures of *Bact. coli* of both strains used in this laboratory and grown in the various conditions set out in Table VIII. Toluene treatment of the bacterial suspension completely inhibits aspartase II but, according to the work of Quastel & Woolf [1926], has no effect on aspartase I. Hence the Q_N determined after such treatment and measured during the initial stages of the deamination while the evolution of NH_3 is linear, gives a measure of aspartase I activity. From Fig. 11 we see that the rate of deamination is constant aerobically in the presence of a concentration of adenosine greater than the critical value and we can take this rate as a measure of the total activity. Assuming for the present that this total activity is the sum of the activities of aspartase I and II, we obtain that of aspartase II by difference. All the relevant figures are set out in Table VIII. The ratio of aspartase II/aspartase I shows that quantitatively the aspartase II is the more important enzyme in all cases. With strain I, aspartase I is negligible in the anaerobic cultures and it is with this strain that the greater part of the work has been carried out. Strain II shows approximately the same total activities for each type of culture as strain I but contains, in every case, a higher degree of activity due to aspartase I so that the ratios are of a lower order throughout.

Table VIII. *Analysis of deaminase activity and distribution of aspartase I and II in Bact. coli*

	Aerobic deamination				Anaerobic deamination				Total activity	Aspar- tase I	Aspar- tase II	Ratio II/I
	Q_N				Q_N							
			Toluene treated				Tol. treat.					
Adenosine	-	+	-	+	-	+	+					
Conditions of growth												
Strain I:												
Anaerobic in broth	138	364	8	7	32	401	7	364	7	357	51	
Broth in flask	150	250	23	6	32	114	4	250	4	246	62	
Aerobic in broth	166	331	28	30	27	348	50	331	50	281	4	
Aerobic on agar	96	121	6	6	17	127	9	124	9	115	13	
2% glucose broth	34	75	12	15	26	83	20	75	20	55	3	
Strain II:												
Anaerobic in broth	75	449	36	36	77	395	80	449	80	369	4.6	
Aerobic in broth	312	437	70	48	114	400	60	437	60	377	6	
2% glucose broth	54	95	32	32	40	106	32	95	32	63	2	

It can now be seen why previous workers should have overlooked the existence of aspartase II. Woolf [1929] grew his cultures in broth in Roux bottles and so would have obtained the highest content of aspartase I. Growth was allowed to continue for 48 hr. by which time, as can be seen from Fig. 2, the total activity would have fallen to a low figure. The organisms were washed 3 times and the suspensions then aerated for 1 hr. so that a considerable "decay" of aspartase II activity would occur. The suspension was kept for use up to a fortnight and it is stated that the activity remained constant for 2 months whereas aspartase II

activity decays to zero within 12–24 hr. Finally, the actual experiments were carried out anaerobically when the small amounts of adenosine present would have no significant effect and also after toluene treatment which inhibits the action of aspartase II.

Effect of formate on deamination of l-aspartic acid by Bact. coli. In the early experiments of this series, a reactivation of a “decayed” suspension was obtained with boiled bacteria or with formate. The action of the boiled bacteria can now be explained since Mesrobianu [1936] has shown that *Bact. coli* contains an appreciable amount of adenosine and is a plentiful source of its precursors. The effect of formate remains to be investigated.

Figs. 14 and 15 show the effects on the anaerobic and aerobic deaminations of the addition of the critical amount of adenosine (1), $M/120$ formate (2), and of the two together (3). Aerobically, the formate has the same accelerating effect as adenosine but anaerobically, after a brief marked acceleration of the rate, the effect of formate is scarcely significant.

Fig. 14.

Fig. 15.

Fig. 14. Anaerobic liberation of NH_3 from l -(-)aspartate by *Bact. coli* (I) in presence of: 1, adenosine 0.03 mg./ml.; 2, $M/120$ formate; 3, $M/120$ formate + adenosine 0.03 mg./ml.; 4, alone.

Fig. 15. Aerobic liberation of NH_3 from l -(-)aspartate by *Bact. coli* (I) in presence of: 1, adenosine 0.03 mg./ml.; 2, $M/120$ formate; 3, $M/120$ formate + adenosine 0.03 mg./ml.; 4, alone.

Fig. 16 shows the effect of increasing formate concentration on the aerobic Q_N . For comparison the effects of similar concentrations of adenosine are marked with a dotted line and it is seen that, to attain a maximum rate, a much higher concentration of formate than of adenosine is required but that the maximum rate reached is the same in both cases. With strain I, the critical concentration of formate would appear to be slightly greater than $M/1500$.

Fig. 17 shows the results obtained in an experiment with the aspartase II fraction of *coli*-juice in which the rate of deamination measured in $\mu\text{g. NH}_3\text{-N}$ liberated per 30 min. per ml. of preparation is determined in the presence of various concentrations of adenosine (1). The deamination is followed aerobically and a maximum rate is obtained with a concentration of 0.125 mg./ml. adenosine. The experiment was then repeated in the presence of $M/6000$ formate (2) with the result that the maximum rate of deamination was reached in the presence of 0.0625 mg./ml. adenosine, so that the critical concentration of adenosine is halved by the presence of the formate. In the absence of adenosine, formate alone has no consistent or appreciable effect on deamination by the fraction.

Fig. 18 shows the results of an experiment similar to the last, carried out on a suspension of the bacteria. The critical concentration of adenosine for maximum rate of deamination was determined and gave the usual value of 1/30 mg./ml. The experiment was then repeated in the presence of $M/6000$ formate and again

Fig. 16. Effect of formate (and adenosine) on aerobic deamination.

Fig. 17.

Fig. 18.

Fig. 17. Rate of deamination of *l*-(-)aspartate by fraction A of *coli*-juice in presence of: 1, varying concentrations of adenosine; 2, varying concentrations of adenosine + $M/6000$ formate.

Fig. 18. Rate of deamination of *l*-(-)aspartate by *Bact. coli* in presence of: 1, varying concentrations of adenosine; 2, varying concentrations of adenosine + $M/6000$ formate.

the optimal concentration of adenosine was approximately halved by this concentration of formate. Reference to Fig. 16 shows that $M/6000$ formate is much less than the critical concentration of formate required by strain I to give the maximum Q_N .

Since the presence of $M/6000$ formate halves the amount of adenosine that must be added to give a maximum Q_N , and $M/1500$ formate will give this maximum Q_N in the absence of added adenosine while the organism already contains a certain amount of adenosine, it is suggested that the presence of sufficient formate decreases the amount of adenosine required until the system is maximally activated by the natural adenosine of the organism.

Products of the action of aspartase II on l-(-)aspartic acid. A preparation of aspartase II was made according to the scheme set out above and the fraction dissolved in water and dialysed overnight. In this way 40 ml. of a solution of

aspartase II were obtained. This was set up in a large Thunberg tube with 25 ml. Na aspartate equiv. to 1 g. *L*-(-)aspartic acid; 50 mg. adenosine dissolved in 15 ml. water, and 20 ml. *M*/5 phosphate buffer at *pH* 7.5. The tube was evacuated and placed in a bath at 40°; 0.2 ml. of the contents was withdrawn every 30 min. and the $\text{NH}_3\text{-N}$ determined to follow the course of the reaction. After 5 hr. when the deamination had almost stopped at c. 50 % completion the reaction was stopped by acidifying with H_2SO_4 .

In a preliminary experiment, acid was added to a concentration of 10 % and the solution extracted with ether in a separating funnel. The ether extract was evaporated to dryness, the residue taken up in water and precipitated with conc. AgNO_3 . The white precipitate obtained was filtered off, resuspended in water and decomposed with H_2S , the Ag_2S filtered off and the clear filtrate evaporated to small bulk. On standing about 20 mg. of colourless crystals were obtained. A portion of these, dissolved in water, gave a white precipitate with AgNO_3 , decolorized KMnO_4 and Br water and gave the reactions typical of fumaric acid. M.P. (dec.) 200° in open tube with sublimation, M.P. 240° in sealed tube, which is considerably lower than the M.P. (287°) of pure fumaric acid. Thus the product was impure and represented a very low yield.

Accordingly the exp. was repeated. After 5 hr. incubation the reaction was stopped by acidifying slightly with H_2SO_4 and boiling to precipitate the protein which was then filtered off. The filtrate was neutralized and evaporated to 15 ml. and then extracted continuously with ether in a liquid extractor for 6 hr. The ether extract was then evaporated to dryness and the residue dissolved in 25 ml. of water.

The water solution was analysed for fumaric, succinic and malic acids according to the method of Needham [1927]. 1 ml. of the solution was neutralized with sat. $\text{Ba}(\text{OH})_2$ to remove sulphate and phosphate; the precipitate was filtered off immediately and washed with water.

97 % alcohol was then added to a final concentration of 36 % and the *pH* adjusted to 7.0. 2 ml. conc. AgNO_3 were then added and the precipitate was filtered off on a Gooch crucible. After thorough washing with 60 % alcohol, the precipitate was redissolved in dil. H_2SO_4 and the total Ag estimated by titration in the cold against 0.01 *N* KCNS using iron alum as external indicator. The AgSCN was filtered off and the filtrate analysed for the above acids.

Malic acid was estimated by the method of Auerbach & Kruger [1923] and it was shown that, under the conditions of the experiment, fumaric acid could be estimated by titration at 40° in the presence of H_2SO_4 against 0.01 *N* KMnO_4 (1 ml. 0.1 *N* KMnO_4 = 1.27 mg. fumaric acid).

Table IX. *Analysis of residue from ether extraction*

Residue dissolved in 25 ml. water	
Total Ag obtained from 1 ml.	= 11.8 mg.
Fumaric acid/ml. estimated against <i>N</i> /100 KMnO_4	= 2.47 mg. $\equiv$ 4.7 mg. Ag
Malic acid/ml. estimated polarimetrically	= 4.1 mg. $\equiv$ 6.6 mg. Ag
Total silver/ml. due to known acids	= 11.3 mg.
Total malic acid in residue	= 102.5 mg. $\equiv$ 102 mg. aspartic acid
Total fumaric acid in residue	= 61.8 mg. $\equiv$ 70 mg. aspartic acid
Total aspartic acid equivalence of residue	= 172 mg.
Total aspartic acid disappeared (from NH_3 determinations)	= 348 mg. } 49 % recovery

From Table IX it can be seen that the total acids of the extract as determined by the Ag salts can be accounted for as malic and fumaric acids. However, the total yield of these acids will account for only 50 % of the aspartic acid that has

disappeared but Needham [1927] showed that malic acid cannot be extracted quantitatively from water solution owing to its unfavourable partition coefficient. Since it would appear that only malic and fumaric acids are produced, these can both be estimated accurately in the actual reaction mixture if this is first deproteinized and evaporated to a small bulk. Table X gives the results of such an experiment and it can be seen that the aspartic acid disappearing is satisfactorily accounted for by the malic and fumaric acids appearing.

Table X. *Products of aspartase II action*

Total aspartic acid disappeared	=285 mg.
Total malic acid formed	=162.5 mg. $\equiv$ 162 mg. aspartic acid
Total fumaric acid formed	=116.0 mg. $\equiv$ 133 mg. aspartic acid
Equivalent total aspartic acid	=295 mg.

Thus the action of the fraction A obtained from *coli*-juice upon *l*-(-)aspartic acid is to produce NH_3 together with fumaric and malic acids. In the extract, the sum of the fumaric and malic acids does not account for the aspartic acid disappearing, but if these are estimated in the reaction mixture itself, then complete equivalence is obtained.

The presence of fumarase in fraction A. The formation of both malic and fumaric acids in the last experiment suggests that the fraction A obtained from *coli*-juice contains fumarase in addition to aspartase II. This was shown to be the case by the following experiment.

40 ml. of a preparation of fraction A were incubated *in vacuo* with 20 ml. phosphate buffer at pH 7.5 and 500 mg. fumaric acid made up into 20 ml. of neutral solution. At intervals of $\frac{1}{2}$, 1, 2, 4, 6 and 24 hr. a 5 ml. sample was withdrawn and added to 10 ml. of 14.2% ammonium molybdate solution. A similar sample was added to 11 ml. of 6% trichloroacetic acid. 1 ml. of glacial acetic acid was then added to the first mixture and both samples were left in the dark for 3-4 hr. The protein was precipitated by both sets of reagents and was filtered off before the final estimation. The filtrate was made up in each case to 22 ml. for convenience and the malic acid estimated by the difference in rotation of the two samples [Auerbach & Kruger, 1923].

Table XI shows the formation of malic acid from fumaric acid in this experiment and thus demonstrates the presence of fumarase in the fraction.

Table XI. *Presence of fumarase in fraction A of coli-juice*

Initial concentration of fumaric acid = 6.25 mg./ml.

Increase in rotation (Hg green) in presence of molybdic acid due to 10 mg. malic acid under experimental conditions = +0.80°.

Time	Increase in rotation in presence of molybdic acid	Malic acid mg./5 ml. sample	Malic acid mg./ml.
0	0	0	0
30 min.	+0.47°	5.25	1.05
1 hr.	+0.87	10.9	2.2
2 hr.	+1.35	16.9	3.4
4 hr.	+2.09	26.1	5.2
6 hr.	+2.11	26.4	5.3

SUMMARY

1. The deaminating activity of *Bact. coli* towards *l*-(-)aspartic acid varies (a) with the conditions of growth of the organism, and (b) with the age of the culture. The latter variation can be shown to be due to an alteration in the

chemical constitution of the growth medium consequent upon the metabolic activity of the cells.

2. Washed suspensions lose their activity upon standing and the rate of loss depends upon the dilution of the suspension.

3. The lost activity can be immediately recovered by the addition of boiled bacteria or formate to the suspension.

4. The recovery obtained with boiled bacteria resembles a coenzyme effect.

5. The coenzyme can be replaced by adenylic acid and its breakdown products, the most active being adenosine.

6. The addition of adenosine to the bacterial suspension results in a greatly increased rate of deamination aerobically or anaerobically.

7. A cell-free juice obtained by crushing and centrifuging *Bact. coli* will deaminate *l*-(-)aspartic acid.

8. A scheme for fractionation of the juice with $(\text{NH}_4)_2\text{SO}_4$ is given: this scheme results in the separation of two enzymes capable of deaminating *l*-(-)aspartic acid anaerobically. These enzymes are provisionally named:

Aspartase I which is unaffected by toluene and/or adenosine.

Aspartase II which is completely inhibited by toluene treatment, requires the presence of a coenzyme for its action, and is activated *in vitro* by adenosine.

9. The distribution of aspartase I and II in cultures of *Bact. coli* (two strains) grown under varying conditions has been determined.

10. Formate produces an activation of aspartase II aerobically but not significantly anaerobically.

11. Formate appears to reduce the amount of adenosine required to activate aspartase II.

12. The action of the aspartase II-fraction of *coli*-juice on *l*-(-)aspartic acid is to produce NH_3 and fumaric and malic acids.

13. The aspartase II-fraction of *coli*-juice also contains fumarase.

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REFERENCES

- Auerbach & Kruger (1923). *Z. Natur. Genusm.* **46**, 97.
Booth & Green (1938). *Biochem. J.* **32**, 855.
Das (1936). *Biochem. J.* **30**, 1080, 1617.
Gale & Stephenson (1938). *Biochem. J.* **32**, 392.
Green & Brosteaux (1936). *Biochem. J.* **30**, 1489.
Harden (1901). *J. chem. Soc.* **79**, 623.
Lutwak-Mann (1936). *Biochem. J.* **30**, 1405.
Mesrobianu (1936). Thesis presented at Strasbourg University. (Paris: Masson.)
Needham (1927). *Biochem. J.* **21**, 739.
Quastel & Woolf (1926). *Biochem. J.* **20**, 545.
Stephenson (1930). *Bacterial Metabolism*. (London: Longmans, Green & Co. p. 275.)
— & Gale (1937). *Biochem. J.* **31**, 1316.
— & Trim. Private communication: to be published shortly.
Straub (1938). *Nature, Lond.*, **141**, 604.
Virtanen & Tarnanen (1932). *Biochem. Z.* **250**, 193.
Warburg & Christian (1938, 1). *Biochem. Z.* **295**, 261.
— — (1938, 2). *Naturwissenschaften*, **26**, 201.
Woolf (1929). *Biochem. J.* **23**, 472.