LII. FACTORS INFLUENCING BACTERIAL DEAMINATION

II. FACTORS INFLUENCING THE ACTIVITY OF *dl*-SERINE DEAMINASE IN *BACTERIUM COLI*

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IN our previous studies on bacterial deamination by *Bact. coli* [Stephenson & Gale, 1937] we measured deaminase activity in Q_N (γ ammonia-N liberated per mg. dry wt. bacteria per hr.). In the cases of glycine and alanine, we showed that only oxidative deamination occurs, that the Q_N is not influenced by the presence of the substrate in the growth medium and is greatest in aerobic growth conditions, being then about 15 for glycine and about 30 for alanine. These values are reduced by about 70 % by anaerobic growth conditions and by about 95 % by the presence of 2 % glucose in the growth medium. In the case of glutamic acid, the highest Q_N (about 30) is obtained in anaerobic growth conditions while the inhibitory effect of glucose in the growth medium is the same as for the other two amino-acids.

The next two amino-acids to be studied were *l*-aspartic acid and *dl*-serine, the deaminations of which have certain features in common:

1. The Q_N is of a different order from those studied earlier and reaches, in some conditions, values of over 1000 in the case of *dl*-serine.

2. The inhibitory effect of glucose in the growth medium is the same as in the cases previously studied.

3. Anaerobic as well as aerobic deamination occurs.

4. The Q_N varies enormously with the age of the culture and with other factors which are studied in this paper.

The data in this communication refer only to *dl*-serine; the study of *l*-aspartic acid will appear later.

Methods. The two strains of *Bact. coli* used were the same as in our previous work. The bacteria were grown on caseinogen tryptic broth, centrifuged and washed once with water, suspended in water and the strength of the suspension determined by the photoelectric turbidimeter [Clifton *et al.* 1935]. The concentration used varied from 0.1 to 1.0 mg. dry wt. per ml. according to the activity of the preparation. For each determination 1 ml. each of 0.1 *M* phosphate buffer at pH 7.5, 3*M*/250 *dl*-serine and bacterial suspension were taken; for aerobic deamination these were shaken in a bath at 40° exposed to the air; for anaerobic deamination they were placed *in vacuo* in a Thunberg tube; 2 ml. of the mixture were used for each determination of NH_3 by the method of Parnas.

Exp. 1: Fig. 1 shows the course of aerobic and anaerobic deamination by bacteria grown aerobically. The deamination is linear till 50 % of the amino-nitrogen has been liberated, when the rate alters. Probably the two isomerides are attacked at different rates.

In all later work where rates were measured, deamination was not allowed to proceed further than 50% theoretical. Fig. 2 shows the effect of pH on the deamination rate.

Fig. 1.

Fig. 2.

Fig. 1. Course of the aerobic and anaerobic deamination of *dl*-serine by *Bact. coli* I, 1 mg./ml.

Fig. 2. Effect of pH on the rate of deamination of *dl*-serine by *Bact. coli* I.

Fig. 3. 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 , (4) growth curve (strain I).

Exp. 2: Fig. 3 shows the variation in Q_N due to culture time and conditions of aeration. In Curve 1 the culture was grown anaerobically in nitrogen for varying periods; in Curve 2 the growth was carried out aerobically in Roux bottles using also liquid medium. In Curve 3 the organism was grown in 2%

glucose broth with chalk and bubbled continuously with nitrogen. Samples were taken at intervals with sterile precautions; the chalk was removed by centrifuging for 2 min. and pouring off the supernatant liquid, from which the organisms were then removed by centrifuging in the usual way. Curve 4 is the growth curve of a culture in N_2 and shows how optimum activity is related to maximum growth.

This experiment shows, in Curve 1, an increase in Q_N from 300 to 900 between 8 and 10 hr. and a subsequent decrease. The aerobic culture shows a similar variation on a smaller scale. Curve 3 shows that the presence of 2% glucose in the growth medium depresses the Q_N to 10 and this low activity does not vary with age. The high Q_N of *dl*-serine as compared with other amino-acids is noteworthy.

Exp. 3: Decay of Q_N on standing. It was early noted that the deaminase activity in washed suspensions decreases on standing. To illustrate this point a culture was grown for 13 hr. anaerobically and the washed suspension made up in water to 0.2 mg. per ml. The spinning and washing occupied about $1\frac{1}{2}$ hr., and the activity immediately following was $Q_N=590$. The activity declined while the suspension at room temperature was exposed to the air as seen in Fig. 4. The experiment was repeated *in vacuo* in Thunberg tubes and a similar decline was noted.

Fig. 4. Decline in deaminase activity on standing suspension of *Bact. coli* I in water at room temperature.

Exps. 4 and 5: Effect of dilution on "decay" of activity. Table I shows that if the suspension is diluted progressively from 1.7 mg./ml. to 1/50 of that strength and the Q_N immediately determined, no falling off due to dilution occurs; if however the suspensions of different strengths are incubated *in vacuo* for 3 hr. at 37° the decay increases with the dilution, being 30% for the undiluted suspension and 88% for the suspension diluted to 1/100.

Table I. *Effect of instantaneous dilution with water*

Suspension of bacteria (mg./ml.)	Dilution	Q_N
1.71	—	670
0.34	1/5	672
0.171	1/10	681
0.085	1/20	670
0.034	1/50	665

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=681)	Loss of activity %
1.71	—	0.171	476	30
0.34	1/5	0.171	369	45
0.171	1/10	0.171	80	88

Exp. 6: Prevention of "decay" of activity. Here Exp. 5 was repeated using as diluent: (1) a suspension of boiled bacteria (about 3 mg./ml.); (2) a 24-hr. autolysate; (3) a 24-hr. diffusate made at 0°. The cells had been removed from

(2) and (3) by centrifuging. Table II shows that all these diluents prevent "decay". This effect is due to a prevention of "decay" rather than the replacement of a substance removed during incubation, since the addition of boiled bacteria to the suspension, after activity has fallen off, has no immediate restorative effect.

Table II. *Effect of the addition of boiled organism to the suspending medium on "decay" of activity during incubation for 3 hr. at 37°*

Incubated suspension of bacteria (mg./ml.)	Suspending medium	Suspension of bacteria for estimation (mg./ml.)	Q_N initial	Q_N after incubation	Loss %
1.76	Water	0.176	861	579	30
0.176	Water	0.176	861	75	92
0.176	Boiled organism	0.176	760	755	0
0.176	Autolysate	0.176	692	578	16
0.176	Autolysate	0.176	692	616	11
0.15	Diffusate	0.15	728	694	5
0.16	Boiled organism	0.16	714	714	0
0.35	Water	0.176	861	169	80
	Boiled organism now added to "decayed" suspension				
0.176	Boiled organism	0.176	169	167	No change

It appears from Exp. 6 that "decay" of activity is due to the gradual diffusion of some substance from the cell; this substance is present in large quantities in boiled suspensions of cells and in boiled autolysates and diffusates from cells; hence when the bacteria are suspended in such preparations, loss by diffusion is checked and the initial activity maintained. If this is the case, the suspension of boiled bacteria from which the factor has been removed by prolonged diffusion, should be unable to prevent the "decay" of an active suspension. This is shown to be the case in the following experiment.

Exp. 7. A suspension of bacteria of about 100 mg. dry wt. was washed at 0° in 900 ml. of water for 12 hr.; the cells were centrifuged and resuspended as before, 3 washings in all being given. The organisms were finally suspended in 5 ml. water and boiled, and the preparation ("B.D.O.") used to dilute a suspension of freshly grown active cells and the whole incubated *in vacuo* for 3 hr. The results are given in Table III. In water the activity falls 74%, in the presence of boiled diffused cells ("B.D.O.") 55%, whilst in the diffusate (prepared from the concentrated first washings) the loss is only 5%.

Table III

Suspending medium	Suspension of bacteria (mg./ml.)	Q_N initial	Q_N after incubation	Loss %
Water	0.15	730	188	74
"B.D.O."	0.15	684	310	55
Diffusate	0.15	728	694	5

Exp. 8: Effect of phosphate in preventing "decay" of activity. The first substance found which could replace the boiled diffusate in preventing "decay" was phosphate. This is shown in Fig. 5. Here it is seen that *M*/100 phosphate buffer effectively prevents decay but *M*/10 as well as *M*/1000 and *M*/10,000 are ineffective.

Fig. 5. Effect of phosphate concentration on "decay" of activity during 3 hr. incubation at 37° (strain I).

Exp. 9: Effect of cozymase (coenzyme I) in preventing "decay". It was thought possible that the effect of boiled bacteria etc. in preventing "decay" might be due to the loss of a coenzyme. We therefore substituted a cozymase preparation containing about 50 % cozymase: this proved effective in a concentration of 1 mg./ml. but not at lower concentrations. The preparation was equally effective after prolonged boiling at pH 12 when the cozymase would be destroyed; the effect was therefore not due to cozymase itself but to a decomposition product or an impurity. Accordingly the substances listed in Fig. 6 were tested and muscle adenylic acid and glutathione were found to be active. Table IV shows that adenylic acid is effective in a concentration of 1 mg./10⁵ ml. To differentiate further between cozymase and adenylic acid, progressive dilutions of each were made and the prevention of "decay" determined; these results are also shown in Table IV. It was found, however, that with strain II, adenylic acid does not prevent "decay".

Table IV. *Effect of adenylic acid in the suspending medium upon the "decay" of activity during incubation for 3 hr. at 37°*

Suspending medium	Concentration of adenylic acid	Suspension of bacteria (mg./ml.)	Q_N initial	Q_N after incubation	Loss %
Water	—	0.163	528	92	80
Adenylic acid	1 mg./100 ml.	0.163	570	560	1.7
	1 mg./10 ³ ml.	0.163	521	500	3.4
	1 mg./10 ⁴ ml.	0.163	528	530	0
	1 mg./10 ⁵ ml.	0.163	520	510	1.7
	1 mg./10 ⁶ ml.	0.147	375	310	17.0

Effect of cozymase in the suspending medium on the "decay" of activity during incubation for 3 hr. at 37°

Suspending medium	Concentration of cozymase	Suspension of bacteria (mg./ml.)	Q_N initial	Q_N after incubation	Loss %
Water	—	0.173	428	173	60
Cozymase	1 mg./10 ⁴ ml.	0.152	320	188	40
	1 mg./10 ⁵ ml.	0.152	307	134	56
	1 mg./10 ⁶ ml.	0.152	281	122	60

Final concentration in all cases = 1 mg./l.

Fig. 6. Effect of various agents in preventing "decay" of activity during 3 hr. incubation in *vacuo* at 37° (strain I).

Exp. 10: Effect of reducing agents in preventing decay. The effect of glutathione (GSH) has already been mentioned. It was further noticed that "decay" occurred in air, in N_2 and in *vacuo*, but not in H_2 ; this is shown in Table V. The action of H_2 must in this case be attributed to its action as a

Table V. Effect of hydrogen in preventing "decay" of activity

	Initial	Q_N
(1) After standing 3 hr. in <i>vacuo</i>		550
(2) After standing 3 hr. in H_2		204
(3) Suspension (1) after standing with H_2 overnight		530
		395

Table VI. Effect of formate system on "decay" of activity

Suspending medium	Suspension of bacteria (mg./ml.)	Q_N initial	Q_N after incubation	Loss %
Water	0.184	801	0	100
$M/2 \times 10^2$ formate	0.184	625	0	100
$M/10^4$ phosphate	0.184	731	0	100
$M/2 \times 10^2$ formate + $M/10^4$ phosphate	0.184	720	475	33

Fig. 7.

Fig. 7. Effect of reducing systems on "decay" of activity during 3 hr. at 37° (strain I).

Fig. 7a.

Fig. 7a. Factors in the "decay" of activity during 3 hr. incubation at 37° (strain II).

reducing agent and not to its anaerobic effect. In a few cases, a suspension whose activity had "decayed" was found to regain some of its activity on standing in H_2 ; this is also shown in Table V. Fig. 7 shows the action of other reducing agents of which GSH is the most effective. Other dehydrogenase systems were also tested (Table VI). Here it is seen that formate alone has no effect but that, in the presence of $M/10,000$ phosphate which alone is inactive, the system prevents "decay". Fig. 7a shows the effect of phosphate, GSH and formate with strain II.

It is necessary here to differentiate between the mode of action of the various substances and systems which prevent "decay". Hitherto the occurrence and prevention of "decay" have both taken place at 37° . If the various systems are tested at 0° it is found (Fig. 8) that adenylic acid alone (with strain I) is effective whereas $M/100$ phosphate and all the reducing systems are now inactive. This suggests that adenylic acid acts by preventing the outward diffusion of some active substance whilst the other systems owe their activity to enzyme action.

Exp. 11: The effect of "age" of culture on subsequent "decay". During the course of the work we sometimes obtained suspensions which did not "decay"

Fig. 8. Loss of activity during 3 hr. at 0° (strain I).

Fig. 9. Effect of growth conditions and "age" on activity (Q_N) and "permeability". (1) Activity of culture grown in N_2 , (2) "Permeability" of culture grown in N_2 , (3) Activity of culture grown in H_2 , (4) "Permeability" of culture grown in H_2 .

when diluted and incubated. This irregularity depended on the time during which the culture was grown. This was tested as follows: the organism was

grown in broth anaerobically in H_2 and samples were removed at intervals, spun and washed. The immediate activity was determined, the suspension then incubated *in vacuo* for 3 hr. and the % loss of activity determined. The results are shown in Fig. 9. Here it is seen that the 10–12 hr. culture loses its activity most readily whilst younger and older cultures are relatively stable. If the organism is grown in N_2 , the loss of activity is less. As a tentative working hypothesis we regard the “decay” as due to a diffusion of a substance out of the cell and differences in the rate of “decay” as due to differences of permeability.

Exp. 12: Fig. 9 also shows the variation of activity with “age” of culture and shows that the period of maximum “permeability” coincides with maximum activity. Since the activities (Q_N) are measured after centrifuging and washing, they are probably lower than those of the cells actually in the culture fluid. This is especially the case with the organism grown in H_2 where the permeability is highest (100 % loss of activity in 3 hr.). The apparent difference in activity of the cultures grown in H_2 and N_2 is therefore an experimental artefact due to the greater loss of activity of the H_2 culture during centrifuging and washing; the difference between the two curves is greatest at the period of maximum permeability, the early and late portions following each other closely. All subsequent experiments were done with cells whose activity and permeability were at their maximum, i.e. 11 hr. cultures grown anaerobically in H_2 .

Exp. 13: Recovery of activity of suspension after “decay”. The first successful experiment in effecting recovery after “decay” is set out in Table VII. After decay at 0° , incubating with boiled cells and $M/100$ phosphate restores 80 % of the original activity; whilst if the same process is carried out at 0° no appreciable recovery occurs. If on the other hand, decay takes place at 37° , instead of at 0° , no recovery can be effected.

Table VII. *Restoration of activity with boiled organism and phosphate*

Bact. coli grown anaerobically in H_2 . Washed suspension = 0.18 mg./ml.

	Q_N
Initial	423
After 3 hr. <i>in vacuo</i> at 0°	0
Boiled organism and $M/100$ phosphate* added. After incubation <i>in vacuo</i> for 2 hr. at 37°	346
Initial	709
After 3 hr. <i>in vacuo</i> at 0°	347
Boiled organism and $M/100$ phosphate* added. After standing <i>in vacuo</i> for 2 hr. at 0°	362

* Final concentrations.

Exp. 14: Factors affecting recovery. In each of 7 Thunberg tubes were put 4 ml. of water and 0.5 ml. bacterial suspension prepared as usual (1.7 mg./ml.; initial Q_N 524). These were evacuated and kept for 3 hr. at 0° . The Q_N was then 287. To the Thunberg tubes (1–7) 0.5 ml. of the following was added: (1) water; (2) boiled bacteria in $M/10$ phosphate; (3) boiled bacteria in water; (4) phosphate to bring to $M/10$; (5) $M/10$ phosphate; (6) $M/100$ phosphate; (7) $M/10,000$ phosphate. The tubes were evacuated and kept at 37° for 2 hr. The results are shown in Fig. 10. The boiled bacteria in absence of phosphate produce no recovery but prevent further decay; boiled bacteria in $M/100$ phosphate produce

complete recovery; $M/100$ phosphate alone produces partial recovery; $M/1000$ phosphate produces no recovery but arrests further decay whilst $M/100,000$ phosphate has no effect.

Fig. 10. Factors in recovery—effect of boiled bacteria and phosphate (strain I).

Exp. 15: Further factors in recovery. The procedure in this experiment was the same as for Exp. 14. Here the effect of boiled bacteria in $M/100$ phosphate is duplicated by $M/30,000$ GSH in $M/100$ phosphate, GSH alone and adenylic acid with and without phosphate being without effect. These results are shown in Fig. 11. Fig. 12 shows the rate of recovery with $M/100$ phosphate and different concentrations of GSH. The flattening of the curves in Fig. 12 is attributable to the effect of the temperature; slow recovery never attains completion owing to the effect of temperature on permeability.

Fig. 11.

Fig. 11. Factors in recovery (strain I).

Fig. 12. Effect of concentration of GSH on rate of recovery (strain I). (1) $M/100$ phosphate, (2) $M/100$ phosphate + $M/3 \times 10^4$ GSH, (3) $M/100$ phosphate + $M/3 \times 10^4$ GSH.

Fig. 12.

Exp. 16: Fig. 13 shows that with $M/100$ phosphate and $M/30,000$ GSH restoration may attain completion before the effect of temperature on permeability sets in. The question arises as to whether the recovery produced by

$M/100$ phosphate alone is due to phosphate only or to phosphate and residual reducing material remaining in the cell after "decay". We attempted to remove reducing substances completely from the cell by allowing "decay" to take place in air instead of *in vacuo*; the procedure then was the same as in Exps. 14 and 15. Fig. 14 shows that in this case, $M/100$ phosphate alone produces no recovery but that $M/100$ phosphate with GSH, or $M/100$ phosphate with boiled bacteria, is effective.

Fig. 13.

Fig. 14.

Fig. 13. Effect of phosphate and phosphate + GSH on recovery (strain I).

Fig. 14. Factors in recovery—recovery after aerobic decay (strain I).

Fig. 15. Factors in recovery—reducing systems (strain I).

Exp. 17: Recovery by other reducing systems. The procedure was the same as in Exps. 14 and 15, and the results are given in Fig. 15. Here recovery was obtained with cysteine and lactate in the presence of phosphate but less completely than with GSH and phosphate. Formate and phosphate produce a

recovery which goes beyond the initial value. It must be remembered however that decay sets in during the initial spinning and washing of the suspension and the extra recovery with formate and phosphate represents a return to the value of the cells in broth culture. In order to test this, a broth culture prepared in the standard way was divided into two parts *A* and *B*. *A* was spun and washed in water in the usual way. To *B* was added formate to *M*/200 and phosphate to *M*/100 before centrifuging, and the same additions were made to the washing water. The results are given in Fig. 16 together with the recovery obtained with

Fig. 16. Factors in recovery—action of reducing systems (strain I).

Fig. 16a. Factors in recovery (strain II). Effect of incubating with various agents for 1 hr. at 37° in vacuo.

M/100 phosphate + GSH and with *M*/100 phosphate + formate. It is seen that, as predicted, loss of activity occurs in two stages: aerobically during the spinning and washing and anaerobically later in the experiment. The GSH-phosphate system recovers the later loss; the formate-phosphate system recovers both losses. Fig. 16 *a* shows the factors in recovery with strain II.

The variability of activity with culture time still awaits explanation. Fig. 3 shows that a 6-hr. culture has an activity far below a 10-hr. culture. We attempted to raise the Q_N of the 6-hr. culture by the addition of formate and phosphate to the culture medium and the washing water (Table VIII). Although this procedure raises the Q_N , the height attained never approaches that of the 10-hr. culture in plain broth. In other words, the early cultures appear to be comparatively impermeable towards the phosphate-formate system.

Table VIII. *Effect of the formate-phosphate system on "young" cultures*

Cultures grown for 6 hr. anaerobically in N_2 :	Q_N
A. Grown in plain broth—washed in water	181
B. Grown in plain broth—washed in the presence of formate and phosphate	179
C. Grown in broth plus $M/200$ formate and $M/100$ phosphate—washed in water	286
D. Grown in broth plus $M/200$ formate and $M/100$ phosphate—washed in the presence of formate and phosphate	229
E. Grown in broth plus $M/100$ phosphate—washed in water	217
F. 10 hr. culture in plain broth (for comparison)	1100

DISCUSSION

The decay of *dl*-serine deaminase appears to be due to a loss from the cell by diffusion of some substance or substances acting as coenzyme. This loss can be prevented by exudate from boiled organism, by $M/100$ phosphate or by keeping the cell in a reducing medium; this suggests that the coenzyme may exist in reduced and oxidized forms and in phosphorylated and non-phosphorylated forms, the reduced phosphorylated form being relatively stable and non-diffusible. On this hypothesis standing in water causes the coenzyme to become oxidized (anaerobically) and to lose phosphate. Recovery is brought about by various reducing systems in the presence of phosphate but not in its absence, the slow incomplete recovery induced by phosphate alone is attributable to some reducing agent remaining in the cell and does not occur if this is removed by aeration, recovery then occurring only with phosphate and a reducing agent (GSH). Recovery occurs at 37° but not at 0° , which suggests that an enzymic process is involved.

The marked effect of very low concentrations of muscle adenylic acid in preventing both decay and recovery with strain I, and in preventing recovery with strain II, is unexplained on this hypothesis. We can also offer no explanation for the effect of high concentrations of phosphate in preventing recovery; it has been suggested that this may be due to removal of magnesium, but we have been unable to confirm this.

Both decay and recovery depend not only on chemical reagents but also on the condition of the cell. Deamination is most active when the cells are grown anaerobically and towards the close of the growth period; it is also then most subject to rapid decay and effective recovery; cultures grown in H_2 decay and recover more rapidly than those grown in N_2 ; we tentatively attribute these differences to changes in permeability. Recovery depends also on temperature; suspensions which decay at 0° can recover but if decay occurs at 37° recovery does not take place; provisionally we attribute this also to an alteration of permeability.

SUMMARY

1. *dl*-serine is deaminated by washed suspensions of *Bact. coli* both aerobically and anaerobically; both isomerides are attacked though probably at different rates.

2. The activity (Q_N) of serine deaminase is very high and varies with the age of the culture from 200 to 1000; it attains its height at 11 hr. and subsequently falls off. It is increased by anaerobic growth conditions and, like other deaminases, is decreased 95 % or more by the presence of 2 % glucose in the growth medium.

3. Washed suspensions lose their activity on standing in water either at 0° or at 37° aerobically or anaerobically.

4. Decay of activity occurs more rapidly in dilute than in concentrated suspensions.

5. Decay of activity (during 3 hr. at 37°) can be prevented by adding boiled suspensions of the organisms or by autolysate or diffusate from which the cells have been removed; also by *M*/100 phosphate, and by various biological reducing systems including GSH, H_2 and formate in the presence of phosphate.

6. If decay of activity occurs at 0° it can be recovered, but not if it occurs at 37°.

7. Agents producing recovery are boiled bacteria, *M*/30,000 GSH, *M*/200 formate; all these require phosphate. Phosphate alone (*M*/100) produces a slow and incomplete recovery if decay has occurred anaerobically but not if it has occurred aerobically.

8. Both decay and recovery depend also on the condition of the cell; cultures grown in H_2 at the peak of their activity decay most rapidly and recover most completely.

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