AMINOACID DEAMINATION BY THERMOPHILIC BACTERIA—PART I

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The studies on bacterial deamination have in general not been carried out as widely as studies on tissue deamination. Harden (1901) reported the observation that *Bacteria coli* cultures in glucose broth deaminate aspartic acid. Quastel and Woolf (1926) showed that *Bact. coli* could bring about the following reactions:

(1) $COOH.CH_2.CH.NH_2.COOH \rightarrow COOH.CH_2.CH_2.COOH + NH_3$
Aspartic acid — Succinic acid

(2) $COOH.CH_2.CH.NH_2.COOH \rightarrow COOH.CH=CH.COOH + NH_3$
Aspartic acid — Fumaric acid

The first of these reactions takes place anaerobically and in the absence of inhibitors whereas in the presence of inhibitors the deamination of aspartic acid proceeds according to the second equation. Virtanen and Tarnanen (1932) found that *Bacteria fluorescens liquefaciens* could deaminate aspartic acid, the optimum pH was 7·0 to 7·5 and no other aminoacid was deaminated. Strickland (1934) has studied the anaerobe *Clostridium sporogenes* and has found that its suspensions deaminate alanine optimally at pH 7·5. Bernheim *et al.* (1935) examined the deamination of aminoacids by resting cell suspensions of *B. proteus*. Webster and Bernheim (1936) have studied the oxidation and deamination of a number of aminoacids by *B. pyocyaneus*. Janke and Tyenthal (1936) have found that *B. coli, B. vulgar, B. mycoides,* and *Pseudomonas flourescens* oxidatively deaminate glycine. Woods and Cliften (1937) report that *Clostridium tetanomorphum* anaerobically deaminates as well as decarboxylates a large number of aminoacids. Stephenson and Gale (1937), Gale and Stephenson (1938) and Gale (1938) have also made a comprehensive study of the deaminating activity of *B. coli*; the optimum pH for deamination of various aminoacids, the effect of addition of various compounds and the decay of activity of washed suspensions was studied. Hills (1940) has studied aminoacid deamination by certain bacteria, while Desnuelle and Fromageot (1939) have studied the anaerobic bacterial degradation of cystine and cysteine.

The present study is concerned with deamination of aminoacids by thermophilic bacteria. These bacteria have the peculiar property of growing

at high temperatures and this renders them an intriguing subject for study. They are fairly widely distributed in soil (Glebig, 1888; Rabinowitsch, 1895; Weinzirl, 1919; and Bergey, 1919), and undoubtedly play a part in several soil and agricultural reactions. In Beccari system of garbage fermentation temperatures above 50° C. are reached and thermophilic bacteria participate in the liquefaction of proteins in the first stage and in the decomposition of the resulting aminoacids in the second stage. In the thermogenic fermentation of tobacco, aminoacids are first formed from proteins and are then deaminated.

Methods of Analysis and General Experimental Methods

In most of the experiments here described the organisms were used in the form of washed suspensions. The bacteria were grown in nutrient broth, reaped by centrifuging at 3,000 r.p.m. for fifteen minutes and washed once with distilled water. The bacterial mass was then made into an aqueous suspension which was rendered as homogeneous as possible by shaking. Equal volumes of this suspension withdrawn after shaking produced nearly the same amount of deamination.

For most experiments it was not necessary to determine the quantity of cells per ml. of the suspension. Where experiments had to be made to compare the relative deaminating ability of cells cultivated in different ways, it was necessary to standardise the suspensions. For this purpose an aliquot volume of the suspension was filtered through a tiny porcelain filter fitted into the side arm of a microbeaker; all the cells were retained in the beaker and their weight was determined by difference after washing with distilled water and desiccating the beaker and contents *in vacuo* over phosphorous pentoxide and caustic soda at room temperature to constant weight. The average deviation in values obtained in duplicate estimations of this nature was less than 3·3% and the differences in deaminating ability of cells grown in different ways, where significant, were at least 50 to 100%. This gravimetric process of standardisation had to be adopted because the suspensions, although macroscopically uniform, still contained aggregates of large number of cells which settled under gravity rather too rapidly to allow of accurate results being obtained by nephelometric methods, besides the disaggregation of the cells in the suspension which also determines the opacity could never be always reproduced. Counting of cells either after staining or on plating also gave inaccurate results for similar reasons. Nephelometric methods and counting procedures could only be applicable in cultures whose age was twenty-four hours or less, because in such cultures the cells had not yet aggregated themselves into film-like structures.

Deamination of aminoacids can be quantitatively followed by estimating either any of the reactants or any of the products of reaction. Were it not for complications due to side reactions estimation of oxygen consumed, ketoacid or other nitrogen-free acid formed, loss of α-amino nitrogen, or ammonia produced could all be used for quantitatively studying deamination. Although suspensions of what may be called " resting cells " were used in the present study, there is always the possibllity that oxygen is being consumed both in the presence and in the absence of aminoacids in reactions quite distinct from pure deamination. The ketoacids produced are often degraded further or used up in the formation of more complex carbohydrate like substances in a manner which is only imperfectly understood at present. On account of these limitations estimation of ammonia produced or α-amino nitrogen disappeared gives the best possible approximation of the extent of deamination. It is unlikely that the ammonia liberated enters into any side reactions and if there is no significant back reaction then the whole of the ammonia produced will be present as such in the reaction mixture. During the investigations on bacterial deamination here described attempts were made to reverse the process of deamination, for example in case of aspartic acid by starting with succinic, oxalacetic or fumaric acids and ammonium chloride but no decrease in free ammonia could be detected at all the hydrogen-ion concentrations. Errors due to back reaction may therefore be ruled out. The rate of ammonia formation in an aminoacid bacterial cell mixture, corrected for the blank value due to autodeamination of the cells themselves therefore represents very nearly the actual rate of deamination of the aminoacid. In the present experiments the rate of formation of free ammonia corresponded to the rate of decrease of α-aminoacids as measured in the Van Slyke apparatus. Both methods gave comparable results, but the former method was standardised for the purpose of detailed study of deamination. Estimation of ammonia was carried out in a microkjeldahl apparatus of the Parnas type. The reacting mixture was brought to pH 10–11 and then steam-distilled for three to five minutes, the distillate being received either in M/100 sulphuric acid or in a saturated solution of boric acid as usual.

The experimental technique employed was very simple. The organisms were cultivated in nutrient broth by incubating at their respective optimum temperatures. The cells were collected by centrifuging the cultures at 3,000 r.p.m. for fifteen minutes. After washing once in the centrifuge the bacterial mass was resuspended in a known volume of distilled water and the suspension made homogeneous by shaking. Known volume of the suspensions and the aminoacid solution made at the stated pH in McIlvaine's

citrate-phosphate buffer were mixed and the increase in free ammonia was estimated after incubation at 40° C. for stated lengths of time. Careful blank experiments were performed simultaneously and proper corrections for auto-deamination of the cells were made. Where delay in carrying out the estimation was unavoidable the reaction was stopped after the stated period by adding 0·100 ml. of 4 M hydrochloric acid to every 10 ml. of the mixture of aminoacid solution and bacterial suspension. This also fixed up the free ammonia so that no loss could take place. The reactions were all carried out in either stoppered hard-glass test-tubes or conical flasks.

Deamination of dl-*aspartic acid,* dl-*alanine, glycine,* dl-*glutamic acid and* dl-*leucine*

The bacteria were cultivated in nutrient broth by incubation at 50° C. for 48 hours and were reaped by centrifuging. The cells were washed and made up into a uniform suspension in distilled water. 5 ml. of this suspension were added to 20 ml. of M/66 solution of the aminoacid made at the required pH in McIlvaine's citrate-phosphate buffer and the mixture was shaken. A 5 ml. portion of the mixture was withdrawn immediately and analysed for ammonia content. The remainder was incubated for 24 hours at 40° C. in well stoppered test-tubes after which the reaction was stopped by adding a drop of pure hydrochloric acid and ammonia estimation was again carried out in duplicate. Blank experiments were conducted at all hydrogen-ion concentrations in two sets. In the first set the bacterial suspension was replaced by 5 ml. of distilled water and in the second the aminoacid solution was replaced by an equal volume of distilled water. The data reported below have been corrected for the two blank values.

The optimum pH for the deamination of *dl*-aspartic acid by *Bacillus thermophilus, B. ærothermophilus* and *B. thermoacidurans* is, therefore 7·7, 7·7 and 8·0 respectively, and for *dl*-alanine 8·4, 7·9 and 8·1 respectively; glycine is deaminated optimally at pH 7·9, 7·5 and 8·3 respectively; *dl*-glutamic acid at pH 7·2, 7·5 and 8·0; and *dl*-leucine at pH 7·4, 7·2 and 7·9 respectively.

Relative rate of deamination of dl-*aspartic acid,* dl-*alanine,* dl-*glutamic acid, glycine and* dl-*leucine*

In order to determine the relative ease with which the various aminoacids are deaminated equal volumes of the different bacterial suspensions were added to solutions of the different aminoacids prepared at their respective pH optimas for deamination. The amount of ammonia liberated on incubation was determined as usual and corrected for blanks. The results are summarised in Table II.

TABLE I

Ammoniacal nitrogen liberated in 24 hours, mgm./5 ml. of cell suspension

pH	*B. thermophilus*	*B. aerothermophilus*	*B. thermoacidurans*
	dl-*Aspartic acid*		
6·5 ..	0·203	0·193	0·163
7·2 ..	0·492	0·410	0·295
7·9 ..	0·662	0·586	0·513
8·6 ..	0·440	0·402	0·405
9·3 ..	0·180	0·175	0·232
10·0 ..	0·076	0·008	0·071
	dl-*Alanine*		
6·5 ..	0·170	0·144	0·105
7·2 ..	0·215	0·372	0·238
7·9 ..	0·300	0·506	0·495
8·6 ..	0·523	0·370	0·442
9·3 ..	0·283	0·130	0·201
10·0 ..	0·092	0·065	0·072
	Glycine		
6·5 ..	0·105	0·104	0·095
7·2 ..	0·189	0·386	0·206
7·9 ..	0·286	0·384	0·368
8·6 ..	0·178	0·112	0·372
9·3 ..	0·108	0·080	0·195
10·0 ..	0·040	0·006	0·082
	dl-*Glutamic acid*		
6·5 ..	0·172	0·125	0·063
7·2 ..	0·286	0·248	0·125
7·9 ..	0·170	0·242	0·205
8·6 ..	0·105	0·128	0·172
9·3 ..	0·062	0·062	0·092
10·0 ..	0·005	0·015	0·022
	dl-*Leucine*		
6·5 ..	0·195	0·201	0·182
7·2 ..	0·482	0·325	0·281
7·9 ..	0·366	0·208	0·314
8·6 ..	0·212	0·116	0·286
9·3 ..	0·085	0·032	0·161
10·0 ..	0·010	0·000	0·008

TABLE II

Aminoacid	Ammoniacal nitrogen liberated in 24 hours by the different bacteria, mgm./5 ml. of cell suspensions		
	B. thermophilus	*B. aerothermophilus*	*B. thermoacidurans*
dl-Aspartic acid ..	0·810	0·630	0·700
dl-Alanine ..	0·460	0·370	0·390
Glycine ..	0·300	0·265	0·280
dl-Glutamic acid ..	0·305	0·350	0·260
dl-Leucine ..	0·665	0·650	0·625

It is therefore obvious that the relative ease of deamination of the different aminoacids does not vary from bacteria to bacteria under study. *dl*-Aspartic acid and *dl*-leucine are always the easiest to deaminate followed by *dl*-alanine, while glycine and *dl*-glutamic acid are the most difficult to deaminate.

Effect of anaerobic conditions on the deamination of dl-*aspartic acid*

In order to find out whether deamination requires the presence of oxygen parallel experiments were conducted under atmospheric conditions and in an hydrogen atmosphere. For experiments under hydrogen atmosphere the aminoacid solutions were deaerated thoroughly by vacuum and bubbled through with pure hydrogen. This process was repeated thrice. The bacterial suspension was also deaerated, measured out and mixed rapidly with the aminoacid solution, the mixture again deaerated and bubbled through with pure hydrogen after which the flasks were sealed off and incubated. Proper corrections were made for blanks. The aminoacid used was *dl*-aspartic acid.

TABLE III

Organism	Ammoniacal nitrogen liberated in 24 hours under atmospheric conditions, mgm./10 ml. of mixture	Ammoniacal nitrogen liberated in 24 hours under hydrogen atmosphere, mgm./10 ml. of mixture
B. thermophilus ..	0·300	0·105
B. aerothermophilus	0·245	0·070
B. thermoacidurans	0·215	0·005

Deamination by these organisms is therefore an oxidative process and is restricted by absence of oxygen. The slight apparent increase in ammoniacal nitrogen under hydrogen atmosphere is probably due to traces of oxygen which could not be driven out in the process of deaeration and bubbling through with hydrogen.

Effect of presence of particular aminoacids in growth medium on the deaminating capacity of cells grown therein

If bacterial enzyme systems are adaptive in nature then it may be expected that if an aminoacid is added to the medium in which the bacteria are cultivated then the ability of cells obtained from such cultures to deaminate the particular aminoacid will be greater as compared to the deaminating ability of cells which have been grown in media without the aminoacid. This question was examined in case of *dl*-aspartic acid and *dl*-alanine with all the three bacteria and the results are summarised in Table IV. Cells obtained from nutrient broth containing *dl*-aspartic acid were tested for deaminating

ability against *dl*-aspartic acid alone, those obtained from broth containing *dl*-alanine were tested against *dl*-alanine only and cells obtained from plain broth were tested, for comparison, both against *dl*-alanine and *dl*-aspartic acid. Blank corrections were made for any increase in ammoniacal nitrogen in absence of the bacterial cells and for auto-deamination of the cells.

TABLE IV

Organism	Nature of medium	Mgm. dry wt. of the suspension per 5 ml.	Mgm. ammoniacal nitrogen liberated by 5 ml. of the suspension		Mgm. ammoniacal nitrogen liberated per 10 mgm. dry wt. of cells	
			dl-Aspartic acid	*dl*-Alanine	*dl*-Aspartic acid	*dl*-Alanine
B. thermophilus ..	Plain nutrient broth	12·66	0·667	0·532	0·527	0·420
	Nutrient broth with 1% *dl*-aspartic acid	13·89	0·987	..	0·718	..
	Nutrient broth with 1% *dl*-alanine	10·15	..	0·886	..	0·872
B. aerothermophilus	Plain nutrient broth	14·15	0·596	0·515	0·421	0·364
	Nutrient broth with 1% *dl*-aspartic acid	10·98	0·685	..	0·624	..
	Nutrient broth with 1% *dl*-alanine	13·20	..	0·697	..	0·528
B. thermoacidurans	Plain nutrient broth	18·78	0·823	0·718	0·438	0·382
	Nutrient broth with 1% *dl*-aspartic acid	16·85	1·057	..	0·627	..
	Nutrient broth with 1% *dl*-alanine	12·16	..	0·965	..	0·793

It is obvious from Table IV that in case of the three thermophilic bacteria presence of any aminoacid in the medium in which the bacteria were grown increases their ability to deaminate this particular aminoacid.

Effect of oxidation-reduction reagents on deamination

It has been mentioned before that deamination of aminoacids by washed suspensions of the thermophilic bacteria is probably an oxidative process because it is suppressed by the careful exclusion of oxygen. Hydroquinone was found, as expected, to suppress the course of deamination of *dl*-aspartic acid by all the four bacteria. Methylene blue was rapidly reduced to the leuco base by mixtures of cell suspensions and the aminoacid, the cells alone possessed only slight capacity to reduce the methylene blue. This shows that

the aminoacid serves directly or indirectly as the hydrogen donating system. In spite of this the speed of deamination was not accelerated by the presence of methylene blue. Care was taken in all these experiments to ensure that the optimum pH was not altered by the addition of methylene blue or hydroquinone. The aminoacid employed was *dl*-aspartic acid at optimum pH.

TABLE V

Effect of hydroquinone on deamination

Organism	Concentration of hydroquinone in the mixture	Mgm. of ammoniacal nitrogen liberated per 5 ml. of cell suspension
B. thermophilus ..	Nil	0·537
	M/200	0·132
B. aerothermophilus ..	Nil	0·519
	M/200	0·337
B. thermoacidurans ..	Nil	0·425
	M/200	0·234

TABLE VI

Effect of methylene blue on deamination

Organism	Concentration of methylene blue in the mixture	Mgm. of ammoniacal nitrogen liberated per 5 ml. of cell suspension
B. thermophilus ..	Nil	0·532
	M/550	0·547
	M/250	0·543
B. aerothermophilus ..	Nil	0·521
	M/550	0·501
	M/250	0·473
B. thermoacidurans ..	Nil	0·435
	M/550	0·411
	M/250	0·389

Effect of narcotics on bacterial deamination

Chloroform and toluene were the two reagents tested. 0·5 ml. of each was added to each mixture consisting of 5 ml. washed cell suspension, 10 ml. M/40 solution of *dl*-aspartic acid and 10 ml. of buffer and the mixture was shaken. Controls received no toluene or chloroform.

Toluene and chloroform therefore destroy the deaminating capacity of freshly prepared suspensions of all three bacteria. The effect of narcotics on an old suspenison is however, not so striking.

TABLE VII

Organism	Mgm. of ammoniacal nitrogen liberated per 5 ml. of cell suspension		
	In absence of chloroform or toluene	In presence of chloroform	In presence of toluene
B. thermophilus—Fresh suspension ..	0·495	0·000	0·072
Do. 5-day old suspension ..	0·473	0·325	0·380
B. aerothermophilus—Fresh suspension ..	0·600	0·013	0·022
Do. 5-day old suspension ..	0·645	0·406	0·387
B. thermoacidurans—Fresh suspension ..	0·586	0·007	0·012
Do. 5-day old suspension ..	0·617	0·484	0·502

DISCUSSION AND SUMMARY

Broadly speaking biological deamination of aminoacids is generally of three types:

(1) Oxidative deamination:

$$R.CH(NH_2).COOH + O \rightarrow R.CO.COOH + NH_3$$

(2) Reductive deamination:

$$R.CH(NH_2).COOH + H_2 \rightarrow R.CH_2.COOH + NH_3$$

(3) Hydrolytic deamination:

$$R.CH(NH_2).COOH + H_2O \rightarrow R.CH(OH).COOH + NH_3$$

Washed suspensions of the thermophilic bacteria *Bacillus thermophilus*, *B. aerothermophilus* and *B. thermoacidurans* possess the property of deaminating a number of aminoacids, for example, aspartic acid, alanine, glycine, glutamic acid and leucine. This deamination is of the oxidative type because it is strongly suppressed by the exclusion of oxygen. This property of oxidative deamination makes these bacteria important factors in soil ammonia formation and in the various biological processes in which ammonia is formed from aminoacids, especially at relatively high temperatures, for example in garbage and silo fermentation, sewage disposal, etc. The optimum pH of deamination varies with the different bacteria under study and with the different aminoacids; but is always in the alkaline region. In natural processes where deaminating ability of these organisms is called into play the reaction of the medium is generally either neutral or slightly acidic. But as soon as a little ammonia has been produced the reaction would start getting more and more alkaline thus favouring the production of more ammonia. As was to be expected the capacity of these bacteria to deaminate any particular aminoacid is enhanced if that aminoacid is present in the growth medium. As the deamination brought about by these bacteria

is of oxidative type it is suppressed in the presence of an hydrogen donating reagent like hydroquinone; but contrary to expectations methylene blue does not favour deamination. Toluene and chloroform destroy the deaminating capacity of fresh cell suspensions of these bacteria but only partially suppress that of five-day old suspensions.

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