128. AMMONIA PRODUCTION BY PATHOGENIC BACTERIA

By GEORGE MASON HILLS[1]

From the Department of Bacterial Chemistry (Medical Research Council), Bland-Sutton Institute of Pathology, and from the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1

(*Received 3 May 1940*)

THE discovery of glutamine as a growth factor for *Streptococcus haemolyticus* [McIlwain *et al.* 1939] and the occurrence of hydrolytic and synthetic glutaminase in those animal tissues, kidney and liver [Krebs, 1935] which contain the most active deaminases [Krebs, 1933], suggested that glutamine might play a part in the metabolism of amino-acids by streptococci, possibly as a carrier of labile NH_3. It was considered desirable therefore to examine the deamination of amino-acids by the organism since no interest has been shown in this field, though a soluble protease has been extracted by Stevens & West [1922]. Foster [1921] showed that NH_3 was liberated coincidently with disappearance of NH_2-N during the period of most active growth on glucose-broth or glucose-serum-broth, though subsequently NH_2-N reappeared without disappearance of NH_3. This was assumed to be due to continued proteolysis without further utilization of amino-acids. Out of 101 substrates tested as H-donators to indigo-tetrasulphonate, 71, including the amino-acids, were found by Farrell [1935] to be inactive. The present work reconciles these findings, since it is shown that out of 22 α-amino-acids only arginine yields significant amounts of NH_3 and the reaction is not oxidative but hydrolytic. As the same behaviour was shown by streptococci of diverse habitat and nutritional requirements, the study was extended to staphylococci. Minor differences were shown but arginine was quantitatively the most important source of NH_3, urea, the next best, being not more than half as active.

The observations of Fildes [1934] and Knight [1936] suggested that this restricted metabolic activity might be related to a habitat in association with the products of animal metabolism with the ultimate development of pathogenicity in many of the organisms examined. Comparative data for representative human and animal pathogens of other genera were lacking; early work on amino-acid breakdown was restricted to chemical identification of the products of putrefaction, often by mixed cultures of unidentified organisms, while the recent work of the Cambridge school, under better controlled conditions, has dealt almost entirely with strict anaerobes and *Bacterium coli* (for bibliography see Stephenson [1939]). Experiments on the meningococcus and the diphtheria and typhoid organisms were therefore carried out. The first two showed even more restricted chemical activity than the gram-positive cocci previously examined and the last, though an active NH_3 producer, resembled *Bact. coli*, forming little NH_3 from arginine.

To find how far the breakdown of arginine was a constant character of gram-positive cocci, experiments were carried out with a strain of staphylococcus trained to grow on a synthetic medium with NH_4^+ salts as the main source of N [Gladstone, 1937, 2]. With such a strain it is possible to alter at will any adaptive

[1] Whole-time worker for the Medical Research Council.

enzymes by controlled variation of the medium with little change in the total growth to complicate interpretation of results. *Bact. typhosum* has also been trained to dispense with a preformed supply of amino-acids [Fildes *et al.* 1933] and the effect of training was also observed in this case.

Technique

(1) *Organisms.* The strains used were:

1. *Str. haemolyticus.* Richards. Group A from Dr L. Colebrook.
2. *Str. haemolyticus.* Kenny. Group B from Dr L. Colebrook.
3. *Str. haemolyticus.* N. Morris. Group B from Dr L. Colebrook.
4. *Str. haemolyticus.* Batty. Group B from Dr L. Colebrook.
5. *Str. viridans.* N.C.T.C. No. 3165.
6. *Str. faecalis.* Freshly isolated by Dr G. P. Gladstone.
7. *Str. lactis.* N.C.T.C. No. 2700.
8. *Staph. aureus.* Strain No. 1 of Gladstone [1937, 2].
9. *Staph. aureus.* Trained from No. 8 [Gladstone, 1937, 2].
10. *N. intracellularis.* N.C.T.C. No. 3375.
11. *Bact. typhosum.* Rawlings. N.C.T.C. No. 160.
12. *Bact. typhosum.* Trained from No. 11 [Fildes *et al.* 1933].
13. *C. diphtheriae.* Mitis strain from Dr A. B. Rosher.

(2) *Media for growth.* The basal medium used for general purposes was, with the addition of glutamine, designed for the growth in bulk of group A strains of haemolytic streptococci in the light of the findings of Fildes & Gladstone [1939] and McIlwain [1939; 1940]. It consisted of:

Bacto-peptone	10 g.	in 250 ml. H_2O
NaCl	5 g.	
KH_2PO_4	4·5 g.	
Na glutamate	1·7 g.	
Cystine	0·05 g.	
N NaOH	28 ml.	
Yeast extract	50 ml.	

The mixture, adjusted to *p*H 7·6, together with various additions usually made after autoclaving, gave 1 l. medium. The organisms were grown in 250 ml. conical flasks, each containing 100 ml. medium final volume. The additions were, per flask, 1 ml. 0·02*M* glutamine, 2·5 ml. 0·5*M* glucose (medium A_G), 5 ml. 0·5*M dl*-lactate (medium A_L), 1 ml. 0·5*M* arginine (medium A_A or A_{AG} if glucose was present in addition) or, for meningococcus (*Neisseria intracellularis*) only, 3 ml. horse serum (medium A_S). The inoculum was 0·2 ml. of a just visibly turbid suspension in buffered peptone-water from a 16–24 hr. slant culture. Meningococcus was incubated in an atmosphere of 5 % CO_2 in air. The total N (Kjeldahl) was 2640 μl./ml. while the NH_3 was very low, 14–18 μl./ml. mainly from the peptone which also supplied 495 μl./ml. NH_2-N (Van Slyke).

The "NH_3" medium of Gladstone [1937, 2] for the trained staphylococcus was used with cystine instead of Na thiolacetate as the necessary source of organic S [Fildes & Richardson, 1937]. The organism was maintained on the same medium (medium B) in 5 % CO_2/air. Since growth was slow the inoculum was 0·5–1·0 ml. of 24–48 hr. culture for each 100 ml. medium. Good growth was obtained in 24 hr. by shaking the 100 ml. cultures in 250 ml. conical flasks at 37° in air (110 swings/min., amplitude 3 cm.). Arginine was added when needed as 1·5 ml. 0·5*M* solution/100 ml. (medium B_A). The trained staphylococcus transferred to medium A or A_G gave good growth in a closed incubator without special pre-

cautions to ensure an adequate supply of CO_2. The trained *Bact. typhosum* was grown on a simpler medium (B'), that of Gladstone [1937, 1] with lactate, no glucose and no $NaHCO_3$.

(3) *Determination of metabolism of washed cells.* The organisms were reaped by centrifuging, washed once with Ringer's solution and resuspended to form a thick suspension. The dry weight was determined by direct weighing on the microbalance of the cells from 0·5 ml. suspension, spun down after dilution with 5 ml. H_2O. The same quantity of suspension was added to neutralized substrate to give 7·5 m*M* (each optical isomer) and phosphate buffer 33·3 m*M* in a final volume of 3·33 ml. The NH_3 was determined initially and at intervals up to 4 hr. on 1 ml. aliquots (0·5 ml. where 2 mol. NH_3 were evolved rapidly) by the method of Parnas & Heller [1924] or that of Conway & Byrne [1933]. The NH_3 was collected in 1 ml. 0·01 *M* H_2SO_4 which was back-titrated with 0·01 *M* NaOH. In the former case the final suspensions were shaken aerobically in tubes ($6 \times \frac{3}{4}$ in.) at 37°. Aliquots were removed at appropriate intervals and washed into the Parnas apparatus with 5 ml. saturated borax. In the latter case, the aliquots were incubated in Conway units already warmed to 37°, with standard acid in the centre well, and 1 ml. saturated K_2CO_3 was added to stop the reaction at the appropriate time and expel the NH_3. It was found advisable to smear lightly the top of the wall of the inner well with semi-solid paraffin fixative to avoid errors due to creeping of solutions. The method was adopted for highly pathogenic organisms like *C. diphtheriae* on account of the simplicity of the apparatus which can be sterilized easily after use by autoclaving. Recoveries of added NH_3 were 97–99 % by either method. Quantities are expressed as μl. gas to facilitate comparison with the usual metabolic quotients (14μg. NH_3-N $\equiv$ $22{\cdot}4\mu$l. NH_3).

Determination of CO_2 output in the breakdown of arginine was by the method of Dickens & Šimer [1931]. Each vessel contained 1·5 ml. Ringer's solution buffered by 25 m*M* $NaHCO_3$ with or without 20 m*M* arginine. Addition of 0·5 ml. organism suspension gave 15 m*M* substrate concentration. The method also showed that there was no O_2 uptake under aerobic conditions.

Identification of the remaining product from arginine is described at the appropriate part of the text.

(4) *Materials.* Synthetic *dl*-amino-acids were used as far as possible to avoid failure to recognize effects due to abnormal optical specificity as shown by the deaminases of animal tissues [Krebs, 1933]. The exceptions were *l*(+)-arginine, *l*(−)-cystine, *l*(−)-histidine, *l*(−)-hydroxyproline, *l*(+)-lysine, *l*(+)-ornithine, *l*(−)-proline and *l*(−)-tryptophan.

Results

The liberation of NH_3 from various substrates by the organisms studied is given in Table 1. The values are average values for a 2–4 hr. period except where there is significant variation with time, when maximum values are given, either initial values ([1]) or, if an autocatalytic effect is shown, final values ([2]) for an experimental period not exceeding 4 hr. Further details are considered with reference to the various organisms.

(1) *Streptococci.* Although glucose was known to inhibit almost completely the formation of deaminases in *Bact. coli* [Stephenson & Gale, 1937], this did not seem to be the case with *Str. haemolyticus* Richards since the amount of NH_3 produced in the medium during growth was but slightly affected (Table 2). The slight deficiency of 18μl. equivalent to about 11μg. NH_3-N may well have been used for synthetic purposes since about 150μg. more organisms were formed per

ml. medium. Similarly with washed suspensions acting on peptone (Table 2) the Q_{NH_3} of glucose-grown organisms, although at first less than with lactate-grown organisms, rapidly rose to a similar level. Owing to the larger crop of organisms the total amount of NH_3-producing enzyme was considerably larger when glucose was present during growth. For this reason, and in order to shorten the lag phase and get more consistent growth, glucose was generally included in the medium for cultivation.

When individual amino-acids were used as substrates, only arginine gave a significant Q_{NH_3} ranging from 48 to 220 μl./mg./hr. with a mean of 119 in 10 experiments. With 21 other amino-acids (the only common ones not tried being hydroxyglutamate and diiodotyrosine) the rate was never more than 2·5% of that with arginine and was, in fact, practically zero within the limits of experimental error. The amino-acids which acted as mutual oxidants and reductants

Table 1. *Liberation of NH_3 from various substrates*

Organism ...	*Streptococcus*						
	1	2	3	4	5	6	7
Medium (see text)	A_G	A_G	A_G	A_G	A_G	A_G	A_G
Glutamine in medium	+	−	−	−	±	±	±
Crop (mg./200 ml.)	28–52	20–47	46	32–43	54	25–104	54–73
Time of growth (hr.)	18	24	24–42	24	42–66	18	18
Q_{NH_3} with:							
0 Substrate	0	—	—	—	0	—	0
I Glycine	2						
Alanine	0						
Valine	1	0	1	1	3	1	0
Leucine	1						
Proline	0						
Hydroxyproline	1						
II *iso*Leucine	3						
*nor*Leucine	1	2	2	−1	1	−1	1
Phenylalanine	0						
Tyrosine	−2						
III Lysine	0						
Histidine	−1						
Ornithine	0	1	0	2	3	0	−1
Tryptophan	1						
Citrulline	2	—	—	—	—	—	—
IV Serine	−1			5			
Threonine	0	1	2	0	1	−2	1
Methionine	−1						
Cystine	1	0	2	1	2	0	2
V Aspartate	0			0		−1	
Glutamate	1	1	3	1	2	1	1
Asparagine	0			1		1	
Glutamine	0	2	2	2	1	2	1
VI Guanidine	−1						
Creatine	1	−1	1	1	1	1	−2
Urea[1]	−1						
(mol. NH_3)	—	—	—	—	—	—	—
Sulphanilamide	3	2	−4	1	1	0	0
Arginine[1] (range)	49–220	170–184	151–218	103–165	83–110	146–219	200–264
(mol. NH_3, mean)	1·85	1·86	1·82	1·55	1·78	1·96	1·77
NH_3 final in medium (range, μl./ml.)	123–128	132–150	—	114–171	129–139	129–145	129–134
Q_{NH_3} with 1% peptone	62–123	—	—	—	—	—	—

[1] Initial value.

} Vertical braces show mixtures of substrates.

Table 1 (*cont.*)

Organism ...	*Staphylococcus*							
	8	9	9	9	9	9	9	9
Medium (see text)	A_G	B	B_A	B_A	A_G	A_G	A	A
Glutamine	–	–	–	–	–	–	–	–
Crop (mg./200 ml.)	47–81	24–78	28	50	58	66	14	30
Time of growth (hr.)	18	24–48	24	48	24	72	18	66
Q_{NH_3} with:								
0 Substrate	—	1	4	2	2	2	3	2
I Glycine, Alanine, Valine, Leucine, Proline, Hydroxyproline	3	2	13	30[2]			24[2]	17[2]
II *iso*Leucine, *nor*Leucine, Phenylalanine, Tyrosine	4	1	7	5			5	2
III Lysine, Histidine, Ornithine, Tryptophan	4	1	4	6	28[2]*	22*	5	4
Citrulline	—	—	—	—			—	—
IV Serine	13							
Threonine	7	4	8	20			12	19
Methionine, Cystine	6							
V Aspartate, Glutamate	7	2	5	6			5	3
Asparagine		—	—	—			—	—
Glutamine	5	—	—	—			—	—
VI Guanidine	2	1	2	3			3	2
Creatine	3							
Urea[1]	42	>200	>350	*ca.* 1300	*ca.* 900	5	>47	29[2]	41
(mol. NH_3)	—	1·96	1·96	2·05	—	2·26	—	—
Sulphanilamide	4	—	—	—	—	—	—	—
Arginine[1] (range)	85–200	3–6	18	20	28	8	217	35
(mol. NH_3, mean)	1·95	—	—	—	1·93	—	1·92	—
NH_3 final in medium (range, μl./ml.)	131–144	—	—	—	19	248	148	249
Q_{NH_3} with 1 % peptone	—	—	—	—	—	—	—	—

[1] Initial value, except where otherwise indicated.
[2] Maximum value reached autocatalytically.
} Vertical braces show mixtures of substrates.
* Citrulline, asparagine and glutamine omitted.

in the reaction of anaerobes discovered by Stickland [1934], also gave no enhanced NH_3 formation when combined in all possible pairs (Table 3). The naturally occurring amides, asparagine and glutamine, yielded no NH_3, nor did guanidine, creatine or urea which were tested on account of their chemical relationship to arginine. The suspensions used in all these cases were highly active when tested with arginine. Sulphanilamide gave no NH_3 and did not affect the breakdown of arginine (Fig. 1).

Other streptococci with less fastidious growth requirements were tested to find if limited power to form NH_3 was associated with the need for glutamine during growth. To reduce labour on account of the possibility of large numbers of negative results, mixtures of amino-acids were used in the first place, individual

Table 1 (*cont.*)

Organism ...	*Meningo-coccus*	*Bact. typhosum*			*C. diph-theriae*
	10	11	11	12	13
Medium (see text)	A_S	A	A_G	B′	A_G
Glutamine in medium	–	–	–	–	–
Crop (mg./200 ml.)	6–18	44–60	70	22–44	22–64
Time of growth (hr.)	24	24	24	24	24–48
Q_{NH_3} with:					
0 Substrate	0–11	1	—	1	−2
I Glycine, Alanine, Valine, Leucine, Proline, Hydroxyproline	8–13	30	—	4	−1 to +4
II *iso*Leucine, *nor*Leucine, Phenylalanine, Tyrosine	0–7	4	—	0	−2 to +7
III Lysine, Histidine, Ornithine, Tryptophan	2–4†	12–29†	—	2	0–2†
Citrulline	—	—	—	—	—
IV Serine	9–11	204–280[1]	0	30–64[2]	3
Threonine		11–57[2]	—	4–10	4
Methionine		−1	—	3	0
Cystine		−1	—		−1
V Aspartate	7	183[1]	55[1]	14–25	7–48[1]
Glutamate		8	—	3	1
Asparagine	—	—	—	—	—
Glutamine	—	—	—	—	—
VI Guanidine, Creatinine, Urea[1]	—	—	—	—	—
(mol. NH_3)	—	—	—	—	—
Sulphanilamide	—	—	—	—	—
Arginine[1] (range)	0–2	10–24	—	−1 to 1	−3 to 11
(mol. NH_3, mean)	—	—	—	—	—
NH_3 final in medium (range, μl./ml.)	28–67	208–216	36–43	—	38–62
Q_{NH_3} with 1% peptone	14–24	—	—	—	32

[1] Initial value.
[2] Maximum value reached autocatalytically.
} Vertical braces show mixtures of substrates.
† Arginine included.

Table 2. *Effects of lactate and glucose in growth medium on NH_3 production by* Str. haemolyticus *Richards*

Medium, with glutamine	A	A_L	A_G
Final NH_3, μl./ml.	146	141	128
Crop, mg./100 ml.	5	7	20
Q_{NH_3}, washed cells in 5% peptone			
0–30 min.	—	480	290
30–90 min.	—	500	455

Table 3. *Absence of Stickland reaction from* Str. haemolyticus *Richards*

Q_{NH_3} in the presence of hydrogen acceptors and donators combined in pairs

		Donators		
Acceptors	—	Alanine	Valine	Leucine
—	0	0	0·5	1
Glycine	2	0	0·5	−0·5
Proline	0	0	0	0
Hydroxyproline	1	0	1	0·5

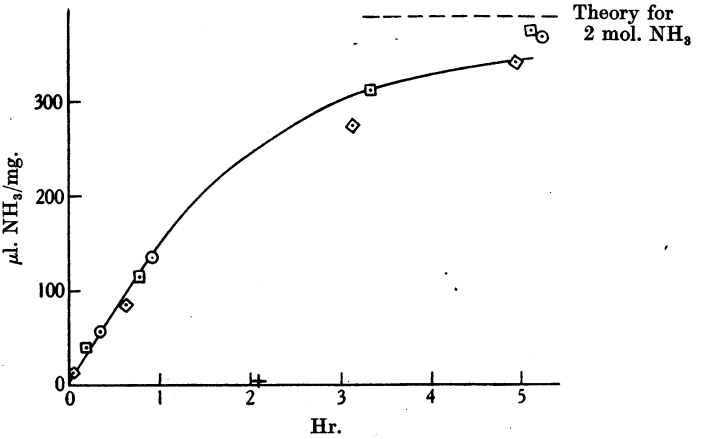

Fig. 1. NH_3 production with arginine and sulphanilamide by *Str. haemolyticus* Richards strain. ⊙ Arginine 7·5 m*M*. ⊡ Arginine 7·5 m*M* + sulphanilamide 7·5 m*M*. ◇ Arginine 7·5 m*M* + sulphanilamide 1·5 m*M*. + Sulphanilamide 7·5 or 1·5 m*M*.

compounds of a group being examined subsequently only if significant amounts of NH_3 were produced. Most mixtures contained only chemically related compounds but the acids which take part in the Stickland reaction were grouped together on account of possible interactions. To reduce the total number of mixtures phenylalanine and tyrosine were included in group II and trytophan in group III. Apart from slight action on serine by one of the haemolytic streptococci of group B similar results were obtained. Growth of the strain of *Str. viridans* was slow but was not improved by glutamine though Fildes & Gladstone [1939] found that the compound was needed by younger strains supplied by Dr Colebrook. The faecal streptococcus freshly isolated from human urine grew better with glutamine at first, but after a few subcultures on nutrient agar the organism dispensed with the need for the addition of this factor to medium A_G. The breakdown of arginine was unaffected by these nutritional changes and no other amino-acids yielded NH_3 in significant amount. Although this organism appeared to have developed the ability to synthesize glutamine during growth none was produced from NH_4^+ glutamate in washed suspension since no NH_3 disappeared.

From the representative strains examined it seemed probable that this breakdown of arginine was a constant character of streptococci. The activity per unit weight of organism was not widely influenced by the presence of arginine or glucose in the growth medium (Table 4), but the mass of growth was increased especially by glucose, so that the enzyme responsible appeared to be constitutive, the total amount formed depending on the amount of organism produced rather

Table 4. *Effect of arginine in growth medium on NH_3 production by* Str. haemolyticus *Richards*

Medium, with glutamine	A	A_A	A_G	A_{AG}
Crop at 20 hr. mg./100 ml.	5	7	12	16
Q_{NH_3}, washed cells, 7·5 m*M* arginine	141	138	155	169
NH_3 in growth medium, μl./ml.				
20 hr.	147	271	118	331
44 hr.	146	323	123	340
Extra due to arginine		177		217
Mol.		1·58		1·84

than directly on the materials in the medium available for its synthesis. The yield of NH_3 from arginine in the medium approached 2 mol. in the presence of glucose and though the amount was less in its absence the reaction may not have been complete. All the streptococci produced nearly 2 mol. NH_3 from arginine in washed suspension in cases where the reaction was followed to completion. The lowest yield was 1·55 mols. with one of the haemolytic streptococci of group B (No. 4). Further reaction after adding more substrate showed that stoppage was not due to destruction of the enzyme or development of an adversely high *p*H.

Manometric experiments with *Str. faecalis* and *Str. haemolyticus* Richards showed that 1 mol. CO_2 was produced with 2 mol. NH_3. A typical experiment is shown in Table 5. Aerobically the Q_{O_2} was negligible and the same reaction

Table 5. *CO_2 and NH_3 production from arginine by* Str. haemolyticus *Richards*

Substrate, 200 μl. per mg. dry cells. Time for completion, 120 min. after 20 min. equilibration. NH_3 from substrate during equilibration, 110 μl. per mg.

Gas phase	Substrate	Metabolism, μl./mg. O_2	CO_2	NH_3	$\frac{NH_3}{Arginine}$	$\frac{CO_2}{NH_3}$
5% CO_2 in 95% O_2	—	−10	+ 10	0	1·81	0·41
	Arginine	− 5	+115	+252		
5% CO_2 in 95% O_2	—	—	+ 8	0	1·79	0·48
	Arginine	—	+126	+248		

occurred anaerobically. A disadvantage of the method used, that of Dickens & Šimer [1931], was that substrate could not be added to the medium after equilibration with gas mixture in the thermostat. The NH_3/arginine ratio therefore referred to the whole of the reaction from the time of immersion of the vessels in the bath, while the CO_2/NH_3 referred to the last 70% of the reaction (in the example given) occurring after equilibration. The figures do, however, suggest that the reaction is:

$$H_2NC(:NH).NH(CH_2)_3CHNH_2COOH + 2H_2O = H_2N(CH_2)_3CHNH_2COOH + 2NH_3 + CO_2. \quad (1)$$

To confirm this equation NH_2-N determinations were made and the formation of ornithine was established by benzoylation followed by isolation of ornithuric acid. *Str. haemolyticus* Richards, from 4 flasks of medium A_G with glutamine, was suspended in $NaHCO_3$ and added to arginine in 0·9% NaCl to give 30 m*M* $NaHCO_3$ and 15 m*M* arginine (Ringer's solution was avoided here and in washing the organisms because of the complication of precipitation of Ca and Mg when the liquid was made alkaline in the subsequent benzoylation). The final suspension was shaken at 37° in 2 Krebs' vessels in equilibrium with 5% CO_2 till the

ERRATUM

Volume 34, No. 7

page 1064, Table 5, first column, second line

for 95% O_2 *read* 95% N_2

reaction was complete as shown by the CO_2 absorption (corrected for no substrate control) in a sample shaken under the same conditions in a Warburg manometer vessel (2–3 hr.). The organisms were killed by acidification with 0·1 vol. *N* acetic acid and heating for 5 min. on the water bath. NH_3 was removed from 4 ml. clear filtrate by evaporation to small bulk with a few drops excess $Mg(OH)_2$ suspension and the sample was acidified with acetic acid and transferred quantitatively to the Van Slyke apparatus for NH_2-N determination. The results, given in Table 6, show clearly that the NH_2-N is doubled in spite of the loss of 2 mol.

Table 6. *Amino-N and NH_3 production from arginine by* Str. haemolyticus *Richards*

	Initial		Final	
	μl./ml.	mol.	μl./ml.	mol.
Arginine	338	1·00	0	0
NH_2-N	365	1·08	715	2·12
NH_3	22	0·06	630	1·87

NH_3, i.e. ornithine must be formed. Complete decomposition of arginine was confirmed by addition of 0·5 vol. 5% flavianic acid to another small sample; no flavianate was precipitated. The remainder of the acid filtrate (45 ml.) was therefore evaporated to dryness, dissolved in a small volume of 2*N* NaOH and freed from NH_3 in a vacuum desiccator. The product of benzoylation (Schotten-Baumann) was transferred quantitatively to a Soxhlet thimble (10 × 50 mm.), which was used as a filter. After drying, benzoic acid was extracted with ether in an all-glass continuous extractor. Subsequent extraction with alcohol gave 211 mg. material, M.P. 179–181°, yield 98%. A single crystallization from 8 ml. 50% alcohol gave 114 mg. fairly pure ornithuric acid, M.P. 180°, yield 67%; mixed M.P. 182° with a specimen, M.P. 185°, prepared in similar yield from *l*(+)-ornithine (Hofmann-La Roche). Ornithuric acid was prepared similarly from the products of the action of the faecal streptococcus on arginine and crystallized to constant M.P. 185–186°, mixed M.P. 184°. All M.P. are uncorrected. The M.P. of ornithuric acid is given variously in the literature, 182–189°.

(2) *Staphylococci.* The main difference between staphylococcus (No. 8) and streptococcus, both growing on the peptone medium A_G, was the urease activity of the former but the rate was less than the rate of attack on arginine. The staphylococcus had also small but definite actions on serine, threonine, glutamine and possibly other substrates. With the trained organism grown on the "NH_3" medium urease activity became predominant, accurate measurement not being made under the conditions chosen owing to disappearance of much of the substrate before taking the second sample. In order to find if the almost complete disappearance of the arginine enzyme was due to the absence of the substrate during growth the organism was grown in the medium B_A with the addition of arginine, but little of the enzyme was produced even if the period of growth was prolonged to 48 hr. and urease activity was still very high. The activity with some amino-acid mixtures was of the same order as that with arginine. If the organism was transferred to the peptone medium for streptococcus, arginine was attacked in washed suspensions after 24 hr. growth with glucose in the medium (A_G), but the enzyme was probably not maximal since practically no NH_3 had been formed during growth. The urease had reverted to a position of less importance quantitatively. If the incubation in the growth medium was prolonged to 72 hr. nearly twice as much NH_3 was liberated as by any other coccus in this

medium, the arginine enzyme had decayed considerably and the urease showed increased activity. In both cases the NH_3 from a complete mixture of all the other compounds used was relatively high (the smaller groups were not tried). In order to enhance this activity, if possible, the organism was grown on the peptone medium without glucose. There was little change, but the usual amount of NH_3 had been liberated during 18 hr. growth and the arginine enzyme was by then highly active. More NH_3 had been liberated in the growth medium after 66 hr. and the arginine enzyme had decayed to 15% of its former value, with some increase in the urease.

(3) *Other organisms.* The Coccaceae so far shown to attack arginine were all gram-positive. For comparison the gram-negative meningococcus was studied. The values were less accurate than the others owing to poor growth of the organism, only 0·15–0·30 mg. dry cells per ml. final suspension being available instead of 1–2 mg. The extreme ranges of values are given rather than the means. No amino-acid mixture gave much NH_3, but as a small amount of NH_3 was liberated in the medium during growth, peptone was used as a standard substrate to show that each suspension used was active. Washed cells had the same activity as unwashed cells and the activity was not enhanced by the addition of serum, which was needed for growth. The feeble activity with amino-acids was therefore not due to damage to the enzyme systems of the delicate organism by Ringer's solution.

Bact. typhosum was studied as a pathogenic member of the Bacteriaceae. The stock strain gave much NH_3 in the medium in the absence of glucose but little in its presence. The organisms were therefore usually grown without glucose. With washed suspensions substrates showing highest activity were serine and aspartate. Arginine, threonine and Stickland's acids (group I) had significant activity, but methionine and cystine were inactive. Glucose in the growth medium inhibited the formation of the serine enzyme completely and that of the aspartate enzyme by 70%. The organism therefore resembled in deaminating power the related *Bact. coli* [Stephenson & Gale, 1937; Gale & Stephenson, 1938; Gale, 1938]. The trained strain showed more feeble activity with little relative change in the rates of attack of the various substrates, excepting possibly the slightly enhanced activity with cystine or methionine.

Arginine thus appeared to be of less importance in gram-negative organisms, quantitatively at any rate, than in the gram-positive cocci. As a gram-positive pathogen of a different order, *C. diphtheriae* was chosen for study. The little NH_3 formed during growth was not affected by the presence of glucose or lactate in the medium. Growth was best with glucose and glucose-grown organisms were used for the experiments with washed suspensions. Little activity was shown except with aspartic acid.

Discussion

The decomposition of arginine studied here appears to be due to a distinct enzyme and not due to the action of urease following that of arginase (using the term in the accepted sense first used by Kossel & Dakin [1904]). The reasons for this are:

(1) The strains of streptococci used have been shown to possess no urease.

(2) The urease of the stock strain of staphylococcus is insufficiently active to account for the rate of breakdown of arginine.

(3) In a strain of staphylococcus trained to grow on an "NH_3" medium the urease activity can be varied at will between very wide limits, the arginine enzyme varying in an inverse manner. The reciprocal relationship suggests that the enzymes may be related though not identical.

The enzyme could be described as an "arginase", but in order to specify the reaction process the name "arginine dihydrolase" is suggested. This distinguishes the enzyme from "arginase", in the accepted sense, which could be described on the same basis as "arginine monohydrolase".

Descriptions of bacterial arginase in the literature may refer often to arginine dihydrolase when the analytical methods used have shown only the increase in NH_2-N (Hino [1924] on *Ps. pyocyanea* and *Ps. fluorescens*) or disappearance of arginine (Kossel & Curtius [1925] on *Ps. pyocyanea*). Both organisms show urease activity [Hino, 1924]; both are gram-negative rods and may be feebly pathogenic. Other organisms which may possess arginine dihydrolase are *Cl. sporogenes* and *Cl. histolyticum*, gram-positive rods which attack arginine more actively than other amino-acids, but differ from the gram-positive cocci in deaminating very actively alanine and glutamate with less action on tyrosine and cystine [Bessey & King, 1934]. *Cl. sporogenes* has also been shown to differ in the mutual oxidation and reduction in pairs of amino-acids [Stickland, 1934]. Arginine moreover yields 3 mol. NH_3 either alone or as a H-donator and ornithine is attacked either alone or as a H-acceptor giving NH_3 and δ-aminovaleric acid [Woods, 1936]. Since urea is not attacked either alone or with H-acceptors or donators, it seems likely that arginine can break down as in the gram-positive cocci to ornithine, which is then attacked further only by the anaerobes.

The mechanism of the action of arginine dihydrolase has not been studied in detail, but citrulline cannot be an intermediate with *Str. haemolyticus* Richards and free urea has also been shown not to be affected. Since the known mechanisms of enzyme reactions generally involve simple stages like the addition or removal of H_2, H_2O or NH_3, it can only be suggested that there is formed a tautomeric form of urea, $HN{:}C(-O^-).\overset{+}{N}H_3$, or its ion, $HN{:}C(-OH).\overset{+}{N}H_3$, such as was postulated by Werner [1923], and instead of being liberated from the enzyme surface in a free state it is further activated to give NH_3 and CO_2 by further reaction with H_2O or OH^-.

Since it has been generally supposed that the deaminases may be involved *in vivo* in synthesis of amino-acids on the basis of the reversibility of enzyme reactions, it was at first surprising that the deaminating power was low in those strains of *Staph. aureus* and *Bact. typhosum* which had been trained to synthesize their amino-acid requirements from NH_3. In the animal, however, the amino-acid oxidases react with O_2 through flavin-adenine dinucleotide [Warburg & Christian, 1938] and as nothing is known of the presence of the nucleotide in the bacteria examined, it may be a limiting factor in deamination. The enzyme may also be linked through coenzyme I with other dehydrogenase systems, such as in the lactic or malic system [Dewan, 1939]. The latter, in conjunction with fumarase, is certainly of importance in the oxidation of *l*(+)-glutamate by *Bact. coli* [Krebs, 1937]. The possibility must also be considered of interaction with α-keto-acids without loss of NH_3, i.e. the transamination reaction of Braunstein & Kritzmann [1937, quoted by Cohen, 1939]. The failure of oxidative deamination in the organisms tested or even the failure of amino-acids to enable streptococci to reduce indigo-tetrasulphonate, may therefore be due to the absence of other factors than the enzyme proper.

The function of arginine dihydrolase in bacteria must remain a matter for future study, but five possibilities may be suggested.

(1) *A neutralization mechanism.* The earlier production of the enzyme by the trained staphylococcus growing on peptone without glucose makes this unlikely, contrasting with the conditions for the formation of the decarboxylating enzymes in *Bact. coli* [Gale, 1940].

(2) *A mechanism for supplying NH_3 for synthesis.* The synthesis of amino-acids, especially those which Gladstone [1937, 2] found more easily dispensable, may be of importance in the natural habitat of the organism where keto-acids with the appropriate C-skeletons may be available for reductive amination, possibly linked with the energy-yielding reactions of carbohydrate metabolism through coenzyme I.

(3) *Ornithine production.* This is suggested by the effects of lack of arginine and addition of ornithine in the growth of *Str. haemolyticus* in a synthetic medium [McIlwain, 1940]. The suggestion of the possibility of the oxidation of ornithine to glutamine by keto-acids in liver [Bach, 1939] affords an interesting speculation as to the relation between the two in streptococci.

(4) *Toxic effect of NH_3 on the host.* Tauber & Kleiner [1931] showed that crystalline urease produces symptoms resembling NH_3 poisoning. The minimum lethal dose per g. in mice gave *in vitro* 1·1 mg./hr. NH_3-N at 20°. Table 1 shows that *Str. haemolyticus* Richards would produce about 0·03–0·14 mg./hr./mg. dry cells at 37°. In comparing these figures we should consider (*a*) multiplication of the cells in the tissues of the host, (*b*) activation of the organism by coenzymes in the body fluids of the host, (*c*) the frequent localization of streptococcal and staphylococcal lesions.

(5) *Relationship to staphylococcal α-haemolysin.* Neither haemolysin [Gladstone, 1938] nor enzyme is produced on the "NH_3" medium. Although arginine is quantitatively the most important amino-acid for haemolysin production (optimum 6·6–20 m*M*) others are also required; nor is the addition of arginine alone to the medium (7·5 m*M*) a sufficient condition for the production of the enzyme. Fildes [1934], discussing bacterial enzyme variation, has distinguished between (*a*) catabolic enzymes rapidly formed through the stimulus of the appropriate substrates and (*b*) anabolic enzymes for the synthesis of an essential nutrient produced through lack of the nutrient only after a lengthy training. The arginine enzyme may therefore be specially associated with pathogenicity since it cannot be classified as an adaptive catabolic enzyme of type (*a*). On the other hand the great activity normally found in untrained organisms does not appear to be essential for the life of a gram-positive coccus, yet full activity is restored in a strain which had lost it by a single subculture on an appropriate medium.

Summary

1. Gram-positive cocci contain an enzyme which attacks arginine in accordance with equation (1) with a Q_{NH_3} *ca.* 50–200.

2. The enzyme has been named arginine dihydrolase to distinguish it from arginase with which it is not identical.

3. With a trained strain of staphylococcus the arginine dihydrolase content can be varied according to the conditions of growth between Q_{NH_3} 217 and 3 while the urease content varies reciprocally between Q_{NH_3} 5 and 1300.

4. Possible functions of arginine dihydrolase in the life of the organism are discussed, especially in relation to pathogenicity.

5. Gram-positive cocci produce no NH_3 aerobically from other amino-acids except, with staphylococcus, serine, Q_{NH_3} 13, and threonine, Q_{NH_3} 7.

6. Meningococcus has, aerobically, Q_{NH_3} 14–24 on 1 % peptone but gives no significant deamination with amino-acids.

7. *Bact. typhosum* deaminates aerobically serine, aspartate and, to some extent, threonine and arginine with Q_{NH_3} 240, 180, 30 and 15 respectively. The

formation of serine deaminase is inhibited 100 % and that of aspartase 70 % by 12·5 m*M* glucose in the growth medium.

8. *C. diphtheriae* has an aerobic Q_{NH_3} 30 with aspartate but gives no significant deamination with other amino-acids.

I am indebted to Dr P. Fildes under whose inspiration and guidance this work progressed and to my colleagues for helpful discussion, especially to Dr G. P. Gladstone who gave active help with the bacteriological work in the early stages. I wish to thank Mr D. E. Hughes for Van Slyke determinations.

REFERENCES

Bach (1939). *Biochem. J.* **33**, 1833.
Braunstein & Kritzmann (1937). *Enzymologia*, **2**, 129.
Bessey & King (1934). *J. infect. Dis.* **54**, 123.
Cohen (1939). *Biochem. J.* **33**, 1478.
Conway & Byrne (1933). *Biochem. J.* **27**, 419.
Dewan (1939). *Biochem. J.* **33**, 549.
Dickens & Šimer (1931). *Biochem. J.* **25**, 973.
Farrell (1935). *J. Bact.* **29**, 411.
Fildes (1934). *Proc. roy. Soc. Med.* **28**, 79.
—— & Gladstone (1939). *Brit. J. exp. Path.* **20**, 334.
—— —— & Knight (1933). *Brit. J. exp. Path.* **14**, 189.
—— & Richardson (1937). *Brit. J. exp. Path.* **18**, 392.
Foster (1921). *J. Bact.* **6**, 211.
Gale (1938). *Biochem. J.* **32**, 1583.
—— (1940). *Biochem. J.* **34**, 392.
—— & Stephenson (1938). *Biochem. J.* **32**, 392.
Gladstone (1937, 1). *Brit. J. exp. Path.* **18**, 67.
—— (1937, 2). *Brit. J. exp. Path.* **18**, 322.
—— (1938). *Brit. J. exp. Path.* **19**, 208.
Hino (1924). *Hoppe-Seyl. Z.* **133**, 106.
Knight (1936). Bacterial Nutrition (M.R.C. Spec. Rep. Ser. no. 210). London.
Kossel & Curtius (1925). *Hoppe-Seyl. Z.* **148**, 283.
—— & Dakin (1904). *Hoppe-Seyl. Z.* **41**, 21.
Krebs (1933). *Hoppe-Seyl. Z.* **217**, 191.
—— (1935). *Biochem. J.* **29**, 1951.
—— (1937). *Biochem. J.* **31**, 2095.
McIlwain (1939). *Brit. J. exp. Path.* **20**, 330.
—— (1940). *Brit. J. exp. Path.* **21**, 25.
—— Fildes, Gladstone & Knight (1939). *Biochem. J.* **33**, 223.
Parnas & Heller (1924). *Biochem. Z.* **152**, 1.
Stephenson (1939). Bacterial Metabolism, p. 135. London.
—— & Gale (1937). *Biochem. J.* **31**, 1316.
Stevens & West (1922). *J. exp. Med.* **35**, 823.
Stickland (1934). *Biochem. J.* **28**, 1746.
Tauber & Kleiner (1931). *J. biol. Chem.* **92**, 177.
Warburg & Christian (1938). *Biochem. Z.* **298**, 150.
Werner (1923). The Chemistry of Urea. London.
Woods (1936). *Biochem. J.* **30**, 1934.