A Preliminary Study of Ammonia Production by *Corynebacterium renale* and some other Pathogenic Bacteria

BY R. LOVELL AND D. G. HARVEY

Royal Veterinary College, London

SUMMARY: Urease is a constituent enzyme of *Corynebacterium renale* and appears to account for the bulk of its ammonia production. *C. renale* also contains an arginase and some amino-acid deaminases, but the former has not been fully characterized. Bovine urine supports the growth of a small inoculum of *C. renale* for a limited time but after growth has reached a maximum it diminishes rapidly and the ammonia and pH values increase. *C. renale* also contains uricase but its precise significance has not yet been determined.

Of the other bacteria studied, *C. ovis* has urease activity similar to *C. renale*, and *C. pyogenes* a stronger initial arginase. *C. equi* contains no appreciable urease or arginase, although it tends to form ammonia from glutamine.

Corynebacterium renale is the cause of a specific cystitis and pyelonephritis in cattle; the lesions in natural infections in cattle and in experimental infections in mice and rabbits after intravenous inoculation are localized in the medulla of the kidney (Lovell, 1946; Lovell & Cotchin, 1946; Feenstra, Thorp & Gray, 1949). This selective localization may, in part, be explained by the metabolic and enzyme activities of the organism, and a study of its ammonia-producing capacity was made because of the old observation that *C. renale* produces ammonia when grown in urine. A preliminary test was made to confirm this by growing a strain of *C. renale* in bovine urine and in peptone water containing 1% (w/v) urea. After 48 hr. incubation at 37° flasks of uninoculated media and those in which the organism had been grown were filtered through Seitz filters and the filtrates examined for free ammonia, urea and creatine. The results are given in Table 1, and show that in bovine urine and in a urea medium *C. renale* breaks down the urea with the formation of free ammonia. The creatine content of the urine remained constant, but largely diminished in the urea medium probably because of the peptone present.

The ammonia-producing capacity of *C. renale* was therefore examined and a few comparative tests made with *C. ovis*, *C. equi*, *C. pyogenes* and *Bacterium coli*.

METHODS

The methods employed were:

(a) Washed suspensions of *C. renale* prepared from growth on agar slopes were seeded into sterile urine, peptone water containing 1% urea, hydrolysed peptone water and peptone water. The urine was sterilized by Seitz-filtration, and the hydrolysed peptone was prepared from a stock solution which consisted of a mixture of 100 g. of Bactopeptone and 800 ml. of conc. hydrochloric acid (A.R.) which had been boiled under a reflux condenser for 48 hr. after which most of the acid had been removed by vacuum distillation; the solution was neutralized to pH 7 by NaOH, diluted to give approximately 1% NaCl;

L-tryptophan was added to give a concentration equivalent to 1 g. for every 100 g. of peptone originally used. After inoculation all cultures were incubated at 37° and periodical examinations made. The number of living bacteria/ml. was determined by plate counts on nutrient agar and estimations made of the ammonia and urea contents.

Table 1. *Content of urea, NH₃ and creatine (in g./100 ml.) of filtrates of urine and urea medium before and after growth of Corynebacterium renale at 37° for 48 hr.*

	Bovine urine		Urea medium	
	Control	After growth	Control	After growth
Urea	2.82	0.12	0.72	Nil
Free ammonia	Nil	1.309	Nil	0.289
Creatine	0.186	0.186	0.21	0.0232

(b) A cell-free extract of *C. renale* was prepared by growing large quantities of *C. renale* on nutrient agar for 48 hr.; the growth was collected, washed with saline and 10 vol. of cold acetone added to 1 vol. of thick washed suspension; the mixture was stirred with a glass rod and filtered through paper and the filter-paper washed with more acetone. The deposit was scraped off the filter-paper, dried over calcium chloride and then ground to a fine powder. Samples of the powder were added to different nitrogenous substrates in a buffered solution consisting of a mixture of 6 ml. 0.1M-NaHCO₃ and 12 ml. 0.1M-KH₂PO₄, the volume of which was made up to 40 ml. with distilled water; when using tyrosine, cystine and uric acid the concentration of KH₂PO₄ and NaHCO₃ was doubled. In order to avoid confusion between possible D- and L-deaminases DL-amino-acids were used wherever possible. Each individual constituent was weighed to give a final concentration of 15 mM. When mixtures of the nitrogenous substrates were used 1 ml. quantities of each substrate was taken as required and 5 ml. of the total taken for estimation purposes.

The ammonia was estimated by either micro- or macro-methods with steam distillation (Kjeldahl) or aeration (Van Slyke). For the nitrogenous substrates and their mixtures the following procedure was adopted. Five ml. of the stock solution was mixed with 5 ml. of an aqueous suspension of the cell-free extract of *C. renale* made by adding 150 mg. of the powder to 60 ml. of distilled water; this was incubated 4 hr. at 37–40°; toluene was added as a preservative. After incubation, the solution was treated with 5 ml. of saturated K₂CO₃ solution in a modified micro-Kjeldahl apparatus and the ammonia distilled into 5 ml. of 2% boric acid + bromocresol blue and the final solution titrated with 0.01N-H₂SO₄. The urea titrations were made by the Van Slyke urease method; the creatine and creatinine by Folin's method as modified by Cole (1933) and his modification of Benedict's method was used for the titration of uric acid. The allantoin titration was that of Larson as described by Hawk, Oser & Summerson (1947).

RESULTS

Growth of Corynebacterium renale in bovine urine and urea medium

The results obtained by growth of *C. renale* in bovine urine are shown in Figs. 1 and 2. In the first experiment (Fig. 1) the initial inoculum was small, about 1500 viable bacteria/ml.; the number increased to 1.5×10^6 at 48 hr. and then diminished till after 6 days' incubation only 100 organisms/ml. were viable. This coincided with a decrease in urea, from 1.25 to 0.25 g./100 ml. at the third day, whilst the ammonia content increased to a little over 0.5 g./100 ml. during the same period. The second experiment (Fig. 2) shows the results with a large inoculum. The initial viable count of 162,000 organisms/ml. fell almost in a straight line to zero; in this sample of urine the urea content was 2.65 g./ml. which fell to 2 g./ml. by the seventh day; there was a corresponding increase in ammonia.

Fig. 1

Fig. 2

Fig. 1. Urease activity and growth of *C. renale* in bovine urine. ——— Logarithmic count of growth; urea in g./100 ml.; - - - - - NH₃ in g./100 ml.

Fig. 2. Urease activity and growth of *C. renale* in bovine urine. ——— Logarithmic count of growth; urea in g./100 ml.; - - - - - NH₃ in g./100 ml.

Whether the initial inoculum of *C. renale* is large or small there is in bovine urine a steady though not always a rapid disappearance of the urea with a corresponding increase of ammonia concentration and pH value, the latter reaching 9.5.

In peptone water containing 1% (w/v) urea similar results are obtained (Fig. 3). With an inoculum of 140,000 viable organisms/ml. there was a steady decrease in viable organisms, a fall in the urea content within 72 hr. and a corresponding increase in ammonia.

A different picture emerged when *C. renale* was grown in peptone water or in acid-hydrolysed peptone water (Fig. 4). In the former medium an inoculum approximating to that used in the urea medium grew and reached its peak at about 48 hr., thereafter falling gradually. Small amounts of ammonia were

present, from 18 to 30 mg./100 ml. In the acid-hydrolysed peptone water the viable organisms were rapidly killed and none were recovered after 48 hr. incubation. The ammonia content was irregular, varying from 6 to 23 mg./100 ml.

Fig. 3

Fig. 3. Urease activity and growth of *C. renale* in peptone water medium containing 1% urea. ——— Logarithmic count of growth; urea in g./100 ml.; - - - - - NH₃ in g./100 ml.

Fig. 4

Fig. 4. NH₃ production and growth of *C. renale* in peptone water and in acid-hydrolysed peptone water. - - - - - Peptone water; ——— acid-hydrolysed peptone water.

Incubation of cell-free extract of Corynebacterium renale in nitrogenous substrates

The results obtained after incubation of the cell-free extract of *C. renale* in different nitrogenous substrates are shown in Table 2. They are compared with those obtained with similar products from *C. equi*, *C. ovis*, *C. pyogenes* and *Bact. coli*. Ammonia production by *C. renale*, although small, occurred with most of the substrates, the one exception being urea, which was rapidly and completely broken down. Prolonged incubation appeared to favour an arginase activity but beyond that ammonia production was slight. *C. ovis* also has urease, but *C. pyogenes* and *Bact. coli* have none; there is some evidence of an arginase of *C. pyogenes* but *C. equi* possesses neither urease nor arginase, it appears to break down (L+)-glutamine. Of the five organisms studied, *C. equi* and *Bact. coli* produced least ammonia when their cell-free extracts were incubated with a mixture of all the nitrogenous substances. This is difficult to reconcile with the results obtained when the strains were grown on the media described by Christensen (1946); in this medium, *C. renale*, *C. ovis* and *C. equi* gave evidence of urease activity, *C. pyogenes* gave a slight change in colour and *Bact. coli* failed to produce this change. When the phenol red was replaced by thymol blue (0.012 g./l.), positive reactions occurred with *C. renale* and *C. equi* and none with *C. ovis*, *C. pyogenes* and *Bact. coli*. In both media *C. renale* gave rapid and intense reactions.

Table 2. *The ammonia production (as $\mu\text{g. NH}_3/\text{mg. dry weight of cell-free enzyme preparation}$) after 4 hr. incubation with various substrates which were single or mixtures of nitrogenous compounds*

(*a* = results obtained with individual nitrogenous compounds; *b*, *c* and *d* = results obtained when different mixtures of compounds were used; . = no estimation made with this individual compound. All the amino-acids were DL except tyrosine, cystine, glutamine and arginine which were L. Cell-free extracts, when incubated in the buffer solution alone, gave slight traces of ammonia only.)

Compound	<i>C. renale</i>				<i>C. equi</i>				<i>C. ovis</i>			<i>C. pyogenes</i>			<i>Bact. coli</i>		
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>a</i>	<i>b</i>	<i>c</i>
Glycine	5				2				.			.			.		
Alanine	5				3				.			.			.		
Valine	2	3			2	2			.			.			.	1	
Leucine	6				2				.			.			.		
Proline	4				2				.			.			.		
Hydroxyproline	4				1				.			.			.		
Isoleucine	4				2				.			.			.		
Norleucine	3				2	5			.			.			.		
Phenylalanine	3	1			3				.			.			.		
Tyrosine	3				3				.			.			.		
Lysine	2				3				.			.			.		
Histidine	0	6	10		2	4	4		.	9		.	8		.	6	
Ornithine	2				9				.			.			.		
Tryptophan	6				3				.			.			.		
Serine	4			8	3		1		.	9		.	10		.		3
Threonine	3	3			2	1			.			.			.	2	
Methionine	2				2				.			.			.		
Cystine	2				1				.			.			.		
Aspartic acid	3				0				.			.			.		
Glutamic acid	3	4			3	9			.			.			.		
Asparagine	3				5				.			.			.	7	
Glutamine	4				19				.			.			.		
Arginine	6				3				5			31			.		
Urea	84				7				83			4			.		
Creatinine	5				4				.			.			.		
Creatine	2	25			2	8			.	41		.	4		.		
Uric acid	3				<1				.			.			.		
Allantoin	2				<1				.			.			.		
Guanidine	2				32				.			.			.		

The arginase activity was studied by incubating 25 mg. of cell-free extract in a solution containing 50 ml. of a solution of 0.05 M arginine, 5 ml. of 0.6 % KH_2PO_4 and 0.45 ml. of 0.01 M- MnSO_4 , and water to 100 ml.; toluene was added as preservative. A control flask omitting the MnSO_4 solution was included and the mixtures were incubated at 37° for 7 days. With the *C. renale* extract (Fig. 5) there was a steady increase in ammonia production, enhanced by the MnSO_4 . Comparable extracts from *C. equi* and *Bact. coli* produced little ammonia, with no significant increase by MnSO_4 . The arginase activity of *C. renale* was further demonstrated by the recovery of ornithuric acid after 3 days' incubation. This was effected by boiling the opalescent solution with 2–3 g. of kieselguhr for 80 sec., filtering and concentrating *in vacuo* to 25–30 ml.; the concentrate was treated with 1 ml. benzoyl chloride and excess NaOH, acidified with 5 % sulphuric acid, shaken with 5 × 20 ml. of ether and allowed

to cool. Impure ornithuric acid (dibenzoylornithine) was filtered off and recrystallized from dilute aqueous ethanol in colourless needles, M.P. 187–188° (uncorr.). Hills (1940) gave the melting-point as 182° and quoted references giving a range of 182–189°. The yield from 1.052 g. of (L+)-arginine was 500 mg. (55 % of theoretical). The once-recrystallized sample contained 7.2 % N; $C_{19}H_{20}O_4N_2$ requires 8.2 % N. Yellow-orange crystalline precipitates

Fig. 5. Decomposition of arginine and production of NH_3 by cell-free extract of *C. renale* with and without added manganese.

were obtained from the *C. equi* and *Bact. coli* flasks when they were clarified and treated with 5 % flavianic acid. These precipitates decomposed at 260–270° with no sharp melting-points. The nitrogen analysis figures indicate that these substances were probably mixtures; *Bact. coli* has already been shown to possess an arginine decarboxylase activity giving rise to agmatine (Gale, 1940 a, b).

*The breakdown of nitrogenous constituents in human urine
by cell-free extracts*

A sample of human urine at pH 7 was divided into portions of 50 ml. and to three of them were added 25 mg. of the cell-free extracts of *C. renale*, *C. equi* and *Bact. coli* respectively. After 40 hr. incubation at 37° the main nitrogenous constituents were estimated in all four samples (Table 3).

Table 3. *Nitrogenous constituents of human urine after incubation with cell-free extracts of Corynebacterium renale, C. equi and Bacterium coli*

	Actual values of control sample (mg./100 ml.)	Percentage recovery when incubated with cell-free extracts of		
		<i>C. renale</i>	<i>C. equi</i>	<i>Bact. coli</i>
NH_3	42.5	1700	80	40
Urea	1165	5	100	—
Creatinine	97.5	99–100	99–100	99–100
Creatine	28.7	99–100	99–100	99–100
Uric acid	10.9	50	99–100	99
Allantoin	3.1	155	99–100	100
pH	7.0	8.8	7.1	7.1

The cell-free extract of *C. renale* attacked urea with a corresponding increase in the ammonia content. There was also a fall in uric acid with an increase in allantoin. Only slight changes occurred with extracts of *C. equi* and *Bact. coli*.

DISCUSSION

The ability of *C. renale* to form ammonia from various nitrogenous substrates undoubtedly lies in its urease activity. In common with the other bacteria studied it forms ammonia from a series of amino-acids, but in this respect its activity is slight. Proteolytic activity is also small and any ammonia produced by this means probably comes from the hydrolysis of amide nitrogen or by a primary proteolytic breakdown of the molecule followed by deaminase activity. This may be compared with the protease isolated from a streptococcus by Stevens & West (1922) and with the action of *C. equi* on glutamine.

We have not yet determined whether the arginase possessed by *C. renale* is a monohydrolase or dihydrolase. One practical difficulty is the wide pH range of the urease present, which we tentatively suggest is 5–9. If the arginase activity is optimal at pH 9 ± 0.5 (Folley & Greenbaum (1948) suggest it is 9.45), then it may be possible to isolate an intermediate product such as urea which may be formed if the arginase is a normal monohydrolase type (Hills, 1940). There is no evidence that *C. renale* forms urea as an intermediate

from compounds containing the $\text{=N}-\overset{\textstyle |}{\text{C}}-\text{N=}$ linkage, such as creatine, creatinine, uric acid and allantoin. It is therefore unlike *C. ureafaciens* (Krebs & Eggleston, 1939). The only other enzyme activity concerned with urinary nitrogenous products, other than amino-acids, is uricase, an enzyme whose activity would be enhanced by the presence of the urea/urease system. Uricase is present in *C. renale* in sufficient amount to cause the disappearance of 50 % of uric acid in human urine at pH 7 after 40 hr. incubation. Further study of this isolated fact might reveal a linkage between the tissue of the host and the metabolic activities of the particular pathogen (Fildes, 1934–5; Knight, 1936). The activity of *C. renale* may be bound up with enzyme systems other than those which produce ammonia. Gale (1940*a, b*) showed that *Bact. coli* possesses a decarboxylase capable of converting arginine into agmatine, and although there is no evidence of agmatine formation by *C. renale*, other natural bases may be revealed which are harmful to the kidney tissue.

The differences in the growth curves in bovine urine from small and large inocula may be due to the rapid increase in ammonia production and pH value, produced by the larger concentration of urease. It is useless to speculate with a medium such as urine, as each sample is different, and it may be that any essential metabolites present are inadequate for small inocula and the growth thereby influenced in that way.

It is hoped to continue the study of the enzyme characteristics of *C. renale*, and one plan consists of varying the constituent nutrients. Hills (1940) noted that the relative concentrations of arginase and urease were sometimes reversed when growing staphylococci on special media. The main object is a study of the factors and conditions responsible for the localization and

multiplication of *C. renale* in the kidney medulla; it may lead to a fuller understanding of its pathogenesis and of the general problem of tissue localization.

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