THE IMPORTANCE OF PROLONGED INCUBATION FOR THE SYNTHESIS OF DIMETHYLNITROSAMINE BY ENTEROBACTERIA

P. J. COLOE AND NANCY J. HAYWARD

*Department of Microbiology, Monash University Medical School,
Prahran 3181, Australia*

THE experimental demonstration that nitrosamines are carcinogenic, mutagenic and teratogenic (Magee, 1969) has raised the question of their potential danger to man, and prompted studies of the circumstances that might expose him to them. Nitrosamines have been found in a limited number of foods for human consumption (International Agency for Research on Cancer, 1972), and have been shown to be produced by bacteria that occur in food (Collins-Thompson *et al.*, 1972; Fong and Chan, 1973). They may be produced *in vivo* from nitrites and either secondary amines (Sander, Schweinsberg and Menz, 1968; Sen, Smith and Schwinghamer, 1969; Lijinsky and Epstein, 1970; Mirvish, 1970) or tertiary amines (Lijinsky, 1974) at the *pH* of the stomach.

The possibility of bacterial production of nitrosamines from secondary amines in the presence of nitrate or nitrite *in vivo* at sites with a *pH* closer to neutrality has been raised. Production *in vitro* has been shown with single strains of four nitrate-reducing members of the enterobacteriaceae, *Escherichia coli*, *E. dispar*, *Proteus vulgaris* and *Serratia marcescens*, from secondary amines at neutral *pH* (Sander, 1968). Hawksworth and Hill (1971) found that 27% of 37 strains of *E. coli* and a proportion of strains of other faecal aerobes and anaerobes were able to produce nitrosamines from nitrate and diphenylamine at a *pH* above 6.5. These authors (Hill and Hawksworth, 1972) consider that the most likely site of *in-vivo* bacterial production of nitrosamines is the urinary tract, because the substrates, nitrate and secondary amines (principally dimethylamine, DMA), are found in urine—and bacteria with both nitrate-reducing and nitrosamine-forming enzymes may be present during infection. The *in-vitro* production of dimethylnitrosamine (DMN) by three strains each of *P. mirabilis*, *P. morganii* and *P. rettgeri* from urine, and the detection of DMN in the urine of two patients with urinary-tract infections caused by *P. mirabilis*, supports this suggestion (Brooks *et al.*, 1972; Thacker and Brooks, 1974). Nitrosamines produced by bacteria during urinary-tract infections may cause cancer in distant anatomical sites (Hill, Hawksworth and Tattersall, 1973; Hawksworth and Hill, 1974).

The present survey was undertaken to define which of the bacteria commonly associated with urinary-tract infection are able to produce DMN. The survey of faecal bacteria by Hawksworth and Hill (1971) appears to show that production of diphenylnitrosamine is not a constant feature of all strains of any species. Similarly, we have found wide variation among strains when we tested bacteria that may cause urinary-tract infections for their ability to produce

Received 28 Oct. 1975; accepted 2 Dec. 1975.

DMN—but only when we used the accepted test incubation period of 24 h. When we extended the test incubation-period to 70 h, almost all of 136 strains of nitratase-positive enterobacteria produced DMN from DMA and nitrate.

MATERIALS AND METHODS

Organisms. A total of 153 strains included 52 *E. coli*, 23 *Klebsiella aerogenes*, two *K. ozaenae*, 36 *P. mirabilis*, 17 *P. vulgaris*, 11 *P. rettgeri* and 12 *P.morganii*. One hundred of these strains were obtained from clinical material submitted to our laboratory and included isolates from specimens of urine (76), faeces (15), pus (three), sputum (one) and blood (one) or from nasal swabs (two), an eye swab and a cervical swab. A further 53 strains were obtained from culture collections at Alfred Hospital, Melbourne (22), National Collection of Type Cultures, London (16), National Institute of Health, Wellington, New Zealand (eight), Monash University Culture Collection (three), Queensland University Culture Collection (three) and Institute of Medical and Veterinary Science, Adelaide (one). Strains used for particular experiments in addition to the general survey were the following: *P. mirabilis* no. B2; *P. vulgaris* no. B14 and *E. coli* no. B78, isolated from urine; *P. vulgaris* no. A15, from a nasal swab; *P. mirabilis* no. B15, from faeces; and *E. coli* no. A2, from pus.

All organisms were identified, and nitrate reduction was determined, by the methods of Cowan and Steel (1965).

Chemicals. DMA in ethanol (33% w/w), DMN, dichloromethane, acetone, sodium nitrite and potassium nitrate were purchased from E. Merck, Darmstadt, Germany. Whenever possible, biochemical-grade chemicals from British Drug Houses were used for the preparation of defined media.

Media. Defined medium was prepared from four solutions which were sterilised and stored separately and mixed when required: 1 litre of the medium contained 780 ml of the basal salts solution and 100 ml of the complex amino-acid solution of Proom and Knight (1955), the former sterilised by autoclaving at 121°C for 15 min. and the latter by filtration through a Gallenkamp Sinta-glass filter of porosity 5; 20 ml of a filter-sterilised vitamin solution containing biotin 1.0 µg, folic acid 2.0 µg, riboflavin 0.1 mg, thiamine hydrochloride 0.5 mg, nicotinic acid 0.5 mg, pyridoxal hydrochloride 0.5 mg and calcium pantothenate 0.5 mg; and 100 ml of a 30% (w/v) glucose solution sterilised by autoclaving at 110°C for 20 min. The defined medium, enriched with 0.09M or 0.022M DMA or 0.1M or 0.025M KNO₃ or 0.1M or 0.025M NaNO₂ or any combination of these, was adjusted to pH 7.4 and sterilised by filtration through sintered glass.

MacConkey Agar (Oxoid) was prepared as directed by the manufacturers. Blood agar contained Nutrient Broth no. 2 (Oxoid) 2.5% (w/v), Powdered Yeast Extract (Oxoid) 0.3% (w/v), Agar no. 3 (Oxoid) 1.2% (w/v), and defibrinated horse blood (Bio-Science Laboratories) 3% (v/v). The agar concentration was increased to 3.6% for firm blood agar to prevent the swarming of *Proteus* spp. Peptone water was prepared from Bacteriological Peptone (Oxoid) as described by Cowan and Steel (1965).

Culture conditions. Volumes of defined medium (20 ml) were dispensed aseptically into 30-ml sterile screw-capped "Universal" bottles, seeded with c. 10⁶ organisms per ml and incubated unshaken at 37°C. MacConkey's agar and blood agar for viable counts were incubated at 37°C for only 12 h to restrict colony size to 0.5–1 mm and so increase the accuracy of counting.

Measurement of growth. Optical densities (OD) of suspensions were measured as extinction values at 500 nm (E₅₀₀) in a Bausch and Lomb Spectronic 20 colorimeter. Viable counts were performed by the method of Miles, Misra and Irwin (1938). Dilutions were prepared in 1% peptone water and one drop of each dilution was placed on each of eight MacConkey's agar plates and one blood-agar plate. The cultures before dilution were streaked on blood agar to check their purity.

Extraction of DMN. Cultures and standard solutions were adjusted to pH 12–13 by

adding 1 ml of 10M NaOH and extracted twice with 10 ml of dichloromethane. The dichloromethane extracts were pooled, saturated with anhydrous K_2CO_3 to remove any water, filtered through Whatman no. 1 filter paper to remove the K_2CO_3 , transferred to a 50-ml round-bottomed flask, evaporated under column reflux at 60°C to remove the dichloromethane, redissolved in 0.5 ml of acetone and evaporated to remove the acetone and any residual dichloromethane. The residue, redissolved in 0.1 ml of acetone, was used for gas-chromatographic analysis. A standard 0.0009M solution of DMN in defined medium was extracted as a control with each batch of test samples.

Detection of DMN. DMN was detected by gas-liquid chromatography by means of a Perkin-Elmer model 881 gas chromatograph equipped with a flame ionisation detector and a glass column [1.83 m (6 ft) $\times$ 6.35 mm (0.25 in.) outside diameter] packed with Chromosorb 103 (Tracor, Analytical Instruments Division, Austin, Texas, USA). The carrier gas was nitrogen and the flow rate 20 ml per min.; the column-oven temperature was 180°C, the detector temperature 210°C, and the injector temperature 200°C. DMN from bacterial cultures was identified initially by comparison of the retention times of unknown peaks with authentic DMN and by mass-spectrometry. In subsequent experiments, DMN was determined both qualitatively and quantitatively by comparison of the retention times and peak areas of extracts of DMN from bacterial cultures with extracts of the standard 0.0009M solution of DMN. The limit of detection of DMN was 0.09 μ moles per 20 ml (4.5 μ M).

Reproducibility of results. Twenty-eight 0.0009M DMN standards analysed during the survey ranged in peak area from 75.4 to 109.2 cm² with a mean of 92.28 cm² and a standard deviation of 8.57 cm². The maximum variation from the mean was 18%. The small final volume of sample (0.1 ml) needed to provide a detectable concentration of DMN without the use of very large volumes of defined medium tended to add to the experimental error inherent in the number of steps required for extraction.

RESULTS

Bacterial production of dimethylnitrosamine in cultures at 24 h

The results from the 153 cultures grown for 24 h in defined medium supplemented with 0.09M DMA and 0.1M KNO_3 are shown in table I. Some strains of all species produced detectable DMN; there was wide variation in amounts

TABLE I
Dimethylnitrosamine (DMN) production in 24-h cultures in defined medium supplemented with 0.09M dimethylamine (DMA) and 0.1M potassium nitrate

Species	Number of strains tested	Number producing* detectable DMN	Number producing >0.09mM DMN	Maximum concentration of DMN produced (mM)	Range of E_{500} readings (turbidity of test cultures)
<i>Escherichia coli</i>	52	38	14	0.36	0.34–3.0
<i>Klebsiella aerogenes</i>	23	23	18	0.32	0.84–1.84
<i>K. ozaenae</i>	2	2	0	0.08	1.46–1.65
<i>Proteus mirabilis</i>	36	26	18	0.35	0.19–0.98
<i>P. vulgaris</i>	17	8	8	0.30	0.035–0.185
<i>P. rettgeri</i>	11	7	5	0.34	0.18–0.74
<i>P. morganii</i>	12	4	1	0.20	0.24–0.96
Any	153	108	64	...	...

* The limit of detection of DMN was 0.09 μ moles per 20 ml, equivalent to 4.5 μ M DMN.

TABLE II
Dimethylnitrosamine (DMN) production in 24-h cultures of the same strains tested on three occasions

Species	Concentration of DMN produced (mm)		
<i>E. coli</i>	0.35	0.36	0.26
<i>P. mirabilis</i>	0.16	0.08	0.12
<i>P. mirabilis</i>	<0.0045	<0.0045	<0.0045
<i>P. mirabilis</i>	0.18	0.16	0.13

of DMN produced by individual strains—ranging from 0.009mm to 0.36mm—and, except possibly for *K. aerogenes*, all species showed a similar variation. Control cultures of five strains of *E. coli*, three of *K. aerogenes*, one of *K. ozaenae*, six of *P. mirabilis*, three of *P. vulgaris*, two of *P. rettgeri* and two of *P.morganii*, in medium either without DMA or without KNO₃, did not form any detectable DMN. There was a wide variation in final turbidity of the test cultures but DMN formation was unrelated to the degree of growth. The final pH of all test and control cultures ranged between 6.0 and 7.4 and this too was unrelated to the amount of DMN produced.

One strain of *E. coli* and three of *P. mirabilis* were tested more than once and found to be consistent in the amount of DMN produced after 24 h (table II).

Time course of production of DMN

One strain each of *P. mirabilis*, *E. coli* and *K. aerogenes*, selected because they produced high yields of DMN, were used to follow DMN production and growth for 48 h (fig. 1). In all three cultures DMN production lagged about 12 h behind growth, starting when the cells were reaching the stationary phase and continuing to increase although the organisms were dying. This was confirmed by more prolonged incubation of three strains of *E. coli* and two strains each of *K. aerogenes* and *P. mirabilis*, chosen because they were typical of strains producing large, medium and small amounts of DMN at 24 h and grown in defined medium enriched with DMN and KNO₃. Samples were taken at intervals up to 100 h; the amounts of DMN produced and the viable counts were determined. Fig. 2 shows the results for *E. coli* strain B78 which produced a large amount and *E. coli* strain A2 which produced a small amount of DMN at 24 h. These results are typical of those obtained with the other cultures tested. DMN production lagged well behind growth, starting when the cells reached the stationary phase and continuing to increase until about 70 h. At least 75% and on average 85% of the DMN was produced in the period from 20–60 h. At 18 h, strain B78 produced almost three times as much DMN as strain A2, but later in the experiment the amounts of DMN were higher for strain A2. At 80 h all strains had produced between 1.0mm and 1.2mm DMN. The amount of DMN present in the cultures did not decrease during the course of the experiment.

FIG. 1.—Growth and dimethylnitrosamine production in defined medium supplemented with 0.09M dimethylamine and 0.1M potassium nitrate by species of enterobacteria: ○-----○ = viable count; ●—● = concentration of dimethylnitrosamine.

FIG. 2.—Growth and dimethylnitrosamine production in defined medium supplemented with 0.09M dimethylamine and 0.1M potassium nitrate by strains of *Escherichia coli* that had given high (no. B78) and low (no. A2) yields when tested at 24 h: ○-----○ = viable count; ●—● = concentration of dimethylnitrosamine.

TABLE III
Dimethylnitrosamine (DMN) production in 70-h cultures in defined medium supplemented with 0.09M dimethylamine and 0.1M potassium nitrate

Species	Number of strains tested	Number producing DMN			Average DMN production (mm)	Range of DMN production (mm)		Number of nitrate-positive strains	Number of cultures viable at 70 h
		Any detectable amount	<0.22 mm	0.22-0.71 mm		Minimum	Maximum		
<i>E. coli</i>	42	42	0	7	35	1.68	0.54	42	5
<i>K. aerogenes</i>	18	18	0	1	17	1.48	0.69	18	4
<i>K. ozaenae</i>	2	2	0	2	0	0.61	0.60	2	0
<i>P. mirabilis</i>	36	34	0	0	34	2.63	0.91	34	12
<i>P. vulgaris</i>	22	20	0	3	17	1.43	0.55	20	7
<i>P. rettgeri</i>	8	8	1	3	4	0.63	0.17	8	2
<i>P. morganii</i>	12	12	1	3	8	0.99	0.216	12	4
Any	140	136	2	19	115	...	...	136	34

These results appear to show that the wide variation in amounts of DMN from cultures at 24 h (table I) may have been due to differences in rate of production of this substance rather than differences in ability to produce it.

DMN production in cultures at 70 h

The results obtained with 140 cultures, including all 45 strains that failed to produce DMN in 24 h (table I), grown for 70 h in defined medium enriched with 0.09M DMA and 0.1M KNO₃ are shown in table III. The yields of DMN were considerably larger than those at 24 h. All strains tested produced detectable DMN except two *P. mirabilis* and two *P. vulgaris* strains; these four strains did not reduce nitrate to nitrite, whereas all the strains that produced DMN in 70 h reduced nitrate to nitrite. There was a wide variation in final turbidity, but DMN formation was unrelated to degree of growth. The final pH of all test and control cultures ranged only between 6.5 and 7.0 and also was unrelated to the amount of DMN produced. Control cultures of seven strains of *E. coli*, four of *K. aerogenes*, one of *K. ozaenae*, five of *P. mirabilis* and two each of *P. vulgaris*, *P. rettgeri* and *P. morganii*, in medium either without DMA or without KNO₃ did not form any detectable DMN. It is of interest that the four species producing the largest yields of DMN, *E. coli*, *K. aerogenes*, *P. mirabilis* and *P. vulgaris*, are also the enterobacteria most commonly found in urinary-tract infections.

The apparently constant capacity of nitratabase-positive strains to produce DMN requires qualification, because DMN can be produced non-enzymically from DMA and nitrite, albeit reportedly in very small amount at pH 6 and not at all at pH 7 (Mirvish, 1970). However, Mirvish incubated the reagents for only 3 h at 25°C and our cultures were held for 70 h at 37°C. Valid controls to test for non-enzymic DMN formation were difficult to devise for two reasons. Firstly, the production of DMN involves two sequential reactions, the reduction of nitrate to nitrite and the subsequent formation of DMN from DMA and nitrite. Only the second reaction can occur non-enzymically in cultures. In controls containing DMA and nitrite, the second reaction started at the moment the reagents were mixed: in the cultures, non-enzymic DMN production could not start until nitrite had been formed. Simple comparison of amounts of DMN in such controls and in cultures in a given time, 24 h or 70 h, could give a clear-cut answer only when the amounts of DMN in cultures were greater than in controls. This answer inevitably underestimated the enzymic capacity of some cultures that produced less DMN than the controls. Secondly, it was difficult to provide controls for the effect of pH because this started at pH 7.4 and fell to between 6.5 and 7.0.

Two sets of controls were chosen, both consisting of uninoculated medium supplemented with 0.09M DMA and 0.1M NaNO₂, providing the maximum nitrite concentration—as if all the nitrate had been reduced immediately the tests cultures were seeded; both sets were incubated for the full 70 h at 37°C, one set at pH 7.4 and the other at pH 6.5. No DMN was detected in either set at 24 h. Some DMN was formed in each at 70 h, 0.71mm at pH 6.5 and

0.22mM at pH 7.4. Whilst it is confidently assumed that all 115 cultures that produced more than 0.71mM DMN (table III) did so enzymically, it is probable that some of the 19 that produced between 0.22mM and 0.71mM also possessed nitrosamine-forming enzymes. This is borne out by the fact that no non-enzymic control yielded any DMN at 24 h and yet six of the 19 cultures produced DMN at 24 h. Thus at least 121 of our nitrate-reducing strains formed DMN in 70 h. Only two test cultures, one of *P. rettgeri* and one of *P. morganii*, gave yields that were lower than the 0.22mM found in controls at pH 7.4 and these were most probably not producing DMN enzymically.

Nitrite toxicity

Of the 140 cultures tested, 106 were dead after 70 h (table III) in medium enriched with DMA and KNO_3 . The substrate associated with the development of lethal conditions appeared to be KNO_3 , because all 140 cultures were viable in medium enriched with DMA alone, whereas, in medium enriched with KNO_3 alone, 118 of the 140 cultures were dead, 101 of them being the same as those that were killed in medium enriched with both DMA and KNO_3 . Nitrite produced from KNO_3 appeared to be the bactericidal agent because all four strains that did not reduce nitrate to nitrite were viable in medium enriched with either DMA and KNO_3 , or KNO_3 alone. To test for possible toxicity of nitrite, one strain of *E. coli* and two of *P. mirabilis* were separately seeded to give 10^6 organisms per ml in defined medium supplemented with NaNO_2 in concentrations from 0.01M to 0.5M. The maximum concentration of nitrite that would permit growth was 0.05M and in higher concentrations no viable organisms could be recovered after 24 h. Since the test medium contained 0.1M KNO_3 and the strains that died reduced nitrate to nitrite, nitrite toxicity is the probable explanation for the death of cultures recorded in table III and observed in the time-course experiments (figs. 1 and 2) as a sudden decrease in viable counts after about 30 h. There was no evidence that DMN was the bactericidal agent. One strain each of *K. aerogenes* and *P. mirabilis*, both of which were dead at 70 h in medium enriched with either DMA and KNO_3 , or KNO_3 alone, were viable at 70 h when grown in defined medium enriched either with 0.9mM DMN or with 9mM DMN, the maximum concentration produced by any culture.

DMN production by nitratase-negative strains

To determine whether nitratase-negative strains could produce DMN from DMA and nitrite, five strains of *P. mirabilis*, including the two nitratase-negative strains B2 and B15, and five of *P. vulgaris*, including the two nitratase-negative strains, A15 and B14, were incubated in defined medium supplemented with 0.022M DMA and either 0.025M NaNO_2 or 0.025M KNO_3 . The low concentration of NaNO_2 was chosen to avoid toxicity, and concentrations of DMA and KNO_3 were made to maintain supplements in the same ratio as in other experiments. No detectable DMN was formed after 24 h by any of

the cultures in either medium; however, after 70 h in defined medium supplemented with NaNO_2 and DMA, all four nitratase-negative strains and four of the six nitratase-positive strains had produced some detectable DMN (table IV). Only the nitratase-positive strains produced detectable DMN after 70 h in medium supplemented with KNO_3 and DMA. There was no detectable DMN formed either in uninoculated medium at pH 6.5 or 7.4, or in inoculated controls without DMA or without KNO_3 or NaNO_2 . After 70 h, all cultures in this series were viable. These results show that the nitratase and nitrosamine-forming enzymes are separate, because the nitratase-negative strains when supplied with nitrite were as active as nitratase-positive strains in producing DMN.

Effect of different substrate concentrations

The percentage conversion of DMA to DMN by each of the six nitratase-positive strains (table IV) was similar in media containing 0.022M DMA and 0.025M KNO_3 to the percentage in media containing 0.09M DMA and 0.1M KNO_3 . The amount of DMN produced appears to represent an equilibrium with the concentration of DMA.

DISCUSSION

It has been suggested that bacteria causing urinary-tract infections may produce nitrosamines from secondary amines and nitrates in urine and, in support of this, bacteria found in urinary-tract infections such as *E. coli* (Sander, 1968; Hawksworth and Hill, 1971 and 1974), *P. vulgaris* (Sander, 1968), *P. mirabilis*, *P. rettgeri* and *P. morganii* (Thacker and Brooks, 1974) have been shown to produce nitrosamines *in vitro*. However, nitrosamine formation

TABLE IV

Dimethylnitrosamine (DMN) production in 24-h and 70-h cultures in defined medium supplemented with 0.022M dimethylamine (DMA) and either 0.025M NaNO_2 or 0.025M KNO_3

Species	Nitratase production	Concentration of DMN produced (mm) in			
		NaNO_2 -supplemented medium		KNO_3 -supplemented medium	
		in 24 h	in 70 h	in 24 h	in 70 h
<i>P. mirabilis</i>	—	< 0.0045	0.114	< 0.0045	< 0.0045
	—	< 0.0045	0.096	< 0.0045	< 0.0045
	+	< 0.0045	0.058	< 0.0045	0.419
	+	< 0.0045	0.057	< 0.0045	0.279
	+	< 0.0045	< 0.0045	< 0.0045	0.254
	+	< 0.0045	< 0.0045	< 0.0045	< 0.0045
<i>P. vulgaris</i>	—	< 0.0045	0.096	< 0.0045	< 0.0045
	—	< 0.0045	0.100	< 0.0045	< 0.0045
	+	< 0.0045	0.019	< 0.0045	0.490
	+	< 0.0045	0.145	< 0.0045	0.481
	+	< 0.0045	< 0.0045	< 0.0045	0.486
	+	< 0.0045	< 0.0045	< 0.0045	< 0.0045

appeared to be an irregularly distributed property among strains of each species. Hawksworth and Hill (1971) found that only 27% of 37 strains of *E. coli* and even lower proportions of other intestinal bacteria formed diphenylnitrosamine; and, although Sander (1968) found that *P. vulgaris* formed diphenylnitrosamine, three strains of *P. vulgaris* failed to form DMN from urine under conditions in which other *Proteus* species produced it (Thacker and Brooks, 1974). Our own experience with 153 strains of *E. coli*, *Klebsiella* and *Proteus* spp. was similar; only 108 produced detectable DMN in 24 h. However, ability to produce DMN appeared to be a constant property of particular strains, and the original object of the present investigation was to attempt to define those strains that produced DMN and were potentially capable of forming this carcinogen when they caused urinary-tract infections.

The results showed that differences in strains are probably of minor significance in comparison with host factors in the urinary tract. When cultures were incubated for 70 h instead of 24 h in defined medium enriched with DMA and KNO_3 , all nitratase-positive strains yielded DMN; even after the most stringent allowance for non-biological formation, 89% of these appear to have done so enzymically. The major difference between strains was in the rate at which they formed DMN. Our tests after incubation for 24 h, when DMN production was only starting, were misinterpreted by us as showing differences in capacity to form DMN. Hawksworth and Hill (1971) incubated their cultures for only 18 h; this presumably explains why only 27% of their strains of *E. coli* appeared able to produce diphenylnitrosamine from nitrate and diphenylamine.

It appears that ability to produce DMN is widely distributed among enterobacteria. Biochemical attributes may be regarded as constant for a species if 80% of strains are positive (Cowan and Steel, 1965) and by this standard a nitrosamine-forming enzyme appears to be a constant property of *E. coli*, *K. aerogenes*, *P. mirabilis* and *P. vulgaris*, and possibly also of *K. ozaenae*, *P. rettgeri* and *P. morganii*, although there is variation among strains in the time of incubation at which the enzyme is first apparent. Some strains are already producing detectable DMN after 16 h and significant yields at 24 h, but others are slower with very small yields at 24 h, although the final yields of the slow producers may be larger than those of the faster ones.

The reverse reaction, breakdown of DMN to DMA by bacteria, has been reported (Hawksworth and Hill, 1971; Rowland and Grasso, 1975); the final yields of DMN probably represent an equilibrium between formation and degradation.

Conditions in the urinary tract analogous to prolonged incubation are likely to be important in determining the potential of an infection to produce carcinogenic nitrosamines. A wide variety of structural abnormalities and associated conditions such as ureteric reflux interfere with normal clearing mechanisms. Infected urine is retained in a semi-continuous culture system, and the infection may persist for many weeks or months. This is much more time than is necessary for even the slower DMN producers to yield the nitrosamine in our in-vitro test model. At the pH of urine, which can be as low as

4.5, non-biological DMN production might also occur—provided that an infecting organism first reduced nitrate to nitrite. On the other hand, in the absence of stasis, it is probable that urinary-tract infections do not pose a risk of DMN production, and procedures such as high fluid intake that tend to flush bacteria from the urinary tract would have the double value of reducing both the numbers of organisms and the time that they spend in the system. The finding that more than 12 h is needed for DMN production under favourably poised conditions *in vitro* and that production is not at a maximum for 70 h puts the importance of DMN produced *in vivo* in association with urinary tract infections into perspective and makes it clear that, unlike the general threat of all urinary-tract infections envisaged by Hill *et al.* (1973) and Thacker and Brooks (1974), infection without structural or functional abnormality would almost certainly not be a potent or persisting source of DMN.

Yields of nitrosamines reported in the literature are small. This is particularly true of the conversion of DMA to DMN, which can be calculated as 0.01 % by *E. coli* in 18 h (Hill and Hawksworth, 1972), 0.09 % by sewage bacteria in 8 days (Ayanaba, Verstraete and Alexander, 1973), and up to 0.05 % by a cell suspension of *E. coli* in 36 h (Ayanaba and Alexander, 1973). Yields of DMN ranged from 0.001mM to 0.03mM. Higher yields have been reported from the other secondary amines found in urine, piperidine and pyrrolidine (Hawksworth and Hill, 1974), but these amines are found in only a fraction of the concentration at which DMA occurs in urine (Hawksworth and Hill, 1972). We also found only small amounts of DMN produced *in vitro* after incubation for 24 h; only 64 out of 153 strains converted more than 0.1 % of 0.09M DMA to DMN and the maximum yield was only 0.36mM. However, with longer incubation the yield of DMN increased, and after 70 h up to 10 % of the DMA in the medium was converted to DMN.

The concentrations of DMA and KNO_3 used in the present investigation were high in comparison with those that might be expected *in vivo*. The normal concentration of DMA in human urine is 0.5mM (Hawksworth and Hill, 1971) and we used 90mM. Urinary nitrate concentration depends on dietary intake and has been found to range from 1mM to 2.6mM (Hawksworth and Hill, 1971) whereas we used 100mM. Although the experiments were artificial to the extent that concentrations of reagents were under the constraint of the sensitivity of the gas chromatograph used to detect DMN, the six nitratase-positive strains used in the experiment with one-quarter the reagent concentrations, 22mM DMA and 25mM KNO_3 , converted similar proportions of the DMA to DMN. Studies that might relate our findings with these test systems more closely to natural circumstances are now indicated.

SUMMARY

Tests with 140 strains representing *Escherichia coli*, *Klebsiella aerogenes*, *K. ozaenae*, *Proteus mirabilis*, *P. vulgaris*, *P. rettgeri* and *P.morganii* in a defined medium supplemented with 0.09M dimethylamine (DMA) and 0.1M potassium nitrate showed that at least 89 % of the 136 strains able to reduce nitrates

produced up to 9mm dimethylnitrosamine (DMN) in 70 h at 37°C. Four nitratase-negative strains produced DMN from DMA in the presence of sodium nitrate. Prolonged incubation was the most important factor in determining DMN production. Stasis and persistent infection in the urinary tract, by simulating prolonged incubation of a culture, may be of importance in determining whether the potential carcinogen, DMN, could be produced *in vivo* by bacterial action on DMA and nitrate in urine.

We thank Miss Nance Quick for excellent technical assistance and Dr A. J. C. Nicholson, Division of Chemical Physics, Commonwealth Scientific and Industrial Research Organisation, for identifying dimethylnitrosamine by mass spectrometry. One of us (N. J. H.) was assisted by a grant from the National Health and Medical Research Council.

REFERENCES

- AYANABA, A. AND ALEXANDER, M. 1973. Microbial formation of nitrosamines *in vitro*. *Appl. Microbiol.*, **25**, 862.
- AYANABA, A., VERSTRAETE, W. AND ALEXANDER, M. 1973. Possible microbial contribution to nitrosamine formation in sewage and soil. *J. natl Cancer Inst.*, **50**, 811.
- BROOKS, J. B., CHERRY, W. B., THACKER, L. AND ALLEY, C. C. 1972. Analysis by gas chromatography of amines and nitrosamines produced *in vivo* and *in vitro* by *Proteus mirabilis*. *J. infect. Dis.*, **126**, 143.
- COLLINS-THOMPSON, D. L., SEN, N. P., ARIS, B., AND SCHWINGHAMER, L. 1972. Non-enzymic *in vitro* formation of nitrosamines by bacteria isolated from meat products. *Can. J. Microbiol.*, **18**, 1968.
- COWAN, S. T. AND STEEL, K. J. 1965. Manual for the identification of medical bacteria, London.
- FONG, Y. Y. AND CHAN, W. C. 1973. Bacterial production of dimethylnitrosamine in salted fish. *Nature, Lond.*, **243**, 421.
- HAWKSWORTH, G. M. AND HILL, M. J. 1971. Bacteria and the N-nitrosation of secondary amines. *Br. J. Cancer*, **25**, 520.
- HAWKSWORTH, G. AND HILL, M. J. 1974. The in-vivo formation of N-nitrosamines in the rat bladder and their subsequent absorption. *Br. J. Cancer*, **29**, 353.
- HILL, M. J. AND HAWKSWORTH, G. 1972. Bacterial production of nitrosamines *in vitro* and *in vivo*. In N-nitroso compounds: analysis and formation, edited by P. Bogovski, R. Preussmann and E. A. Walker, IARC Sci. Publ. no. 3, Lyon, p. 116.
- HILL, M. J., HAWKSWORTH, G. AND TATTERSALL, G. 1973. Bacteria, nitrosamines and cancer of the stomach. *Br. J. Cancer*, **28**, 562.
- INTERNATIONAL AGENCY FOR RESEARCH ON CANCER. 1972. Report of the subcommittee on analytical methods for N-nitroso compounds. In N-nitroso compounds: analysis and formation, edited by P. Bogovski, R. Preussmann and E. A. Walker, IARC Sci. Publ. no. 3, Lyon, p. 92.
- LIJINSKY, W. 1974. Reaction of drugs with nitrous acid as a source of carcinogenic nitrosamines. *Cancer Res.*, **34**, 255.
- LIJINSKY, W. AND EPSTEIN, S. S. 1970. Nitrosamines as environmental carcinogens. *Nature, Lond.*, **225**, 21.
- MAGEE, P. N. 1969. *In vivo* reactions of nitroso compounds. *Ann. N.Y. Acad. Sci.*, **163**, 717.
- MILES, A. A., MISRA, S. S. AND IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.
- MIRVISH, S. S. 1970. Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. *J. natl Cancer Inst.*, **44**, 633.
- PROOM, H. AND KNIGHT, B. C. J. G. 1955. The minimal nutritional requirements of some species in the genus *Bacillus*. *J. gen. Microbiol.*, **13**, 474.

- ROWLAND, I. R. AND GRASSO, P. 1975. Degradation of N-nitrosamines by intestinal bacteria. *Appl. Microbiol.*, **29**, 7.
- SANDER, J. 1968. Nitrosaminsynthese durch Bakterien. *Hoppe-Seyler's Z. physiol. Chem.*, **349**, 429.
- SANDER, J., SCHWEINSBERG, F. AND MENZ, H. P. 1968. Untersuchungen über die Entstehung cancerogener Nitrosamine im Magen. *Hoppe-Seyler's Z. physiol. Chem.*, **349**, 1691.
- SEN, N. P., SMITH, D. C. AND SCHWINGHAMER, L. 1969. Formation of N-nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Fd Cosmet. Toxicol.*, **7**, 301.
- THACKER, L. AND BROOKS, J. B. 1974. In vitro production of N-nitroso-dimethylamine and other amines by *Proteus* species. *Infect. Immun.*, **9**, 648.