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Analysis by Gas Chromatography of Amines and Nitrosamines Produced In Vivo and In Vitro by *Proteus mirabilis*

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Reproducible procedures suitable for routine analysis of small quantities of body fluids and culture media were developed by use of gas-liquid chromatography, heptafluorobutyric-anhydride derivatives, and electron-capture detectors. *Proteus mirabilis* was isolated from two clinical cases of bacteriuria, and the alkaline-extractable products elaborated by this organism in vivo were compared with those produced in vitro. N-nitrosodimethylamine, a potent carcinogen, was produced along with other amines by *P. mirabilis* in all urines tested (both in vivo and in vitro) and in cooked-meat medium. This compound was not produced in vitro by a strain of *Escherichia coli* tested on the same substrates. The significance of these findings was evaluated, with emphasis on the carcinogenic activity and toxicity of N-nitrosodimethylamine and on the use of gas-liquid chromatography as a tool for the diagnosis of urinary-tract disease.

In previous work with anaerobic bacteria [1–3] and with *Neisseria* [4, 5], Brooks et al. demonstrated that analysis of metabolic products by gas-liquid chromatography (GLC) was an effective means, when used in conjunction with other data obtained in the laboratory, for differentiating organisms at the species level. Reproducible techniques were introduced that permitted analysis for amines and other bacterial products on a practical basis. Nevertheless, GLC would be more valuable if bacterial metabolites could be detected in body fluids and identifying patterns established. However, some problems exist; one is the variation in composition of body fluids, such as urine, and the possible effects that such variation may have on the production of bacterial products. Other problems are encountered in extraction of the bacterial metabolites from body fluids and in manipulation and transformation of these metabolites, which renders them detectable by GLC at a level significantly above that of the background components present in the control samples and reagents.

Amines appear to be a group of compounds worthy of investigation, since several workers have demonstrated the potential carcinogenicity of nitrosamines [6] and the possibility that these amines are produced in vivo during infection by certain organisms [7]. In addition, the spectrum of amines detected by a gas chromatograph equipped with a flame-ionization detector was valuable for distinguishing several microorganisms [1, 3]. Electron-capture (EC) detectors, which are sensitive and highly selective, should be of even greater value for analysis of amines, since these substances form derivatives that are very sensitive to detection by electron capture. Anggard et al. [8] demonstrated that heptafluorobutyric-anhydride (HFBA) derivatives had high electron-capturing ability. Other investigators [8, 9] have made derivatives of various catecholamines and related amines in attempts to improve their detection by electron capture. Brooks et al. [10] introduced a technique for preparing electron-capturing derivatives of some biologically important nitrosamines by using pyridine as a catalyst. The procedure is practical for routine analysis and can be used to form electron-capturing derivatives of amines and alcohols. The

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present study was done to develop practical methods for employing EC detectors in the analysis of bacterial amines in body fluids and in cultures and to correlate, if possible, in-vivo and in-vitro production of these compounds. We also investigated the effects of differences in the composition of the growth substrates on the metabolic profiles of the organisms.

Materials and Methods

Organisms. Strains of *Proteus mirabilis* (Center for Disease Control stocks no. 40-512-1 and 40-2701-1) were isolated from the urine of two patients with bacteriuria. A strain of *Escherichia coli* (06 H:—, CDC no. 40-513-1) was obtained from stock cultures and was of a serogroup commonly associated with urinary-tract infections. Identification of both organisms was made by standard biochemical procedures [11].

Specimens, growth media, and cultural procedures. A female patient (KCC, aged 50) otherwise in good health presented with a history of discomfort, pain low in the back, and cloudy urine of two days' duration. She had been treated for six previous episodes of bacteriuria during the past 11 years. No bacteriologic studies were performed during the first episode. Quantitative studies were not done during the next two attacks, but the predominant organism was *P. mirabilis*. Quantitative counts during the last three infections established the existence of bacteriuria due to *P. mirabilis* with colony counts in excess of 10^6/ml. Thus, it appeared that *P. mirabilis* was the causative agent of chronic bacteriuria in this patient.

A midstream specimen of urine was obtained after the urogenital area was thoroughly cleansed and before treatment was begun. The bacterial count was $>10^6$ *P. mirabilis* (40-512-1)/ml. The patient was treated with ampicillin for eight days. Four days later a second urine specimen revealed a count of 4×10^3 *P. mirabilis*/ml. Additional specimens obtained several weeks after recovery (postinfection urine) were free of *Proteus* and, together with specimens from a noninfected male, were used as substrate for in-vitro studies. A single specimen of urine, obtained from a second patient, had a lower count of *P. mirabilis* (40-2701-1) ($<10^5$/ml).

The urine specimens used for substrates were boiled for about 1 min, cooled, and incubated to test for sterility. Twenty milliliters of specimen were inoculated with either *P. mirabilis* 40-512-1 or *E. coli* 40-513-1 with a platinum loop and incubated aerobically for either 20 hr or four days at 37 C. The same organisms were inoculated into 8 ml of cooked meat with glucose (CMG) as described elsewhere [12] and incubated for four days at 37 C.

Preparation of amines for GLC analysis. Twenty milliliters of urine or 8 ml of CMG spent medium were acidified to about *p*H 2.0 with 0.5 ml of 50% H_2SO_4 (v/v) and extracted with 20 ml of nanograde chloroform (Mallinckrodt); moderate shaking and centrifugation separated the chloroform and aqueous layers. The chloroform layer was withdrawn and discarded or saved for further analysis. The residual aqueous sample containing the amines was made basic (above *p*H 10) with 8 N NaOH. The sample was next reextracted with 20 ml of chloroform into a 50-ml beaker and concentrated by air to about 0.5 ml. The concentrate was then drawn up into a disposable pipette, and the chloroform layer (bottom) was displaced into a dry 12×75-mm culture tube. The aqueous layer (top) left in the pipette was discarded. The extract was further concentrated to about 0.06 ml and HFBA derivatives were prepared; one drop (about 0.01 ml) of a mixture of one part pyridine to three parts chloroform was used as a catalyst, and two drops (about 7 μliter) of HFBA were used as the derivatizing agent [10]. One-tenth milliliter of ether was used as a solvent for the HFBA derivatives [10]. The sample was cooled at 4 C for a few minutes to help prevent evaporation of the ether; then 1–1.4 μliter of the 100-μliter sample was used for GLC analysis of the urine samples, and 0.35 μliter was used for analysis of the CMG samples. The sample injection volume was increased about 10% for the polar (TABSORB) column. All HFBA derivatives were analyzed on the day of preparation on both OV-1 and TABSORB columns, usually in alternate order. Care was taken to prevent evaporation of the ether from the syringe before the sample was injected, first by measuring the amount to be injected, then by pulling the sample up into the syringe before injection. In addition, the needle was protected from the hot air that escapes from the oven around the injector ports on the model 5000 Barber-Coleman gas chromatograph by wrapping the column at the injector

port with a few turns of asbestos. The syringe was cleaned after each injection by flushing with 4% HCl, distilled water, methanol, and two different batches of ethyl ether. The solvents were used in the order given.

Tests for purity of the solvents were essential, because some lots contained EC compounds other than chloroform. Therefore, each lot of nanograde chloroform was tested by concentrating 20 ml of the solvent to 0.06 ml. The chloroform concentrate was then processed in the same manner as that described for urine extracts, and 1.4 μliter of the sample was used for analysis. Diethylether was tested by injection of 1.4 μliter of the solvent as purchased.

Preparation of standards. The HFBA derivatives of known standards were prepared as described for urine samples. The amine standard contained the following mixture: 2.02 mmoles of ethylamine, 1.52 mmoles of propylamine, 0.78 mmoles of isobutylamine, 1.64 mmoles of n-butylamine, 1.27 mmoles of isoamylamine, 9.65 mmoles of pyrrolidine, 1.13 mmoles of n-hexylamine, 3.46 mmoles of di-n-butylamine, 3.66 mmoles of β-phenylethylamine, 16.9 μmoles of putrescine, and 17.2 μmoles of cadaverine. Before treatment with HFBA, 0.05 ml of the standard was added to a test tube and diluted with 0.05 ml of chloroform.

Allylamine, secondary butylamine, 1-amino-2-propanol, 1,3-diaminopropane, and tyramine were prepared individually for analysis. Full-scale deflection can be obtained for these amines by preparing individual samples as follows: 668 μmoles of allylamine, 1.44 mmoles of secondary butylamine, and 300 μmoles of 1,3-diaminopropane each in 6 ml of chloroform; and 10 μmoles of tyramine in 2 ml of chloroform. Five-hundredths milliliter of 1-amino-2-propanol was added to 30 ml of chloroform. One-tenth milliliter each of these diluted amines was treated with HFBA, and 0.1 μliter of the 100-μliter sample was used for both the standard mixture and the individual standards for GLC analysis. Although indole and tryptamine may be included in the standard mixture, they are frequently impure, and it is better to analyze for them individually. Five hundred μmoles of indole and 200 μmoles of tryptamine were each added to 20 ml of water and processed in the same manner as urine samples. A 1-μliter injection of a 100-μliter sample was used for GLC analysis.

HFBA-pyridine derivatives of nitrosamine were prepared in the same manner as amines. With a 1-μliter injection of sample, 10 μmoles extracted at *p*H 10 gave full-scale response.

Analysis by GLC. A Barber-Colman gas chromatograph (model 5000) was equipped with a tritium 300-mCi EC detector. The instrument was operated with two U-shaped columns (0.3 cm inside diameter by 7.3 m length). One column (nonpolar) was packed with Chromosorb W 80/100 mesh (AW-DMCS H.P.) coated with 3% (OV-1, Applied Science Laboratories). Another column (polar) was packed with TA33 TABSORB (Regis Chemical Co.). The instrument was operated isothermally for 5 min at 90 C, then programmed for a linear increase of 5 C per min to 220 C. The temperature of both detector and injector was 220 C, the electrometer attenuation was 32, the range setting was 10, and the balance setting was 10^4. Nitrogen was used as the carrier gas at a flow rate of 36 ml/min. The recorder was operated with an input signal of 1 mv and a chart speed of 30 inches/hr. HFBA derivatives of amines were tentatively identified by comparison of retention times of unknown compounds on both polar and nonpolar columns with the retention time of HFBA derivatives of known standards. A third column, which had moderately polar characteristics, was packed with 3% OV-17 (Applied Science Laboratories) on Chromosorb W 80/100 mesh (AW-DMCS H.P.) and was used in some instances to gain additional evidence for the production of N-nitrosodimethylamine (N-NDA).

Results

Standards. A wide variety of biologically important amines were resolved well on both OV-1 and TABSORB columns as illustrated in figure 1, curves A, B, and C. The monoamines tested gave a full-scale response with an injection of 2–24 nmoles, with the exception of pyrrolidine, which required 92 nmoles. About a 10-fold increase of sample (from 0.1 to 1.0 μliter) was required to obtain full-scale response when the monoamines were extracted from water. Beta-phenylethylamine was exceptional in that almost all of this amine was extracted. Putrescine and cadaverine added to chloroform were detected full scale in about 410 pmole quantities, and 1,3-diaminopropane

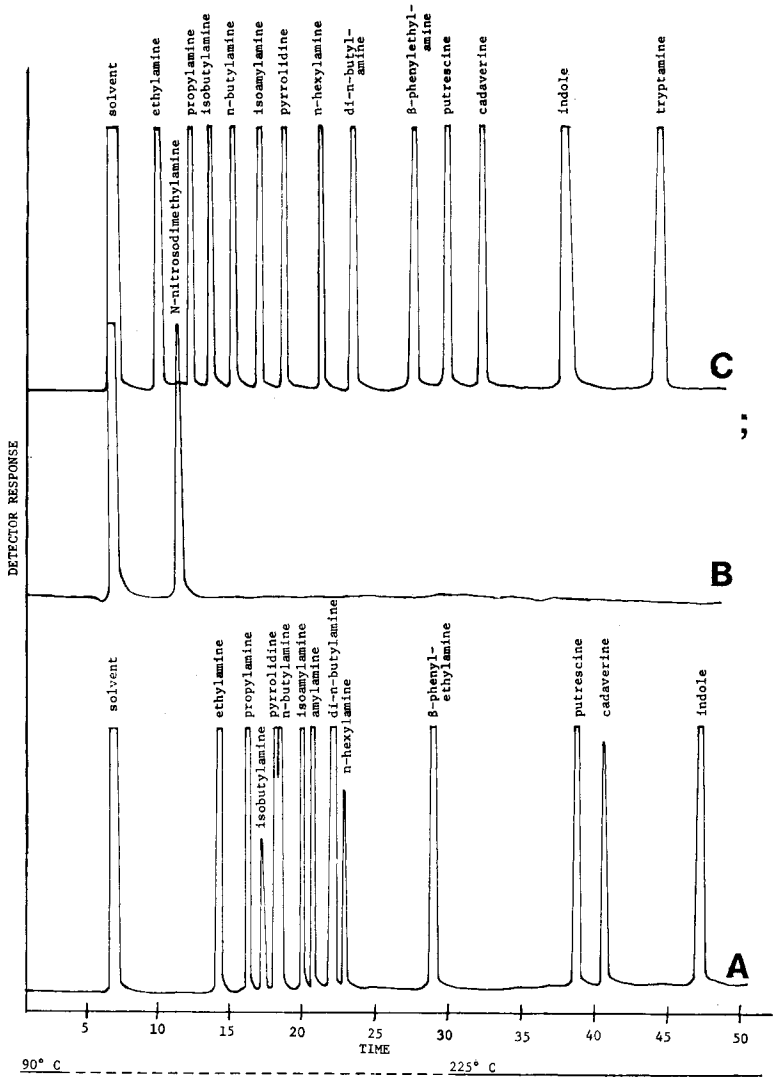

Figure 1. Gas chromatograms of mixtures of standard amines treated to form heptafluorobutyric-anhydride derivatives (electron-capture detector). (**A**) Analysis of a standard amine mixture on a TABSORB (polar) column. (**B**) Analysis of dimethylnitrosamine made on a 3% OV-1 column. (**C**) Analysis of a standard amine mixture made on 3% OV-1 column.

added to chloroform was detected in quantities of about 1 nmole. However, when putrescine and cadaverine were extracted from water, about 200 nmoles were required for full-scale deflection with the volume of sample injection increased from 0.1–1.0 μliter. One μmole of tyramine extracted from water gave a full-scale response with a 1-μliter injection of sample. The detector was relatively insensitive to column bleed, and a good baseline was maintained across the entire temperature span. There was no noticeable change in the detector response after use for seven months, even though the detector temperature and final column temperature were the same, and nine or 10 samples were analyzed each day. Molen [13] reported similar results using tritium detectors for analysis of steroids where both column and detector were operated at temperatures between 220 C and 225 C.

Urine specimens. After growth for 20 hr in the urine obtained after infection, the strain of *P. mirabilis* 40-512-1 isolated from the infected urine produced several of the same compounds detected in the infected urine (figure 2, curves B and C). Compare N-NDA, pyrrolidine, β-phenylethylamine, putrescine, cadaverine, and peaks 18,

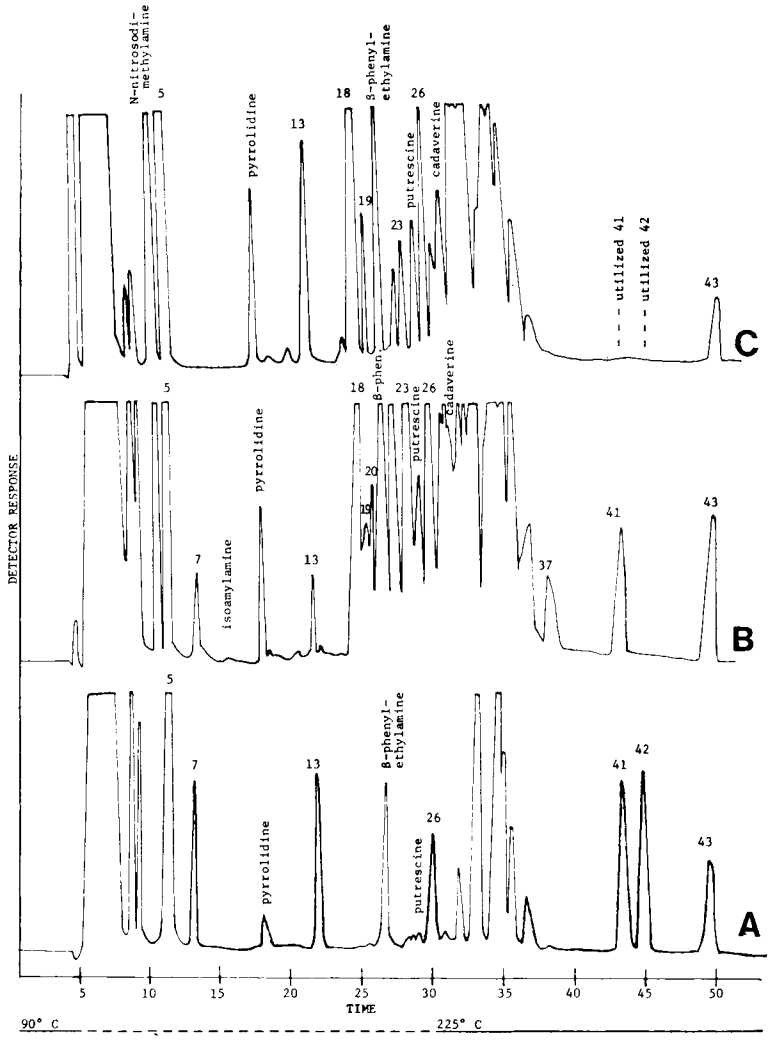

Figure 2. Gas chromatograms of basic chloroform extracts of urine treated to form heptafluorobutyric-anhydride derivatives. The analysis was made on a 3% OV-1 column using an electron-capture detector. (**A**) Control urine taken from a patient several weeks after infection with *Proteus mirabilis*. (**B**) Urine taken from a patient infected with *P. mirabilis* 40-512-1 (>10^6/ml). (**C**) Urine taken several weeks after recovery from infection with *P. mirabilis* 40-512-1. The urine was heated to 100 C for 1 min and incubated to check sterility; then it was inoculated with *P. mirabilis* and incubated for 20 hr at 37 C. Note that heating of the control urine (curve A) did not noticeably change the profile of the control from that of the unheated urine; therefore, curve A can be considered a control for both heated and unheated postinfected urine.

19, 23, 26, and 42 (utilized). With the exception of peak 26, β-phenylethylamine, and trace amounts of putrescine and pyrrolidine, these compounds were not present in urine taken several weeks after infection, as can be observed on the 3% OV-1 column (figure 2, curves A and B) and on TABSORB (figure 3, curves A and B). However, when analyzed on an OV-1 column, the urine sample taken during acute infection and the sample of postinfection urine inoculated with *P. mirabilis* gave profiles very different from the one obtained from control urine obtained after infection. Compare curves B and C with A in figure 2 (3% OV-1 column). The urine of the second patient from which *P. mirabilis* 40-2701-1 was isolated gave a profile similar in many ways to that obtained from the first patient. Compare curves B and C in figure 3 (TABSORB column). N-NDA, pyrrolidine, β-phenylethylamine, putrescine, cadaverine, and peaks 11, 14, 20, 28, 59, and 60 were present in both profiles. (Note that numbering of peaks on the TABSORB column

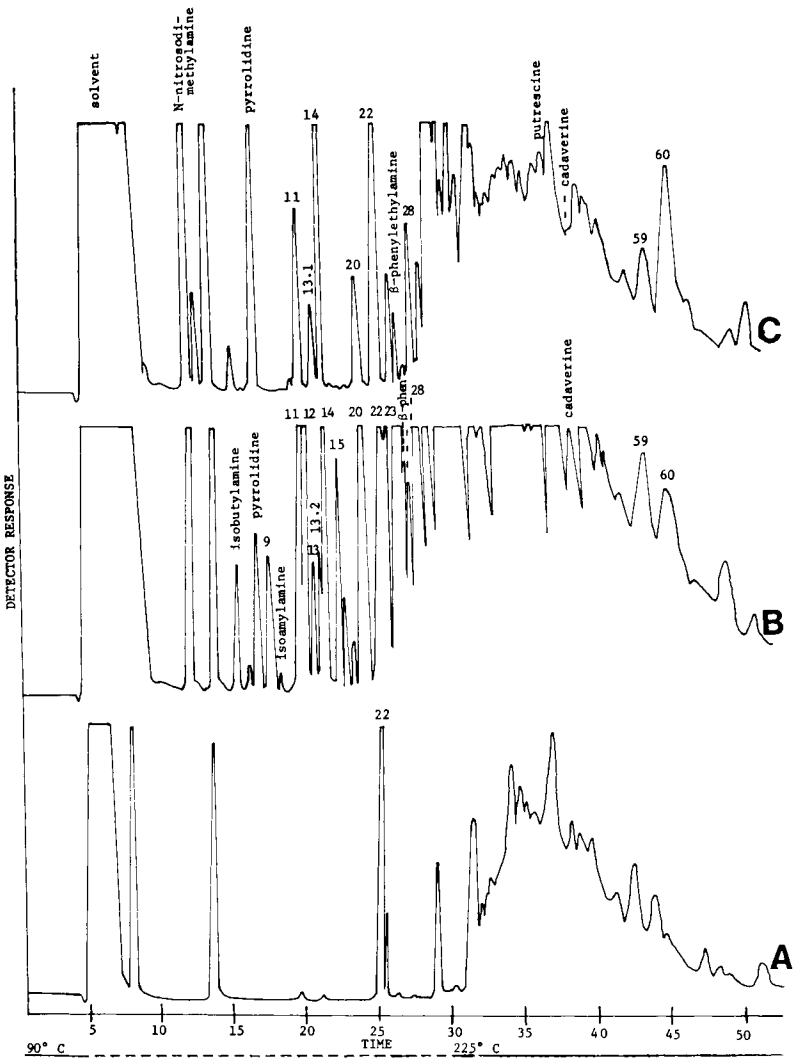

Figure 3. Gas chromatograms of basic chloroform extracts of urine treated to form heptafluorobutyric anhydride derivatives. The analysis was made on a TABSORB (polar) column using an electron-capture detector. (**A**) Urine control sample taken several weeks after patient recovered from an infection with *Proteus mirabilis* 40-512-1 (**B**) Urine taken during time patient was infected with *P. mirabilis* 40-512-1 ($>10^6$/ml). (**C**) Urine taken from a second patient with a low-level infection with *P. mirabilis* 40-2701-1 ($<10^5$ organisms/ml). Note that the number assigned to a peak on the TABSORB column does not necessarily correlate with the same number on the 3% OV-1 column, and that the solvent peak in the control (curve A) is smaller because more of the chloroform was removed by evaporation and not because of a smaller injection of sample.

does not necessarily correspond to numbers of peaks on the 3% OV-1 column.) Striking differences are also apparent when the profiles of the urines from the two infected patients are compared with the profile of the urine taken from one of the patients after recovery. Compare curves B and C with A in figure 3 (TABSORB column).

Differences in the GLC profiles were observed when control urine specimens obtained after infection were inoculated with *P. mirabilis* and incubated for 20 hr and for four days. Compare curve C in figure 2 with curve B in figure 4 (3% OV-1 column). After four days the peak of N-NDA has increased, peaks 18 and 26 remain about the same, peaks 12 and 15 are new, and some of the peaks are reduced in size.

When the same specimen of urine was inoculated with *E. coli* and incubated for four days, the GLC profile was different from that of *P. mirabilis* (3% OV-1 column, curves A and B, figure 4).

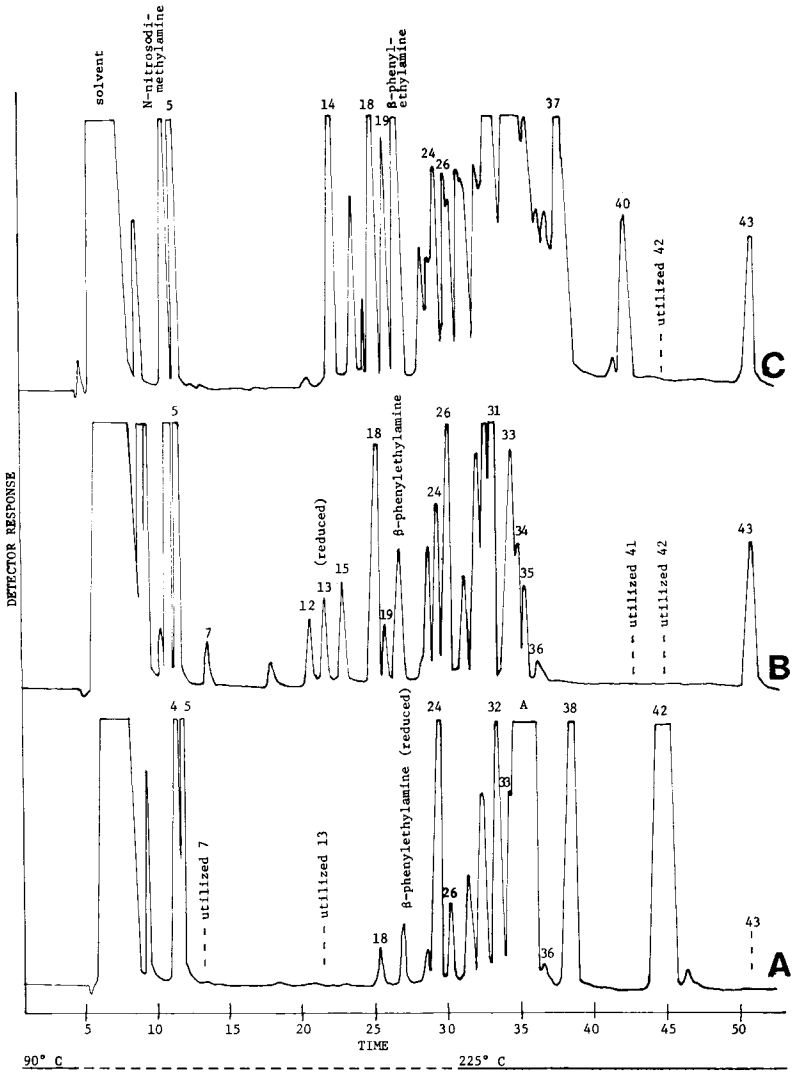

Figure 4. Gas chromatograms of basic chloroform extracts of urine treated to form heptafluorobutyric-anhydride derivatives. The analysis was made on a 3% OV-1 column using an electron-capture detector. (**A**) Postinfected urine control taken from a female patient several weeks after recovery from an infection with *Proteus mirabilis* 40-512-1. The urine was boiled for 1 min, tested for sterility, inoculated with *Escherichia coli* 40-513-1, and incubated for four days. (**B**) Postinfected urine (same as used in curve A) incubated with *P. mirabilis* 40-512-1 for four days. (**C**) Urine taken from a noninfected male, heated, checked for sterility, and incubated with *P. mirabilis* 40-512-1 for four days. (*Utilized* refers to complete removal of a component and *reduced* refers to partial removal.) Note that there are some similarities in the profiles of the two different urines incubated with *P. mirabilis* 40-512-1, and that these differ significantly from that produced by E. coli 40-513-1. Observe also that Peak 4 does not have the same retention time as N-nitrosodimethylamine on OV-1 or on the TABSORB (polar) column.

N-NDA was not produced. Peaks A, 38, and 42 are noticeably different. Peaks 12, 13, and 15 are missing from *E. coli,* and peak 18 is greatly reduced. Peak 42 was present in moderate amounts in the uninoculated urine (figure 2, curve A), but it was greatly increased after the growth of *E. coli.* Unidentified compounds represented by peaks 41 and 43 were used by *E. coli,* whereas *Proteus* used compounds represented by both peaks 41 and 42 but not 43. Even though some differences existed in the composition of the urine taken from two individuals, one male (curve C, figure 4) and one female (curve B, figure 4), several of the same compounds were produced in both urines by

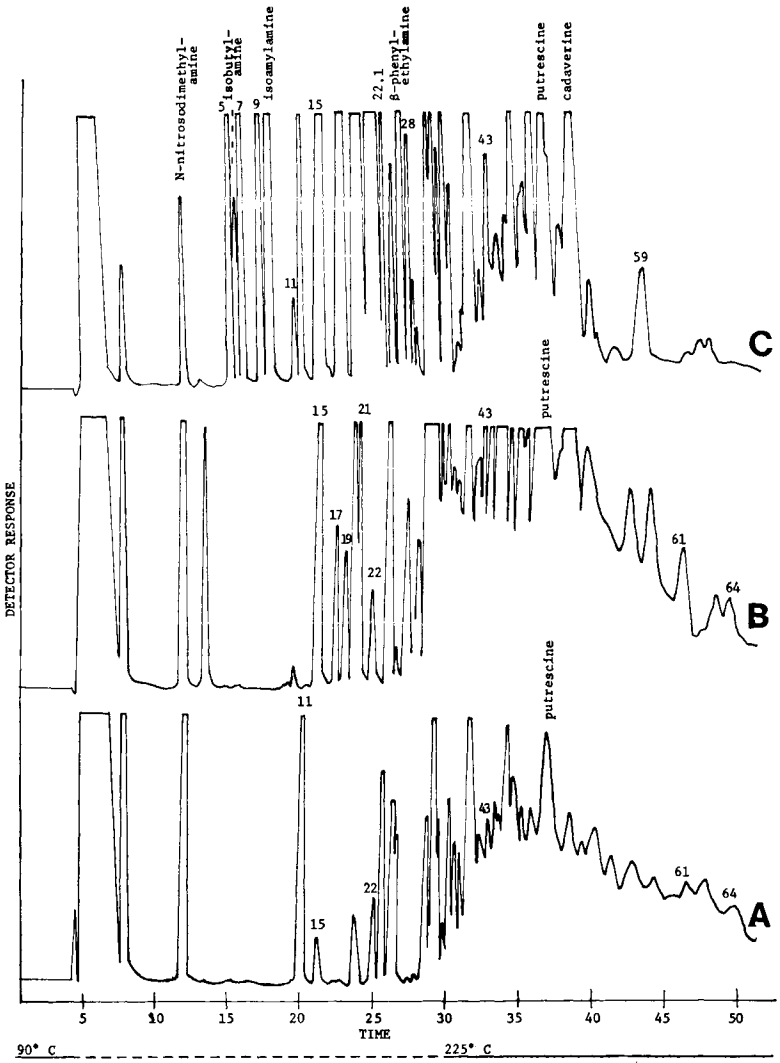

Figure 5. Gas chromatograms of basic chloroform extracts treated to form heptafluorobutyric-anhydride derivatives. The analysis was made on a TABSORB (polar) column using an electron-capture detector. (**A**) Urine taken from a female patient several weeks after infection with *Proteus mirabilis* 40-512-1. The urine was boiled for 1 min, checked for sterility, and incubated with *P. mirabilis* for four days. (**B**) Urine from a noninfected male was treated as in curve A and incubated with *P. mirabilis* 40-512-1 for four days. (**C**) Cooked-meat medium inoculated with *P. mirabilis* 40-512-1 and incubated for four days. Note that even though the substrate is varied in composition, some of the same compounds are produced by the organism on all substrates.

P. mirabilis. Compare N-NDA, β-phenylethylamine, peaks 18, 19, 24, and 26. The compound represented by peak 42 was used in both urines. In addition, when *P. mirabilis* 40-512-1 was grown for four days on CMG and in two different urines (TABSORB column, figure 5), several of the same compounds were produced; observe specifically N-NDA, putrescine, and peaks 15 and 43. Peak 11 is present in one urine (curve A) and in CMG (curve C). Using a flame-ionization detector and established methods [1], we were able to demonstrate the production of isobutylamine, isoamylamine, β-phenylethylamine, putrescine, and cadaverine after four days of growth on CMG (figure 6, curves A and B). It is also apparent that under these conditions *E. coli* and *P. mirabilis* produce different metabolic profiles. It has been demonstrated by other workers using

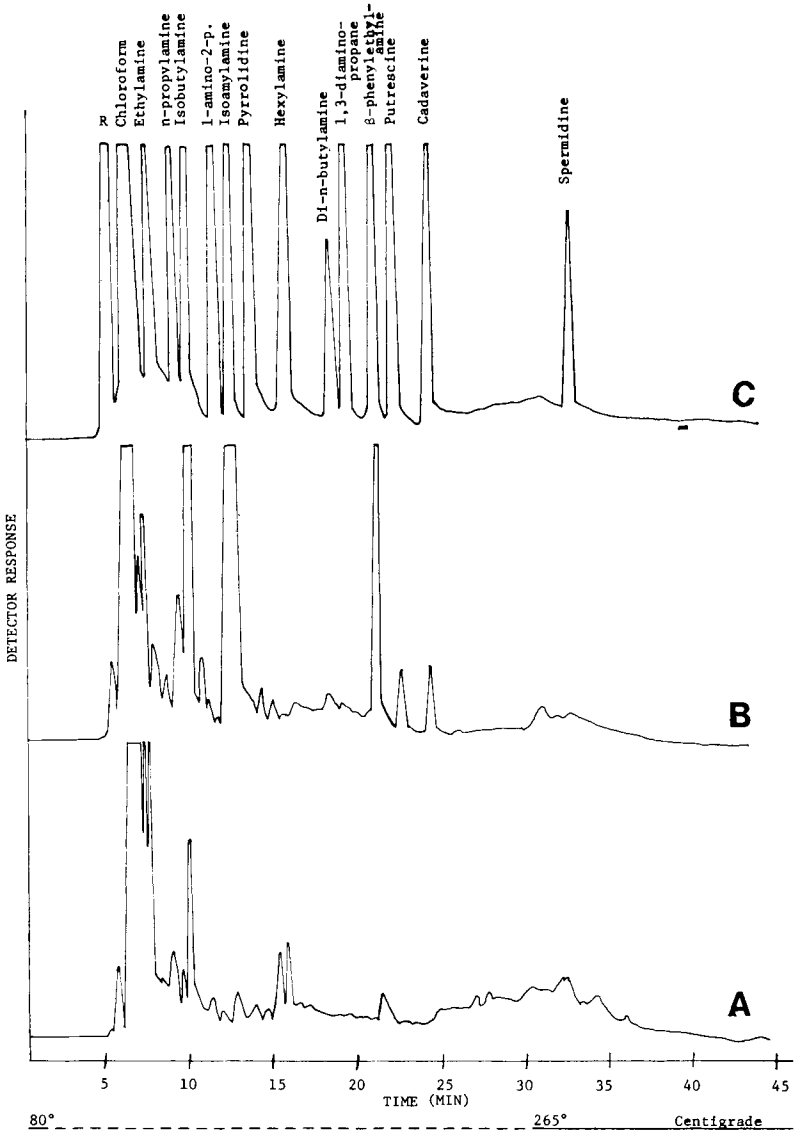

Figure 6. Gas chromatograms of a standard mixture of amines and basic chloroform extracts of spent-culture cooked-meat medium (CMG). Both the extracts and standard amine mixture have been treated to form trifluoroacetic-anhydride derivatives. Analyses were made on a 3% OV-1 column using flame-ionization detectors. (**A**) *Escherichia coli* 40-513-1 incubated for four days on CMG. (**B**) *Proteus mirabilis* 40-512-1 incubated for four days on CMG. (**C**) A standard amine mixture. Note that the amine profiles of the two organisms are distinctly different.

different methods that some species of *Proteus* possess decarboxylases for L-valine and L-leucine, from which isobutylamine and isoamylamine are produced [14], and that *Proteus* species decarboxylate ornithine [11], from which putrescine could be produced. However, the amines produced both in vivo and in vitro in urine and many of those produced in CMG were not detected by flame-ionization detectors. Compare figure 6, curve B, with figure 5, curve C.

Discussion

The data presented here strongly suggest that metabolic profiles, although subject to change when the substrate is modified, are consistent

enough to produce identifying chromatograms in complex media such as urine and CMG. Further work is indicated to determine whether or not the procedure presented here can be used to distinguish various types of urinary-tract disease. Evidence presented by other workers [15] indicates that dietary changes have relatively small effect on the profile of the volatile urinary substances and that individual patterns are very similar. If this is true, the profiles observed here may be rather characteristic of infection with *P. mirabilis*; furthermore, the results obtained here should be confirmed and patients infected with several species of microorganisms tested. The metabolic patterns in the urine of patients infected with these organisms might present distinguishing criteria. Although at the time of this study a cooperative patient infected with *E. coli* was not available for extended in-vivo studies such as those described for *P. mirabilis,* urine from such a patient should be readily available for future studies.

Hawksworth and Hill [7] suggested that in geographic areas where intake of nitrate was high, certain bacteria known to convert secondary amines into nitrosamines in vitro also might form nitrosamines in vivo by a reaction between secondary amines and nitrites produced by the organism or by the host. The same workers report in-vitro conversion by microorganisms of secondary amines to nitrosamines at neutral *p*H, and in more recent studies [16] they predict that the most likely site of bacterial nitrosylation of secondary amines would probably be the urinary bladder of people with urinary-tract infections. The fact that N-NDA was detected in the urine of two different individuals infected with *P. mirabilis,* in urine taken after infection and inoculated with this organism, in inoculated normal urine from a third person, and in CMG medium seems to confirm this prediction and suggests that the necessary concentration of nitrate may be present in the urine even in areas considered to be low in dietary nitrate consumption. Jasmin and Riopelle [17] induced renal tumors in a high percentage of rats that received six successive daily doses of 1.5 mg of N-NDA. A single exposure often produced renal tumors in these animals. Magee and Barnes [6] listed the relatively acute oral toxicity of 24 nitroso compounds for rats; N-NDA was the second most toxic compound in the list. N-NDA was one of the major amines consistently produced by *P. mirabilis*. In the absence of independent data from mass spectrometry and infrared analysis, we are tentatively reporting the identification of N-nitrosodimethylamine in urine. We feel, however, that a high degree of confidence can be placed in this tentative identification for the following reasons. (*1*) Known HFBA derivatives of N-NDA, when added in small quantities to the derivatized extracts of urine samples in which *P. mirabilis* had been cultured, increased the size of the N-NDA peak and had the same retention time on 3% OV-1, 3% OV-17, and TABSORB columns. (*2*) N-NDA in the derivatized extract of urine from a patient with *P. mirabilis* bacteriuria gave the same retention time on both 3% OV-1 and TABSORB columns. (*3*) Since the TABSORB column has separation characteristics quite different from those of the 3% OV-1 column (figure 1, curves A and C), it is unlikely that two different compounds would behave in identical fashion on the two columns.

N-NDA was recovered in smaller amounts than many other amines; it required a concentration of almost 10 μmoles/20 ml of sample with a 1-μliter injection, as determined with known N-NDA or HFBA derivatives. The compound was extractable with chloroform under acidic, neutral, and basic conditions. However, only about one-third as much was extracted under acidic conditions as under neutral or basic conditions; the latter two were equally effective. In any case a single extraction, as used in this study, was not sufficient to recover all of the compound. Detection of the nitrosamines depended upon the formation of HFBA derivatives, and the reaction mechanism for the formation of HFBA derivatives with nitrosamines has not been determined; however, the fact that N-NDA does form derivatives with HFBA and pyridine has been confirmed by mass spectra [10].

A rough calculation of the amount of N-NDA to which one of the patients studied here was exposed during recurrent bacteriuria due to *P. mirabilis* was 0.8 g. This is based on the observation that 10 μmoles of N-NDA/20 ml of urine (0.5 μmoles/ml) gave full-scale deflection of the recorder pen when 1 μliter of the extracted and derivatized sample was injected. The following assumptions were made. (*1*) The average volume

of urine produced daily = 1,500 ml. (*2*) The average duration of significant bacteriuria before treatment or before reduction of high count of *P. mirabilis* = three days. (*3*) Then, 1,500 ml × 0.5 μmoles N-NDA/ml × three days = 2,250 μmoles or 166 mg of N-NDA.

Thus, during a single episode of bacteriuria due to *P. mirabilis,* this patient may have been exposed to 166 mg of N-NDA. Since one patient in this study had experienced five episodes of bacteriuria due to this organism, she had a potential exposure to 0.8 g of N-NDA over a period of 11 years. The demonstration that this compound may be produced in vivo in human urine by the action of bacteria suggests the need for exploring its potential for inducing tumors or for initiating other mechanisms of tissue damage.

The procedure described in this paper has been used in another study (unpublished data) to analyze for amines and for hydroxy-containing compounds, such as alcohols, in body fluids other than urine and in spent culture media. Alcohols form electron-capturing derivatives with HFBA just as amines do. Although alcohols can be obtained in the first extraction with organic solvents (*p*H 2), one extraction often will not completely remove them, and they will appear (usually in small amounts) in the basic extract.

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