Journal of Chromatography, 338 (1985) 51—59
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2414

AVAILABILITY OF 6-HYDROXYNICOTINIC ACID FOR RAPID IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* AND *SERRATIA MARCESCENS*

SANSHIRO SHIRAISHI and NOBUO SAKAMOTO

The Third Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466 (Japan)

KENJI MAEDA

Nagoya University Branch Hospital, 1-20, Daiko-minami 1-chome, Higashi-ku, Nagoya 461 (Japan)

and

TOYOKAZU OHKI*, MASAHARU HOSOI, KAZUHIRO OHTA and NAOKI YAMANAKA

The Bio-Dynamics Research Institute, 1-3-2, Tamamizu-cho, Mizuho-ku, Nagoya 467 (Japan)

(First received June 18th, 1984; revised manuscript received October 5th, 1984)

SUMMARY

Gas chromatography—mass spectrometry has been used to identify specific metabolites produced by Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Escherichia coli* in a defined medium. 6-Hydroxynicotinic acid was detected in spent culture media of *Pseudomonas aeruginosa* and *Serratia marcescens*, but could not be detected in those of *Klebsiella pneumoniae* and *Escherichia coli*. The production of 6-hydroxynicotinic acid was recognized by the addition of nicotinic acid in urine with *Pseudomonas aeruginosa* or *Serratia marcescens*, but not without the addition of nicotinic acid.

Among 10^5 *Pseudomonas aeruginosa* per 1 ml of urine (criteria for the diagnosis of urinary tract infection), 0.15 μg of 6-hydroxynicotinic acid was detected in urine at 4 h incubation with nicotinic acid at the optimum pH of 6.9, 38°C. The production of 6-hydroxynicotinic acid was proportional to the number of the bacteria and displayed a time dependency. These results suggest that the availability of 6-hydroxynicotinic acid might make for more rapid identification of bacteria than current methods.

0378-4347/85/$03.30

INTRODUCTION

Serious infection by pathogenic microorganisms has been diminished by newly developed antibiotics, but the significance of Gram-negative microorganisms such as *Pseudomonas aeruginosa* has increased from the viewpoint of attributable microorganisms of opportunistic infection. Many studies have been carried out for rapid identification of such bacteria using gas chromatography [1—3] or other methods [4, 5], since the present routine diagnostic methods are time-consuming. At present the results of culture take one day and, in the case of growth, information about the identity is given on the second day. It would be desirable to reduce the long time interval and to provide the answer within one working day.

In the present study, the specific metabolites of *P. aeruginosa* and *Serratia marcescens* are identified, and the possibility of clinical diagnosis within 6 h using gas chromatography—mass spectrometry (GC—MS) is discussed.

MATERIALS AND METHODS

Bacteria

The bacteria used in this study, *P. aeruginosa* strain P101, strain P102, *S. marcescens* strain S108, *Klebsiella pneumoniae* strain K105, and *Escherichia coli* strain E101, were isolated from urine samples of patients suffering from urinary tract infections. The identity of the culture specimens was confirmed by conventional and serological methods [6, 7].

Reagents

6-Hydroxynicotinic acid (6HN) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Gasukuro Kogyo (Tokyo, Japan). Nicotinic acid and *p-n*-amylbenzoic acid (ABA) were the products of Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents were of the highest purity available commercially.

Identification of 6HN in spent culture media

Organisms, $1 \cdot 10^8$—10^9, of *P. aeruginosa* strain P101, *S. marcescens* strain S108, *K. pneumoniae* strain K105, or *E. coli* strain E101 were inoculated into Mølar-Hinton broth (Eiken, Tokyo, Japan) and incubated at 38° C for 20 h. After sterilization by filtration, 3 ml of the medium to which 20 μg of ABA (internal standard) had been added were acidified to pH 1 with 6 *M* hydrochloric acid and saturated with sodium chloride. Organic acids were extracted twice with 9 ml of ethyl acetate for 10 min, dehydrated over anhydrous sodium sulphate, and evaporated to dryness with a rotary evaporator and a stream of nitrogen. Organic acids were derivatized with 200 μl of BSTFA at 65° C for 30 min, and 2 μl of the sample were subjected to GC—MS.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a 5710A gas chromatograph (Hewlett-Packard) equipped with a 30-m OV-101 capillary column, a JMS D-300 mass spectrometer (JEOL) and a JMA 2000 data processing

system (JEOL). The carrier gas was helium. Electron-impact ionization (EI) mass spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 300 μA, ion source temperature 200°C, and accelerating voltage 3 kV. Chemical ionization mass spectra were recorded using methane as a reactant gas, and with an ionizing energy of 200 eV. The other conditions were the same as for EI.

Detection of 6HN in urine

Nicotinic acid solution was prepared by dissolving 100 mg of nicotinic acid in 100 ml of 0.2 *M* potassium dihydrogen phosphate—disodium hydrogen phosphate buffer at pH 7.0 and sterilized by filtration. Urine excreted from a healthy man was immediately sterilized by filtration. A 1-ml aliquot of the sterilized urine was incubated with *P. aeruginosa* strain P101 ($1 \cdot 10^7$) and/or 2 ml of nicotinic acid solution (1 mg/ml) at 38°C for 3 h. After centrifugation, 1 ml of the supernatant to which 10 μg of ABA had been added was acidified to pH 1 and saturated with sodium chloride. The organic acids were extracted twice with 3 vols. of ethyl acetate, dehydrated, dried and trimethylsilylated using the same procedure as with the culture media.

Quantitative determination of 6HN

The calibration curve for 6HN in urine was obtained by adding a known amount of standard to 1 ml of water. After addition of 10 μg of ABA (internal standard), 6HN was extracted using the sampe procedure as with the urine sample. Calibration curves relating the concentration of 6HN were obtained from the mass chromatogram. Ion *m/z* 268 was used for the quantitation of 6HN, and ion *m/z* 264 for the monitoring of an internal standard, ABA.

Recovery of 6HN

Quintuplicate estimations of recovery were carried out using 10 μg of 6HN, which were added to 1 ml of phosphate buffer. 6HN was extracted using the same procedure as with the urine sample and quantitated by mass chromatography. Recovery of 6HN was 61.6 ± 7.0% (mean ± S.D., $n = 5$).

pH Dependency of 6HN production

Each pH solution of nicotinic acid was prepared using 0.2 *M* potassium dihydrogen phosphate—disodium hydrogen phosphate and sterilized by filtration. A 1-ml volume of sterilized urine was added to 2 ml of the phosphate buffer containing nicotinic acid and incubated with *P. aeruginosa* strain P101 ($1.1 \cdot 10^7$) at 38°C for 3 h. 6HN was extracted twice with 3 ml of ethyl acetate from 1 ml of medium (see *Detection of 6HN in urine*) and analysed. The ratios of peak heights of 6HN to ABA were obtained on the mass chromatogram of each pH.

Production rate of 6HN

Experiment 1. A 1-ml volume of the sterilized urine to which 2 ml of the phosphate buffer containing nicotinic acid had been added, was incubated with *P. aeruginosa* strain P101, $1.0 \cdot 10^7$, $1.0 \cdot 10^6$, $1.0 \cdot 10^5$, $1.0 \cdot 10^4$, or $1.0 \cdot 10^3$, for 1, 2, 4, or 6 h at 38°C.

The ratios of peak heights of 6HN and ABA on the mass chromatogram of each case were obtained, and the production of 6HN in each case was quantitated using the standard curve.

Experiment 2. Production rate of 6HN by *P. aeruginosa* strain P102 was estimated using the same procedure as for Experiment 1. A 1-ml volume of the sterilized urine to which 2 ml of the phosphate buffer containing nicotinic acid had been added, was incubated with $1.2 \cdot 10^8$, $1.2 \cdot 10^7$, $1.2 \cdot 10^6$, or $1.2 \cdot 10^5$ *P. aeruginosa* strain P102 for 1, 2, 4, or 6 h at 38°C.

RESULTS

Fig. 1a—d shows the gas chromatograms of trimethylsilyl (TMS) derivatives of organic acids in media incubated with (a) *P. aeruginosa* strain P101, (b) *S. marcescens* strain S108, (c) *K. pneumoniae* strain K105, and (d) *E. coli* strain E101; Fig. 1e shows a control medium. The peaks were identified by comparing their mass spectra with those of the trimethylsilylated authentic compounds or the mass spectra in the literature.

Peak 63 was detected in spent culture media of *P. aeruginosa* and *S. marcescens*, but could not be detected in those of *K. pneumoniae* and *E. coli*. The EI mass spectrum of peak 63 is shown in Fig. 2 (upper spectrum). The chemical ionization mass spectrum using methane as a reactant gas showed that the molecular ion of the compound was 283. High-resolution mass spectrometry of the *m/z* 283 ion showed an exact mass of 283.1005, an error of 0.0056 and a probable composition of $C_{12}H_{21}NO_3Si_2$. These data revealed the original composition of $C_6H_5NO_3$ and the structure of hydroxynicotinic acid. The EI mass spectrum of the TMS derivative of 6HN is shown in Fig. 2 (lower spectrum). Peak 63 was identified as trimethylsilylated 6HN, because peak 63 and trimethylsilylated 6HN showed identical retention times and identical mass spectra.

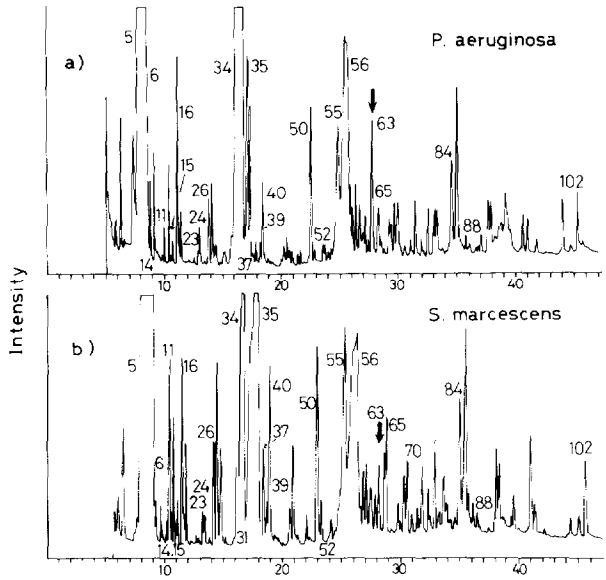

Fig. 1.

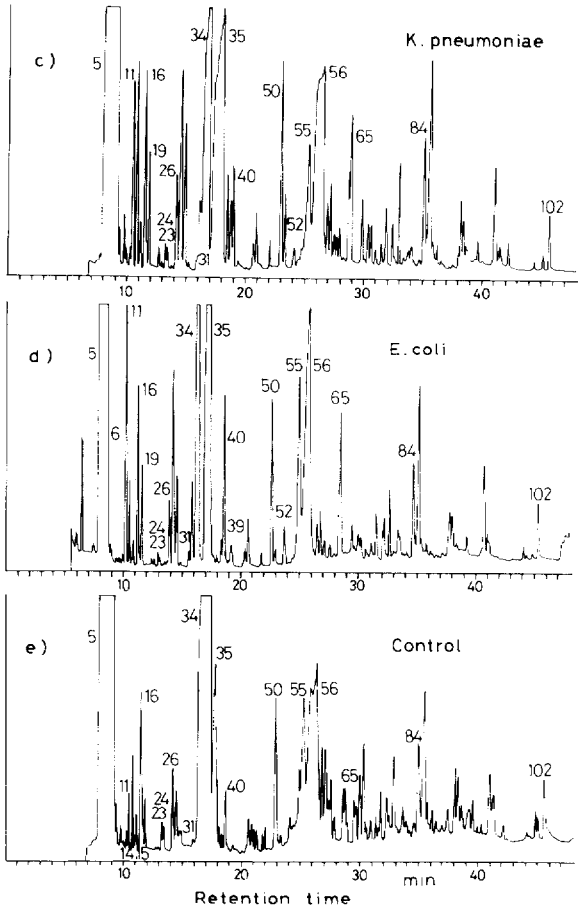

Fig. 1. Gas chromatogram of TMS derivatives of ethyl acetate extracts of: (a) *P. aeruginosa* strain P101 spent culture medium; (b) *S. marcescens* strain S108 spent culture medium; (c) *K. pneumoniae* strain K105 spent culture medium; (d) *E. coli* strain E101 spent culture medium; (e) control medium. Peak identification: 5 = lactic acid; 6 = glycolic acid; 11 = 2-hydroxybutyric acid; 14 = *p*-cresol; 15 = ethyleneglycol; 16 = 3-hydroxybutyric acid; 19 = 2-hydroxyisovaleric acid; 24 = 3-hydroxyvaleric acid; 26 = benzoic acid; 31 = nicotinic acid (minor of two components); 34 = glycerol; 35 = succinic acid; 39 = glyceric acid; 40 = fumaric acid; 50 = 2-deoxytetronic acid; 56 = pyroglutamic acid; 63 = 6-hydroxynicotinic acid; 84 = internal standard (ABA); 88 = 4-hydroxymandelic acid; 102 = palmitic acid. The column temperature was programmed from 100° C to 250° C at 3° C/min.

Fig. 3 gives the gas chromatogram of TMS derivatives in urine incubated with nicotinic acid and/or *P. aeruginosa* strain P101. 6HN could not be detected in urine incubated with only *P. aeruginosa* (b), but was found in urine incubated with both *P. aeruginosa* and nicotinic acid (a). These results indicate that *P. aeruginosa* metabolized nicotinic acid to 6HN within 3 h. (As shown in Fig. 1, the Mϕlar-Hinton broth contains nicotinic acid.)

Fig. 4 shows the pH dependency of the 6HN production. The pH of the urine to which 2 ml of each of the buffer containing nicotinic acid were added, was not changed before or after incubation. The optimum pH of 6HN production was at 6.9.

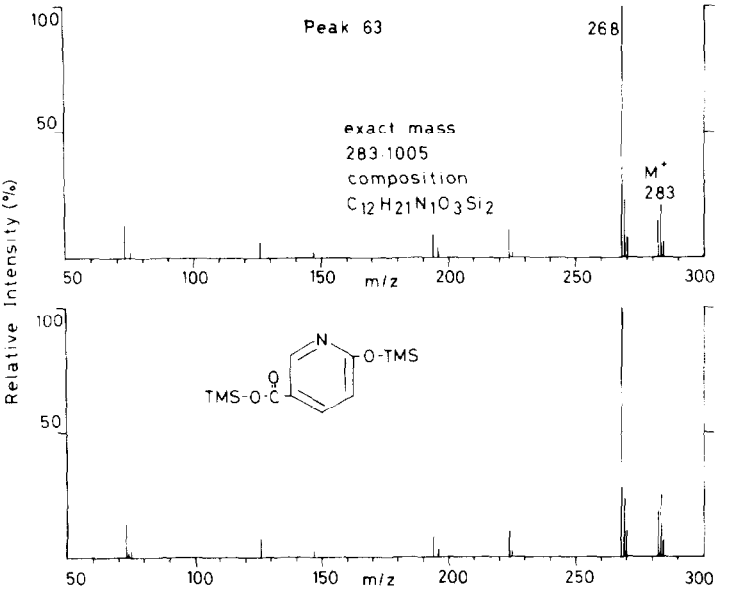

Fig. 2. EI mass spectra of trimethylsilylated 6-hydroxynicotinic acid (lower spectrum) and of peak 63 in Fig. 1a (upper spectrum).

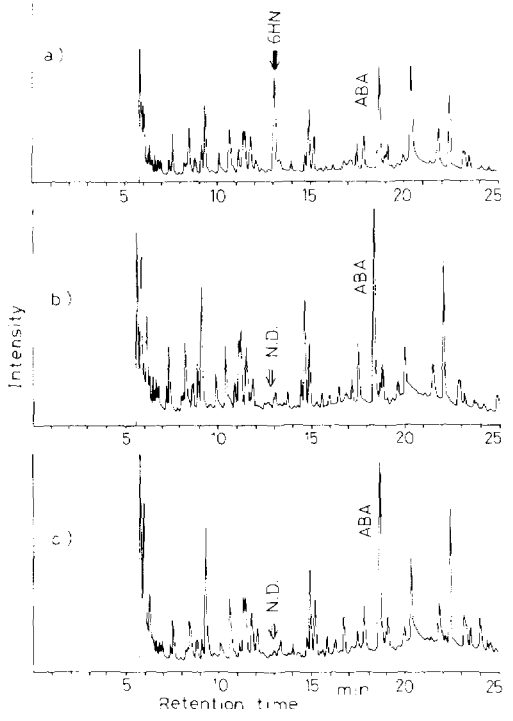

Fig. 3. Gas chromatogram of TMS derivatives of organic acids in urine incubated with (a) *P. aeruginosa* strain P101 and nicotinic acid, (b) *P. aeruginosa* strain P101, (c) nicotinic acid, at 38°C for 3 h. The column temperature was programmed from 150°C to 230°C at 3°C/min. 6HN = 6-hydroxynicotinic acid, ABA = *p-n*-amylbenzoic acid (internal standard), N.D. = not detected.

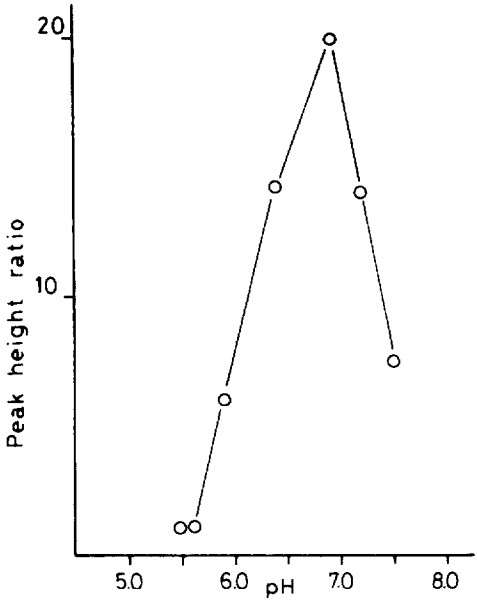

Fig. 4. pH Dependency of 6HN production. A 1-ml volume of urine was incubated with $1.1 \cdot 10^7$ *P. aeruginosa* strain P101 at 38° C for 3 h.

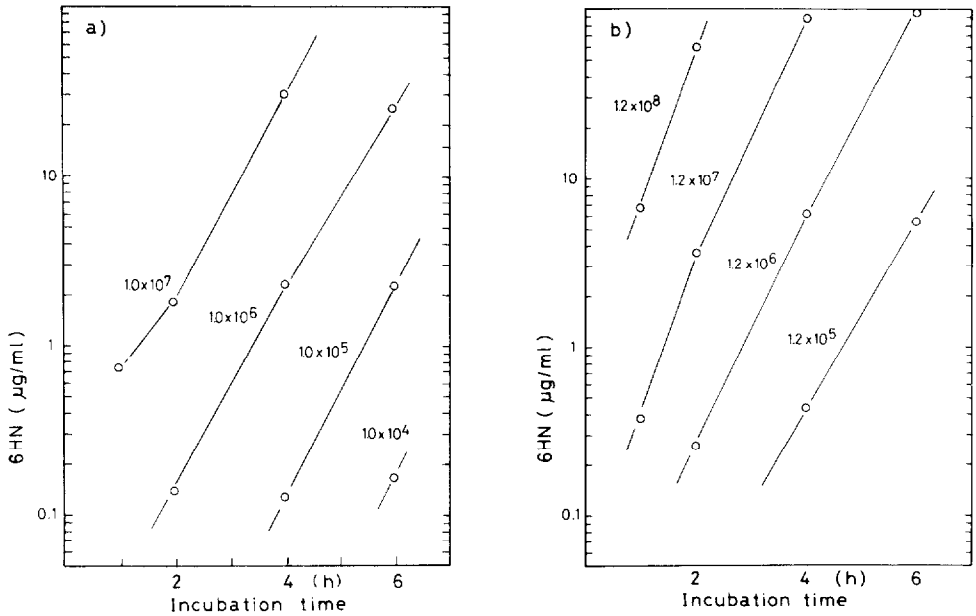

Fig. 5. Production rate of 6HN (a) by *P. aeruginosa* strain P101, (b) by *P. aeruginosa* strain P102. A 1-ml volume of urine to which 2 ml of 1 mg/ml phosphate buffer pH 7.0 containing nicotinic acid had been added, was incubated with *P. aeruginosa*.

Fig. 5a shows the production rate of 6HN by *P. aeruginosa* strain P101. In $1.0 \cdot 10^5$ bacteria, 0.15 µg of 6HN could be detected at 4 h incubation and 2.2 µg at 6 h incubation. In $1.0 \cdot 10^7$ organisms, 0.75 µg of 6HN could be detected

at 1 h incubation. The production of 6HN was proportional to the number of strain P101 and evidenced a time dependency.

Fig. 5b shows another set of experiments of the production rate of 6HN by *P. aeruginosa* strain P102. In $1.2 \cdot 10^5$ organisms, 0.43 μg of 6HN could be detected at 4 h incubation and 5.5 μg at 6 h incubation. In $1.2 \cdot 10^8$ bacteria, 6.7 μg of 6HN could be detected at 1 h incubation and 59.5 μg at 2 h incubation.

DISCUSSION

In this study 6HN was first discovered to be produced by *P. aeruginosa* or *S. marcescens* by means of GC—MS. 6HN has been known to be an intermediate in oxidative degradation of nicotinic acid by some bacteria such as *Clostridium* species [8, 9], *Bacillus* species [10], and *Pseudomonas* fluorescens [11]. There has, however, been no report about the production of 6HN concerning the clinically important microorganisms.

Although 6HN could not be detected in urine incubated with *P. aeruginosa* or *S. marcescens* because of the very low concentration of nicotinic acid, the substrate, the production of 6HN was recognized by the addition of nicotinic acid in urine, proportional to the number of bacteria, and time-dependent. *P. aeruginosa* strain P102 was found to possess the ability to produce 6HN as well as strain P101. These results suggest the availability of 6HN for rapid identification of bacteria.

A typical urinary tract infection reveals the presence of 10^5 or more microorganisms per ml of urine. Our results show that, in the presence of $1 \cdot 10^5$ *P. aeruginosa*, it was necessary to detect urinary 6HN at 4 h incubation, and 0.15—0.43 μg of 6HN could be detected.

Hayward [12] applied head-space gas—liquid chromatography (HS-GLC) to rapid diagnosis of urinary tract infection. The advantages of HS-GLC are that the sample preparation is more quick and simple to perform than our GC—MS method. The adavantage of our method is that specificity of 6HN is excellent, which is important to characterize the bacteria. Although 6HN was found to be helpful for the detection of *P. aeruginosa* or *S. marcescens*, other factors are necessary for the identification of the bacteria. Further characterization of the bacteria, including 6HN-negative bacteria such as *E. coli* or *K. pneumoniae*, is now in progress in our laboratory.

CONCLUSIONS

(1) 6HN was detected in spent culture media of *P. aeruginosa* and *S. marcescens*, but could not be detected in those of *K. pneumoniae* and *E. coli*.

(2) *P. aeruginosa* or *S. marcescens* synthesized 6HN from nicotinic acid.

(3) Detection of 6HN, infected urine or blood incubated with nicotinic acid may be clinically useful for the rapid identification of bacteria.

REFERENCES

1 D.C. Edman and J.B. Brooks, J. Chromatogr., 274 (1983) 1.
2 L. Larsson, P.-A, Mårdh, G. Odham, M.-L. Carlsson, J. Clin. Pathol., 35 (1982) 715.
3 C.W. Moss, J. Chromatogr., 203 (1981) 337.
4 S.T. Bagley and R.J. Seidler, Appl. Environ. Microbiol., 36 (1978) 536.
5 V.A. Lamb, H.P. Dalton and J.R. Wilkins, Amer. J. Clin. Pathol., 66 (1976) 91.
6 N.R. Krieg and J.G. Holt (Editors), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 1984, p. 164.
7 N.R. Krieg and J.G. Holt (Editors), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 1984, p. 480.
8 I.J. Harary, J. Biol. Chem., 227 (1957) 815.
9 I.J. Harary, J. Biol. Chem., 227 (1957) 823.
10 J.C. Ensign and S.C. Rittenberg, J. Biol. Chem., 239 (1964) 2285.
11 D.E. Hughes, Biochem. J., 60 (1955) 303.
12 N.J. Hayward, J. Chromatogr., 274 (1983) 27.