Reaffirmation of the validity of enzymatic cleavage of lithocholic acid from N-ε-lithocholyl-L-lysine and N-α-CBZ-N-ε-lithocholyl-L-lysine

Padmanabhan P. Nair, George Kessie, and Vincent P. Flanagan

Lipid Nutrition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, and the Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205

Summary N-ε-lithocholyl-L-lysine or N-α-CBZ-N-ε-lithocholyl-L-lysine when incubated overnight at 37°C with 3 K units of clostridial cholanoylaminoacid hydrolase (from *Clostridium perfringens* ATCC 19574) in the presence of disodium EDTA (0.1 M), β-mercaptoethanol (0.1 M), and sodium acetate buffer, pH 5.6, released free lithocholic acid. The latter material was isolated by thin-layer chromatography and identified by combined gas-liquid chromatography-mass spectrometry in the full scan and selected-ion mode. In order to maintain its activity, the enzyme was always stored in 1.0-ml aliquots at temperatures below −20°C and each aliquot when thawed was used immediately; any left over enzyme was never reused. Contrary to the observations of Yanagisawa et al. (*J. Lipid Res.* 1984. **25:** 1263-1271) the results of this study reaffirm the validity of the original observations on the enzymatic cleavage of lithocholic acid from tissue-bound form.—**Nair, P. P., G. Kessie, and V. P. Flanagan.** Reaffirmation of the validity of enzymatic cleavage of lithocholic acid from N-ε-lithocholyl-L-lysine and N-α-CBZ-N-ε-lithocholyl-L-lysine. *J. Lipid Res.* 1986. **27:** 905-909.

Supplementary key words tissue-bound lithocholate • cholanoylaminoacid hydrolase • *Clostridium perfringens* ATCC 19574

In 1967, Nair et al. (1) described the isolation of a strain of *C. perfringens* (ATCC 19574) that elaborated a group of closely related enzymes that cleaved the C-N bond in bile acid conjugates. We subsequently reported the partial purification and characterization from this organism of the enzymes associated with the preferential cleavage of the conjugates of glycine and taurine (2, 3). The proteins associated with this activity were collectively referred to as cholylglycine hydrolase. In recent years, both crude and partially purified cholylglycine hydrolases have received wide acceptance as a tool in the analytical methodology of bile acids in biological systems.

Although the enzyme is primarily used for the isolation of free bile acids from their glycine and taurine conjugates, the broad substrate specificity exhibited by crude extracts of *C. perfringes* ATCC 19574 was recognized as a valuable attribute (3). Later studies revealed that the broad spectrum of activity in these preparations represented several closely related isoenzymes with different catalytic and thermal stability characteristics (4). During the course of our studies on the composition of tissue bile acids, we found that extraction of liver tissue with 95% ethanol containing ammonium hydroxide did not always yield the expected amounts of lithocholate. Treatment of the residue with crude extracts of *C. perfringens* yielded additional amounts of lithocholate, an observation that led us to postulate the existence of covalently linked tissue-bound lithocholate (TBL) (4). Degradative studies and comparison with synthetic standards revealed that lithocholate in TBL in linked to the protein via the N-ε-NH_2 groups of lysine residues (5, 6). Subsequently, other investigators verified these observations and validated the analytical methodology employed by the original investigators (7-9). However, in a recent report, Yanagisawa and coworkers (10) questioned the existence of TBL based upon their apparent inability to hydrolyze synthetic NELL using "cholylglycine hydrolase" from commercial sources. The present study was undertaken to reaffirm the validity of the original observations and to describe the appropriate conditions for the enzymatic cleavage of NELL as well as of its peptide-simulating congener, N-α-CBZ-N-ε-lithocholyl-L-lysine (NACELL) using selected ion monitoring coupled with gas-liquid chromatography-mass spectrometry where both retention times and full scans of derivatized lithocholic acid were obtained.

MATERIALS AND METHODS

Reagents

Isooctane, isopropanol, acetic acid, ethyl acetate, methanol, and acetone were ACS grade reagents from Fisher Chemical Co., Fairlawn, NJ. Silica gel G thin-layer chromatographic plates, 250 μm thickness, were obtained from Analtech Inc, Newark, DE.

Crude cholanoylaminoacid hydrolase

Clostridium perfringens ATCC 19574 maintained on trypticase soy blood agar (2) was grown anerobically at 37°C for 16 hr in freshly prepared Brewer modified thioglycollate broth without indicator (Cat. no; 11720, BBL Microbiological Systems, Cockeysville, MD) and harvested by centrifugation at room temperature. The cells were washed five times with physiological saline at 4°C, suspended in ice-cold distilled water, and sonically disrupted in a Rosette cooling cell maintained at 0-4°C. The sonicated cells were centrifuged at 10,000 *g* for 60 min at 4°C and the supernatant was stored in 1.0-ml

Abbreviations: LCA, lithocholic acid; NELL, N-ε-lithocholyl-L-lysine; NACELL, N-α-CBZ-N-ε-lithocholyl-L-lysine; GLC-MS-SIM, gas-liquid chromatography-mass spectrometry-selected ion monitoring; TBL, tissue-bound lithocholate; BSA, bovine serum albumin; TLC, thin-layer chromatography.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

aliquots at −70°C. The enzyme was assayed using glycocholate as the substrate (2). The enzyme, stored at temperatures below −20°C was thawed and used immediately. Left over enzyme was never refrozen or reused, because of losses in activity against lysine conjugates.

Cholylglycine hydrolase

Two commercial preparations of cholanoylaminoacid hydrolase were purchased from Sigma Chemical Co, St. Louis, MO (cholylglycine hydrolase, partially purified, Cat. no: C-4018, Lot no: 20F-6833 and cholylglycine hydrolase, crude, Cat. no: C-3636, Lot no: 32F-6823). These preparations were also stored below −20°C until they were used.

N-α-CBZ-N-ϵ-lithocholyl-L-lysine (NACELL) and N-ϵ-lithocholyl-L-lysine (NELL)

NACELL and NELL were synthesized by the classical mixed anhydride procedure (11) as described earlier (5) or via the N-hydroxysuccinimide ester of lithocholic acid (12). The compounds were purified by TLC before use as a substrate.

Thin-layer chromatography

Lithocholic acid (LCA) and its conjugates were chromatographed on silica gel G, using isooctane–isopropanol–acetic acid 2:2:1 (v/v/v) as the mobile phase. LCA, NACELL, and NELL gave R_f values of 0.81, 0.78, and 0.13, respectively. In addition to being ninhydrin-positive, NELL, upon mass spectral analysis, showed the presence of the molecular ion at m/z 504 and a peak at m/z 487 considered to be due to the loss of an -OH (5).

Enzymatic hydrolysis of NACELL and NELL

Since LCA in the tissue-bound form is linked to protein via the ϵ-NH_2 groups of lysine residues in which the α-NH_2 groups are in peptide linkage, it is considered important to use a derivative that simulated TBL. NACELL was therefore used as a second substrate to test the effectiveness of the enzyme under conditions resembling those with the native product.

The substrates (either NACELL or NELL), 0.5 mg dissolved in 100 μl of ethanol, were incubated overnight at 37°C in a mixture of 0.2 ml of disodium EDTA (0.1 M), 0.2 ml of β-mercaptoethanol (0.1 M), 5.0 ml of sodium acetate buffer, 0.1 M (pH 5.6), and 0.3 ml of the crude enzyme (minimum activity of 10 K units/ml) prepared in our laboratories. Incubation mixtures without either the enzyme or substrate were processed in parallel to serve as controls. In experiments using enzymes from Sigma, solutions containing the desired units of activity were added to the incubation mixture.

At the end of the incubation period, the mixture was diluted with 15 ml of distilled water, acidified to pH 2.0 with conc. HCl and extracted five times with 40-ml aliquots of ethyl acetate. The extract was washed free of acid with distilled water, residual water was removed with anhydrous sodium sulfate, and the extract was evaporated to

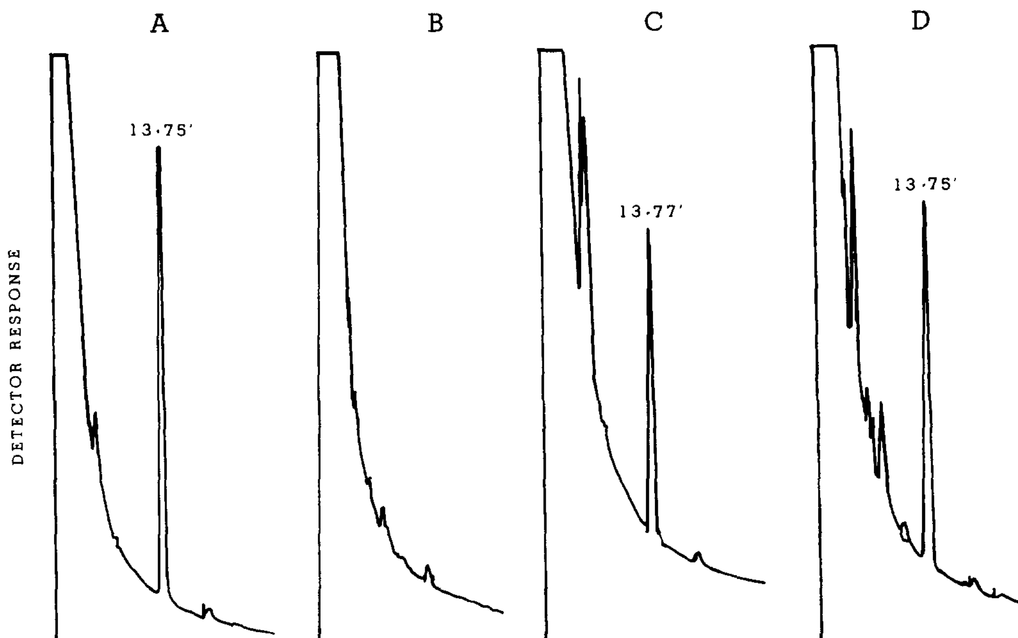

Fig. 1. Gas-liquid chromatographic identification of lithocholic acid (LCA) following enzymatic hydrolysis of NELL and NACELL with crude cholylglycine hydrolase (1300 units) under standard conditions (see text). A, Authentic reference standard of lithocholic acid methyl ester, trifluoroacetate. B, Substrate control without enzyme. C, LCA released from NACELL. D, LCA released from NELL. The yields from four replicate experiments gave a mean value of 66.4 ± 1.19%.

Downloaded from www.jlr.org by guest, on June 19, 2012

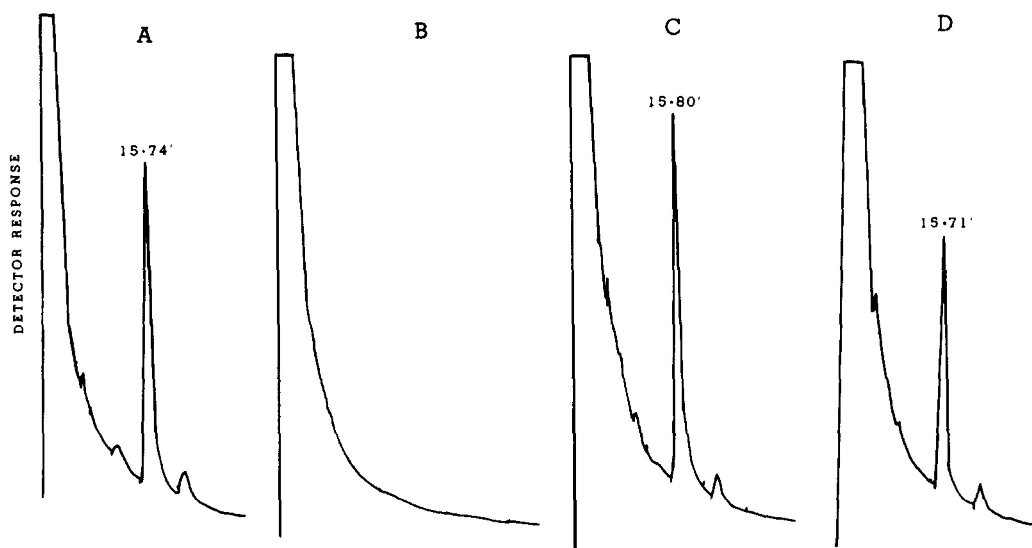

Fig. 2. Gas-liquid chromatographic identification of LCA following enzymatic hydrolysis of NELL with crude (530 units) and partially purified (525 units) cholylglycine hydrolase from Sigma Chemical Co. Conditions for hydrolase are described in the text. A, Authentic reference standard of lithocholic acid. B, Substrate control without enzyme. C, Hydrolysis with partially purified enzyme (Sigma). The yields from four replicate experiments gave a mean value of 38.2 ± 0.87%. D, Hydrolysis with crude enzyme (Sigma). The yields from four replicate experiments gave a mean value of 79.8 ± 1.47%.

dryness. Free LCA in the residue was isolated by TLC as described above. The zone corresponding to LCA, R_f 0.81, was scraped off and extracted with a mixture of acetone-benzene-methanol 1:0.9:0.1. Aliquots of the recovered LCA were used for GLC after conversion to the corresponding methyl ester trifluoroacetate (13) or subjected to GLC-MS-SIM (14).

Combined gas-liquid chromatography-mass spectrometry and selected ion monitoring

LCA methyl ester was prepared with diazomethane and then converted to the trimethylsilyl ethers with N,O bis (trimethylsilyl) trifluoroacetamide and pyridine (1:1). All electron impact (EI) spectra were recorded at an ionization energy of 70 eV on either a Finnigan 3200 F quadrupole equipped with a Promin unit for selected ion recording or a Hitachi RMU-6E mass spectrometer as described previously (14). Gas chromatographic columns were 5 ft and 6 ft long and 2.0 mm ID silanized glass, containing 1% OV-17 on gas-chrom Q, 100/120 mesh operated at 250°C. The ions chosen for selected ion monitoring (SIM) were M/Z 372 and 357, probably due to the loss of M-Me3 SiOH and M-(Me3SiOH + CH_3).

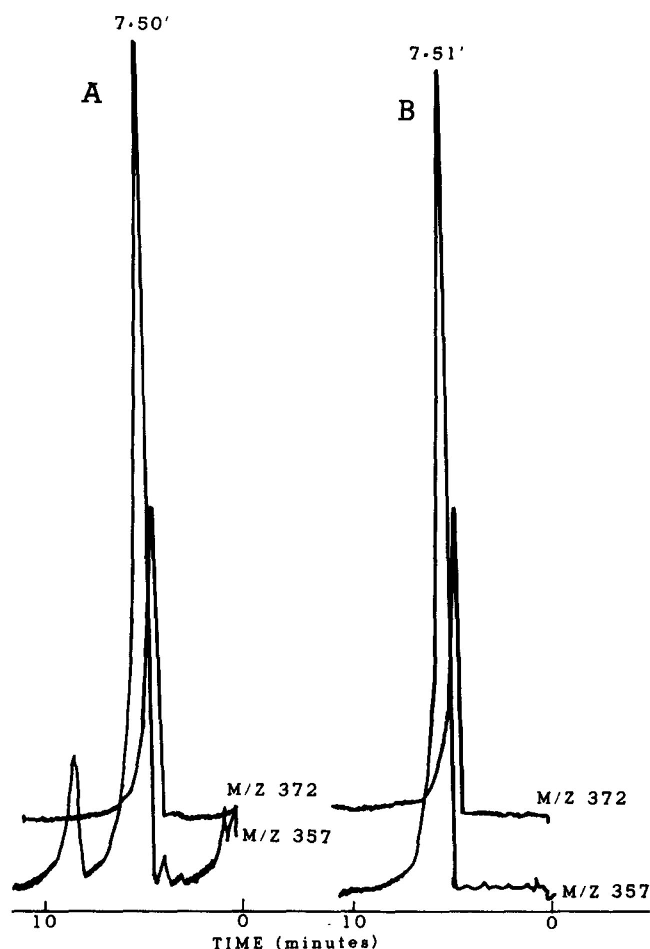

Fig. 3. Selected-ion monitoring of LCA released from the enzymatic hydrolysis of NELL monitored at M/Z 372 (M-90) and M/Z 357 [M-(90+15). A, Product of enzymatic hydrolysis of NELL. B, Authentic reference standard of lithocholic acid.

RESULTS

Enzymatic cleavage of NACELL and NELL

Incubation of either NACELL or NELL with crude clostridial cholanoylaminoacid hydrolase under standard conditions gave a TLC band corresponding to LCA. Isolation of this material followed by GLC showed a peak

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB JOURNAL OF LIPID RESEARCH JLR

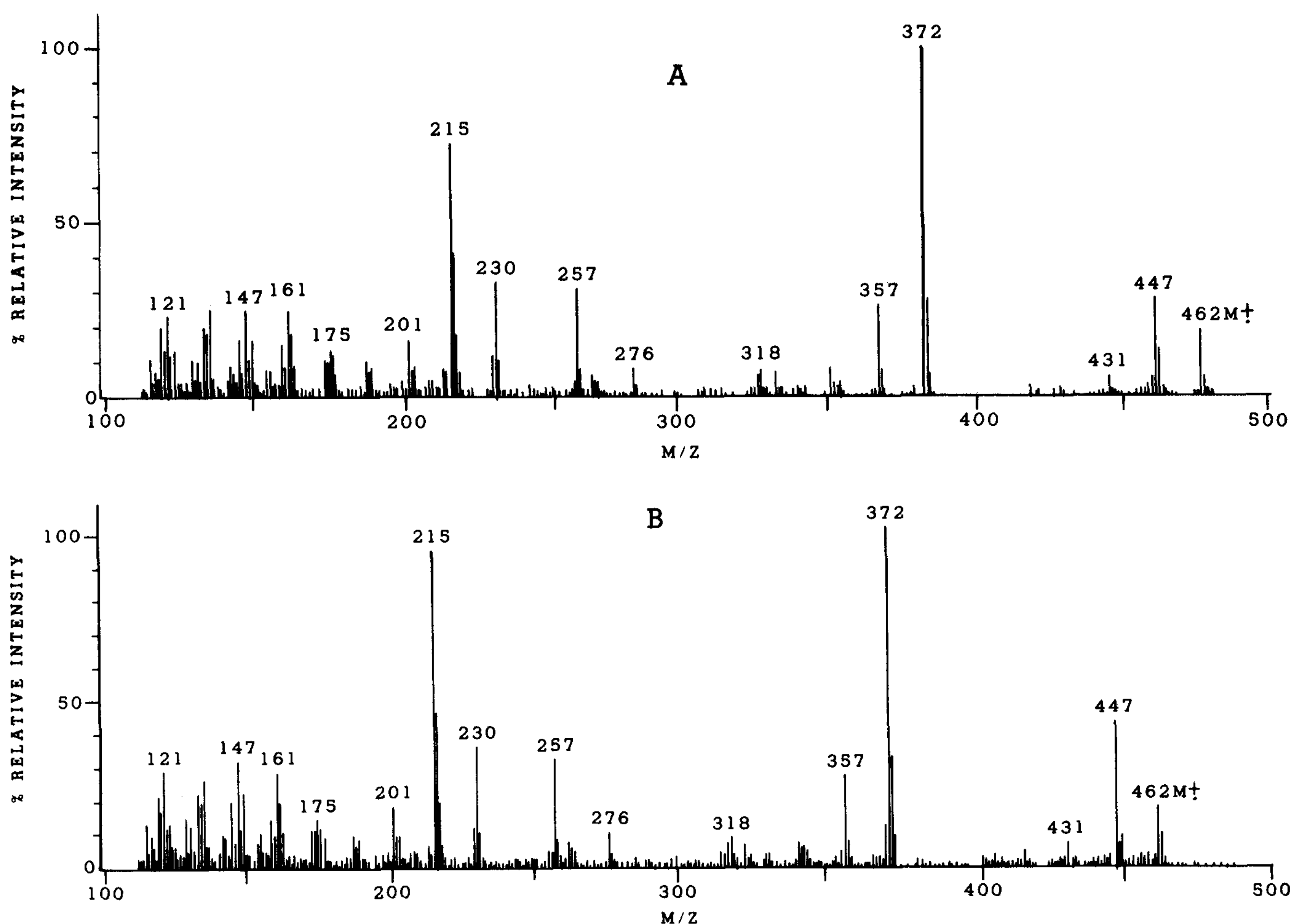

Fig. 4. Mass spectrum of lithocholic acid methyl ester trimethyl silyl ether. A, LCA released from NELL following hydrolysis with crude cholanoylaminoacid hydrolase. B, Authentic reference standard of LCA.

with a retention time identical to that of authentic LCA recovered from the TLC plate (**Fig. 1**). Control incubations performed in the absence of the enzyme did not show any free bile acid (Fig. 1B). The results showed that enzymatic cleavage of both the N-α- protected and free lysyl conjugates resulted in the release of free LCA.

Similarly, incubation of NELL with both partially purified and crude preparations of cholylglycine hydrolase (Sigma) resulted in deconjugation of the substrate (**Fig. 2**). No free bile acid was detected when the incubations were carried out in the absence of the enzyme. The data showed that enzymes from all three sources were active in cleaving the lysyl conjugates of LCA.

GLC-MS-SIM

GLC-MS-SIM of the product of enzymatic hydrolysis was monitored at M/Z 372 (M-90) and M/Z 357 [M-(90+15)], the two characteristic signals obtained from methyl lithocholate-TMS ether (**Fig. 3**). The ratio of the ions 357/372 is in excellent agreement with the authentic compound as are their retention times. As seen in this representative tracing by SIM, enzymatic hydrolysis of NELL consistently showed the release of free LCA, an observation that is consistent with published reports from this group as well as others (5-8). Mass spectrometric analysis further confirmed the identity of the free bile acid as lithocholate (**Fig. 4**).

DISCUSSION

The peptide-bond cleaving activity of extracts of *C. perfringens* ATCC 19574 represents a cluster of closely related cholanoylaminoacid hydrolases possessing overlapping substrate specificities and varying degrees of thermal stability (3, 4). Among these hydrolases, NELL hydrolase is relatively unstable at temperatures above 0°C and as a general rule the enzyme should be stored below -20°C in small aliquots in order to preserve its activity.

Yanagisawa et al. (10) cast doubt about the existence of

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 19, 2012

TBL on the basis of their failure to obtain hydrolysis of NELL. They assumed that cholylglycine hydrolase represented the peptide-bond cleaving activity of a single protein species, contrary to reports indicating the existence of more than one molecular species (3, 4). Since our experiments were carried out under identical conditions for 18 hr with the same batch of enzyme (Sigma, Lot no; 20F-6833) used by Yanagisawa et al., the differences in our results are perhaps attributable to inactivation of the specific enzyme species. It should be pointed out that NELL hydrolase is a relatively minor component in commercial preparations of cholylglycine hydrolase. As a result, any generalized loss of activity could lead to the disappearance of detectable NELL hydrolase activity.

Yanagisawa et al. (10) used glycolithocholic-bovine serum albumin (GLCA-BSA) as a model compound to demonstrate the release of "bound-lithocholate" assuming that GLCA-BSA simulates TBL. This is misleading, since the C-N bond in this compound is between the bile acid and glycine, unlike that in TBL where the bile acid is linked directly to protein via the ϵ-NH_2 groups of the side chains of lysine residues. Enzymatic hydrolysis of GLCA-BSA is therefore similar to the hydrolysis of glycine conjugated bile acids and is not mechanistically similar to that of TBL. NACELL, in which the N-α-NH_2 is linked to a CBZ group, was therefore used in this study to simulate TBL. In addition, we have also used labeled lithocholic acid directly linked to BSA to demonstrate the enzymatic release of the bile acid from its protein-bound form (data not included). Furthermore, synthetic substrates such as NELL and NACELL are relatively insoluble compounds and are therefore less readily cleaved than the more soluble protein-bound native substrate.

In this report we have described the appropriate conditions of storage and use of clostridial cholanoylaminoacid hydrolases from *C. perfringens* ATCC 19574 for the enzymatic cleavage of both NELL and its N-α- protected congener, NACELL. LCA recovered in this manner is identical to authentic standards by such sensitive criteria as GLC-MS-SIM. The findings from this study also reaffirm the validity of the experimental basis for TBL described in reports from three separate laboratories (5, 7, 8).

Manuscript received 16 October 1985.

REFERENCES

1. Nair P. P., M. Gordon, S. Gordon, J. Reback, and A. I. Mendeloff. 1965. The cleavage of bile acid conjugates by cell-free extracts from *C. perfringens*. *Life Sci.* **4:** 1887-1892.
2. Nair, P. P., M. Gordon, and J. Reback. 1967. The enzymatic cleavage of the carbon-nitrogen bond in $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholan-24-oyl glycine. *J. Biol. Chem.* **242:** 7-11.
3. Nair, P. P. 1969. Enzymatic cleavage of naturally occurring bile salt conjugates. *In* Bile Salt Metabolism. L. Schiff, J. B. Carey, Jr., and J. M. Dietschy, editors. Charles C. Thomas, Springfield. 172-183.
4. Patnaik, R., and M. Sokolow. 1975. Characteristics of clostridial cholanoylaminoacid hydrolase. *Gastroenterology.* **68:** 966 (abstract).
5. Nair, P. P., A. I. Mendeloff, M. Vocci, J. Bankoski, M. Gorelik, G. Herman, and R. Plapinger. 1977. Lithocholic acid in human liver: identification of ϵ-lithocholyl lysine in tissue protein. *Lipids.* **12:** 922-929.
6. Nair, P. P., R. Solomon, J. Bankoski, and R. Plapinger. 1978. Bile acids in tissues: binding of lithocholic acid to protein. Lipids. **13:** 966-970.
7. Maruyama. I., T. Maeyama, R. Kumashiro, K. Tanikawa, T. Shinka, and I. Matsumoto. 1980. Computerized gas chromatography-mass fragmentographic analysis of lithocholic acid in liver tissue. VI International Bile Acid Meeting, Falk Symposium No. 29. Freiberg, West Germany. Abstract #72.
8. Gelb, A. M., C. K. McSherry, J. R. Sadowsky, and E. H. Mosbach. 1982. Tissue bile acids in patients with colon cancer and colonic polyps. *Am. J. Gastroenterol.* **77:** 314-317.
9. Turjman, N., and C. Jacob. 1981. Distribution of tissue-bound lithocholic acid in human neoplastic tissues. *Federation Proc.* **40:** 1683 (abstract).
10. Yanagisawa, J., Y. Akashi, H. Miyasaki, and F. Nakayama. 1984. Critical evaluation of the existence of so-called tissue-bound lithocholate in human liver tissue by selected ion monitoring. *J. Lipid Res.* **25:** 1263-1271.
11. Vaughan, J. R., and R. L. Osato. 1952. The preparation of peptides using mixed carbonic carboxylic acid anhydrides. *J. Am. Chem. Soc.* **74:** 676-678.
12. Nair, P. P., G. Kessie, and N. Turjman. 1985. Synthesis of N-ϵ-lithocholyl-L-lysine. A component of tissue-bound lithocholic acid. *J. Steroid Biochem.* **23:** 573-576.
13. Okishio, T., and P. P. Nair. 1966. An improved column for gas-liquid chromatography of substituted cholanic acids. *Anal. Biochem.* **15:** 360-363.
14. Matusik, E. J., V. B. Reeves, and V. P. Flanagan. 1984. Determination of fatty acid methyl esters—elimination of tissue-derived contamination and artifacts and preparation of a support-coated acid-modified polyester liquid phase. *Anal. Chim. Acta.* **166:** 179-188.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH