Journal of Chromatography, 133 (1977) 327–334

CHROM. 9772

ISOLATION AND PURIFICATION OF LITHOCHOLIC ACID METABOLITES PRODUCED BY THE INTESTINAL MICROFLORA

MORRIS I. KELSEY* and SHARYN A. SEXTON
NCI Frederick Cancer Research Center, P.O. Box B, Frederick, Md. 21701 (U.S.A.)
(Received August 31st, 1976)

SUMMARY

Lithocholic acid metabolites produced by the intestinal microflora of rats can be isolated from other endogenous lipids using Sephadex LH-20 column chromatography. Analyses of individual metabolites collected from the column by silica gel coated glass fiber paper chromatography result in the resolution of epimeric 3-hydroxy derivatives. In addition, glass fiber paper chromatography is more sensitive and requires less development time than conventional glass-coated thin-layer plates. Further confirmation of the identity of metabolites is achieved by gas–liquid chromatography, which separates both methyl and ethyl esters of lithocholic and isolithocholic acids.

INTRODUCTION

The ultimate goal of this study was to isolate and identify physiological metabolites of lithocholic acid that could be evaluated for their mutagenicity using the rapid *in vitro* assay developed by Ames *et al.*[1].

Although methods have been reported for the isolation and identification of fecal sterols and bile acids[2], such procedures are tedious and include saponification and extraction steps which may alter the nature of the metabolites obtained. Therefore a rapid, mild methodology was required for the isolation of bile acid metabolites which are formed by the intestinal microflora.

The use of lipophilic Sephadex for the separation of biological compounds soluble in organic solvents has many advantages including ease of manipulation, mild conditions, good recovery of labile compounds and calibration of re-usable columns[3]. Sephadex LH-20, which is a hydroxypropylated derivative of Sephadex G-25, has been used to separate neutral sterols[4], steroid hormones[5,6] and conjugated salts of steroids[7] and bile acids[8,9]. Although similar methodology for the isolation and characterization of bile acids was utilized in previous studies[10,11], this report will describe an improved modification of this protocol in greater detail.

* To whom correspondence should be addressed.

MATERIALS AND METHODS*

Reference standards

Lithocholic acid (LA), isolithocholic acid (IL), 3-keto-5β-cholanic acid (3-keto) and deoxycholic acid (deoxy) were obtained from Omni Research (Mayaguez, Puerto Rico, U.S.A.). The 5β-cholanic acid (5β) was purchased from Schwartz/Mann (Orangeburg, N.Y., U.S.A.).

The preparation of methyl and ethyl esters of LA metabolites has been described previously[10].

Preparation of rat mixed fecal cultures

Fischer 344 rats, obtained from the Animal Farm at the Frederick Cancer Research Center, had been maintained on standard Purina lab-chow for at least six months. A single fecal pellet (0.3 g wet weight) was collected into a small jar which had been previously gassed with oxygen-free CO_2 and was then placed in 180 ml of brain heart infusion (BHI) medium that was prepared by procedures described in the V.P.I. Anaerobe Laboratory Manual[12]. After 2 h of anaerobic incubation at 37° most of the pellet was dispersed, and an additional 5-h incubation resulted in complete dispersion. A 30-ml aliquot from this stock culture was used for inoculation with 1.86 μmoles (700 μg) of lithocholic acid in 1 ml of ethanol. The incubation was continued anaerobically for 48 h at 37° along with two controls: lithocholic acid in BHI and cells in medium without substrate.

Extraction techniques

After the designated incubation periods, cell suspensions of organisms were allowed to equilibrate to room temperature before acidifying to pH 1 with 12 *N* hydrochloric acid. Five volumes of chloroform–methanol (2:1) were used to extract the bile acid metabolites, and the organic layer was washed once with a volume of water corresponding to the original volume of cell suspensions. Removal of the organic solvent at 30–35° using a Buchler (Fort Lee, N.J., U.S.A.) rotary evaporator equipped with a Dewar condenser gave a residue which was analyzed as described below.

Sephadex LH-20 chromatography

Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.) was swollen in isooctane–chloroform–methanol (2:1:1) and packed by gravity into a column (45 × 1 cm I.D.) to give a bed height of 30 cm and a column volume of ap-

* The following names and abbreviations for chemicals and methods have been used throughout the text: lithocholic acid (LA) = 3α-hydroxy-5β-cholan-24-oic acid; methyl lithocholate (ML) = methyl-3α-hydroxy-5β-cholan-24-oate; ethyl lithocholate (EL) = ethyl-3α-hydroxy-5β-cholan-24-oate; isolithocholic acid (IL) = 3β-hydroxy-5β-cholan-24-oic acid; methyl isolithocholate (MIL) = methyl-3β-hydroxy-5β-cholan-24-oate; ethyl isolithocholate (EIL) = ethyl-3β-hydroxy-5β-cholan-24-oate; 3-keto-cholanoic acid (3-keto) = 3-keto-5β-cholan-24-oic acid; Me-3-keto = methyl-3-keto-5β-cholan-24-oate; 5β-cholanoic acid (5β) = 5β-cholan-24-oic acid; Me-5β = methyl-5β-cholan-24-oate; deoxycholic acid = 3α,12α-dihydroxy-5β-cholan-24-oic acid; methyl deoxycholate (methyl deoxy) = methyl-3α,12α-dihydroxy-5β-cholan-24-oate; BHI = brain heart infusion; LH-20 = Sephadex LH-20; TLC = thin-layer chromatography; GFP = glass fiber paper; GLC = gas–liquid chromatography; GLC–MS = combined gas–liquid chromatography and mass spectrometry.

proximately 28 ml. Extracts or reference standards were dissolved in a minimal volume of the above solvent, and the same solvent was used to elute the compounds in fractions of 1–2 ml. Fractions collected were assayed by TLC or GFP chromatography as described below. Methylated standards were eluted in the following column volumes: 5β (47–53%), 3-keto (53–58%), and LA and IL (63–79%).

Thin-layer chromatography

Prescored glass plates coated with silica gel G (Analtech, Newark, Del., U.S.A.) at a thickness of 250 μ were used for analytical purposes when using the solvent system benzene–dioxane–acetic acid (100:10:1)[13]. Visualization of spots was achieved by charring the plates which had been sprayed with the solution ethanol–sulfuric acid–water (2:2:1) (Fig. 1).

Glass fiber paper chromatography

GFP sheets (ITLC-SG) coated with silica gel G (Gelman, Ann Arbor, Mich., U.S.A.) were used to assay lithocholic acid metabolites. The solvent system used was isooctane–diisopropyl ether–acetic acid (75:30:0.5), and visualization of spots was achieved as described under Thin-layer chromatography. However, recently we have experienced some problems with non-uniformity in the ITLC sheets and suggest that each lot be tested with standards before using routinely. For example, Fig. 2 shows a typical separation of acid/ester derivatives on the ITLC sheet which was developed in isooctane–diisopropyl ether–acetic acid (75:30:0.07). These separations were normally achieved with the higher concentrations of acetic acid (0.5 ml), but this particular batch required a more non-polar system to achieve similar separations.

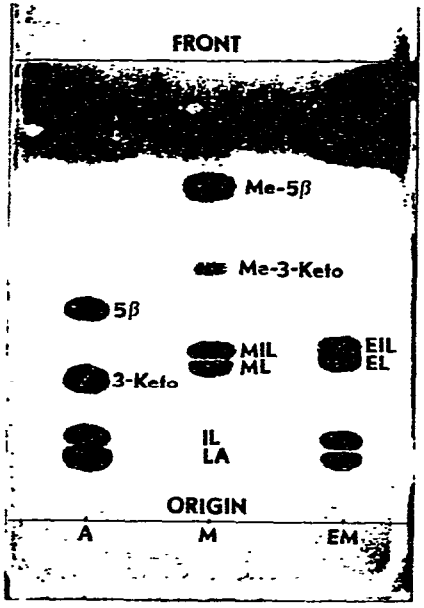
Gas–liquid chromatography

Analyses by GLC were performed on a Shimadzu Model 4 BM gas chromatograph (American Instrument, Silver Spring, Md., U.S.A.) using silanized, coiled glass columns (182 × 0.3 cm I.D.). The GLC phase used was 1% QF-1 coated on Gas-Chrom Q (100–200 mesh), which was obtained from Supelco (Bellefonte, Pa., U.S.A.). Operating conditions included oven and injection port temperatures of 235° or 210°, as indicated, and a detector oven at 280°. Helium gas was used as a carrier at a flow-rate of approximately 60 ml/min.

RESULTS

Separation of the expected LA metabolites by TLC (Fig. 1) resulted in a slight separation of the epimeric LA and IL and their respective methyl and ethyl esters using the solvent system described above[13]. With GFP chromatography, however, a much better resolution of the epimeric derivatives was obtained (see Fig. 2), and the 3-keto and 5β-metabolites were clearly separated from the hydroxylated compounds. The difference in sensitivity of the two methods should also be mentioned since 5 μg or less can be detected on the glass fiber sheets, whereas 20–25 μg of sample is required for good visualization by TLC.

This GFP system was used to routinely assay crude and purified extracts derived from incubations with [^{14}COOH]-LA since the regions of interest could easily be cut out from the sheet and counted with little or no quenching[10,11]. Such techniques



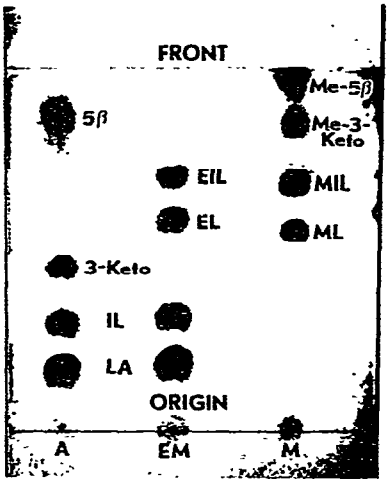


Fig. 1. Separation of lithocholic acid metabolites by TLC using silica gel G plates developed in benzene–dioxane–acetic acid (100:10:1). Mixtures of bile acid standards in methanol were spotted to give approx. 25 μg of each compound. The acid mixture (A) consisted of LA, IL, 3-keto, and 5β. The methylated standards (M) consisted of ML, MIL, Me-3-keto, and Me-5β. The EM mixture consisted of LA, IL, EL, and EIL.

Fig. 2. Separation of lithocholic acid metabolites by GFP sheets developed in isooctane–diisopropyl ether–acetic acid (70:30:0.07). LA metabolites were spotted to give 5 μg of each compound.

are more rapid than TLC since GFP chromatography requires approximately 15 min development time *versus* 70 min with TLC. In addition, recovery of radioactive metabolites from Gelman sheets is easier and more quantitative than from TLC plates from which silica gel must be scraped into scintillation vials. The silica gel also results in more quenching of the sample than the GFP strips[14]. The safety advantage of GFP sheets over TLC plates should be kept in mind when trying to keep chemical or radiological contamination to a minimum.

In addition to GFP chromatography, assay of the crude extracts by GLC was performed initially on a 1% QF-1 column maintained isothermally at 235°. Fig. 3a shows a rather complex profile for the methylated crude extract of LA + rat intestinal flora (see Materials and methods for details) with LA metabolite peaks eluted from 5.6–7.2 min. The control of cells without substrate (Fig. 3b) shows some small peaks in this region of interest also.

Purification of the crude extract by LH-20 chromatography resulted in the isolation of partially purified bile acid metabolites whose behavior on GLC gave retention times relative to methyl deoxycholate (methyl deoxy) of 0.48, 0.53, 0.62, corresponding to MIL, ML and EL, respectively[10]. The metabolites were collected in 2-ml fractions and appeared in fractions 7–9 corresponding to 50–64% of the total column volume.

Re-chromatography on the same LH-20 column resulted in a mixture (referred

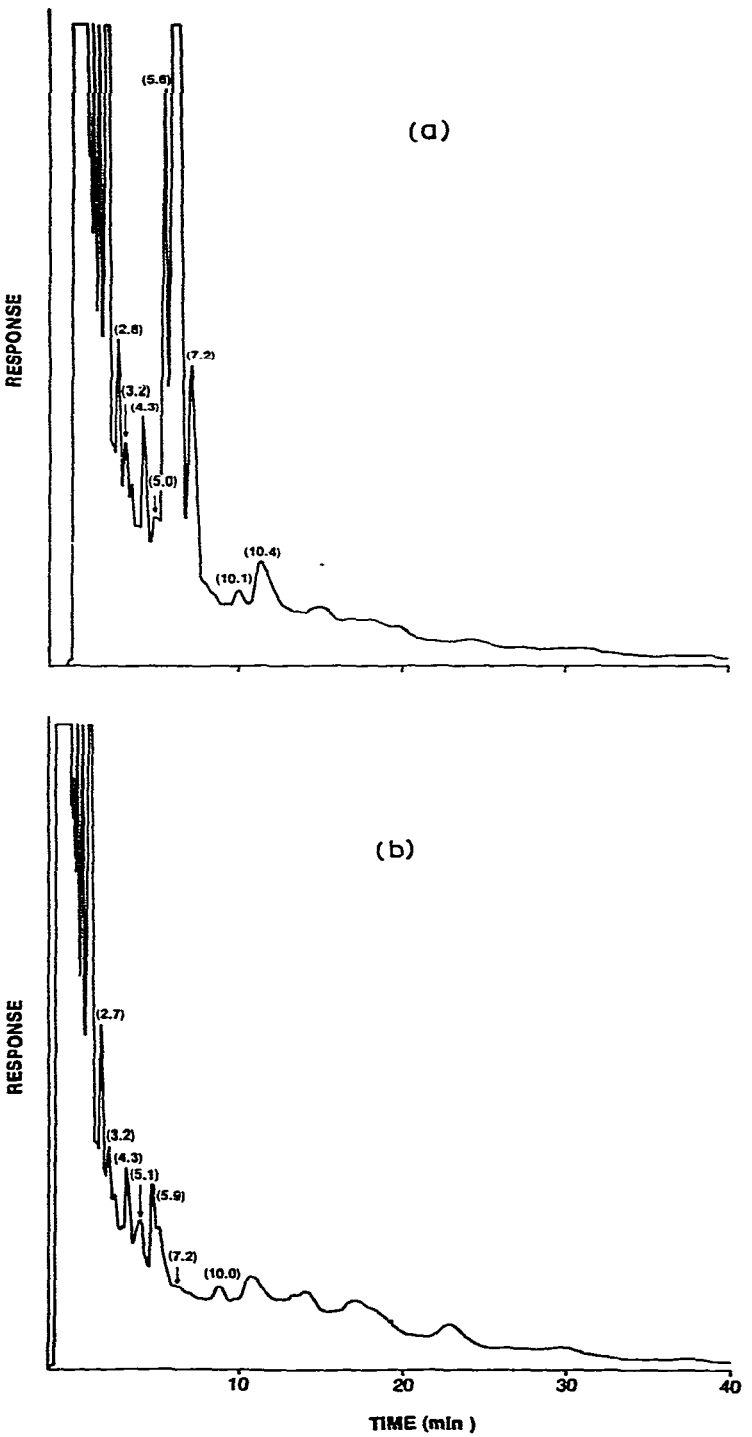


Fig. 3. (a) GLC analysis of a methylated extract of LA incubated with rat intestinal microflora. The stationary phase used was 1% QF-1 maintained isothermally at 235°. Bile acid metabolites had absolute retention times of 5.6–7.2 min. (b) Methylated extract of intestinal flora incubated without LA. GLC conditions were identical to those described for (a).

to as R113-101-1) of highly purified bile acid methyl esters which appeared to be free of the more volatile contaminants seen previously as shown by GLC (Fig. 4a). A synthetic mixture (R113-101-2) consisting of MIL, ML and EL gave an identical profile under the same conditions (Fig. 4b). Further confirmation of the identity of the bile acids in R113-101-1 was obtained by GLC–MS[11].

Although the GLC conditions above were capable of detecting the major metabolites of LA, EL and EIL[10], the presence of large amounts of LA (RRT = 0.53) in the extract masked the small amount of EIL (RRT = 0.58) in the sample. When a mixture of LA derivatives was run at 210°, a better resolution of the metabolites was obtained, as indicated in Fig. 5. Using these improved conditions, both the EIL and EL can be more easily detected and identified by GLC and GLC–MS[11].

DISCUSSION

The use of Sephadex LH-20 chromatography in purifying bile acid metabolites produced from incubations of LA with rat intestinal microflora appears to be an effective and practical analytical method in separating the bile acids from other lipids present. Fractions can be analyzed rapidly by GFP chromatography, and further confirmation of identity is obtained by GLC and GLC–MS.

The development of multiple chromatographic techniques for the isolation, purification and identification of metabolites of LA has facilitated the isolation and identification of EL[10] and also EIL[11], which are new microbial bile acid derivatives.

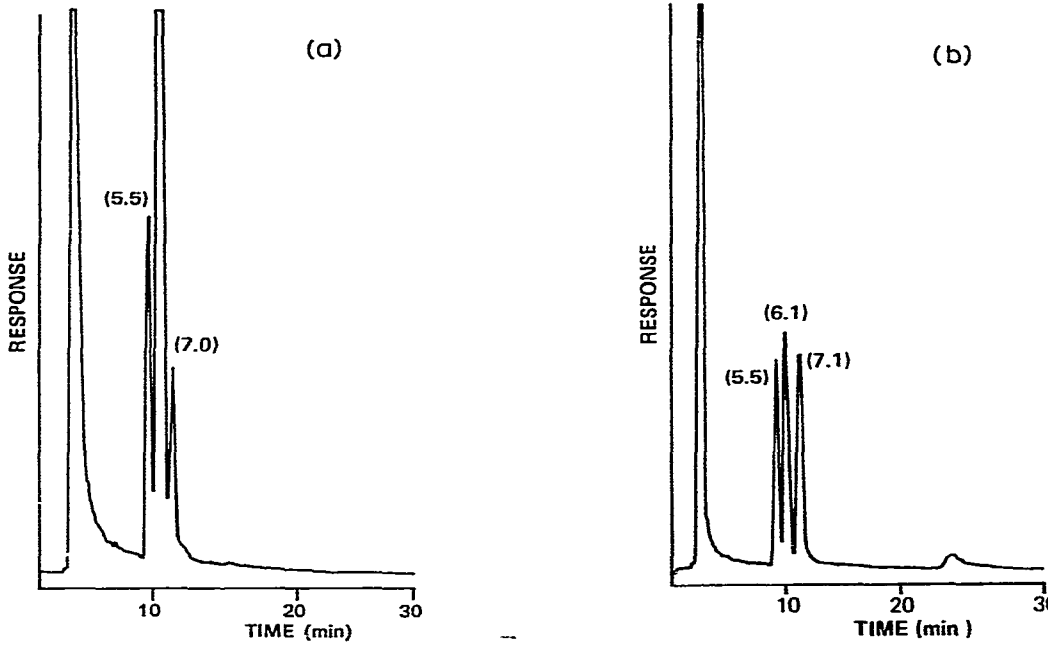


Fig. 4. (a) GLC analysis of Sephadex LH-20 purified extract of LA incubated with rat intestinal microflora (R113-101-1). GLC conditions were identical with those described for Fig. 3a. (b) GLC profile of a standard mixture of MIL, ML and EL (R113-101-2) with absolute retention times of 5.5, 6.1 and 7.1 min, respectively. The conditions used were identical with those described in Fig. 3a.

Frequently, the extracts obtained from LA incubation with mixed fecal flora contain endogenous fecal sterols and other lipids which are eluted from the LH-20 column in the same region as the bile acid metabolites of interest, but no endogenous bile acids are detected by present methodologies[13], presumably due to the small amount (0.3 g wet weight) of fecal material used. However, re-chromatography of the methylated extract on LH-20 appears to remove most of these contaminants[14], as can be seen in the differences in Figs. 3a and 4a. It is difficult to distinguish the biological extract from the reference sample (Figs. 4a and b).

More recently we have extended this approach of purifying methylated crude extracts on larger open columns of LH-20 (150 ml column volume) in order to better separate fecal sterols and other components from bile acids without the need for recycling chromatography[13].

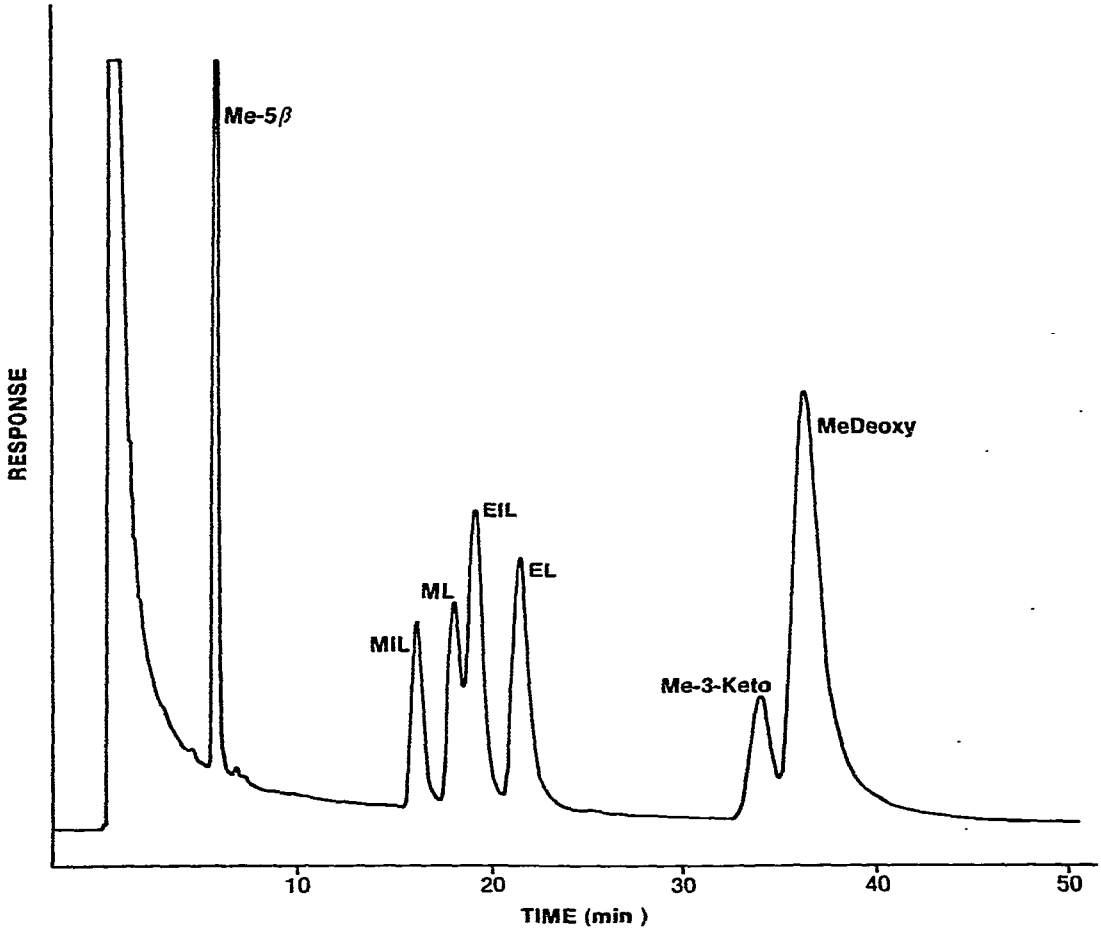


Fig. 5. Separation of methyl and ethyl esters of LA by GLC on 1% QF-1 maintained isothermally at 210°. Abbreviations and other GLC conditions are described in Materials and methods. Methyl deoxycholate was coinjected with the LA metabolites to provide relative retention time values of 0.16 (Me-5β), 0.45 (MIL), 0.50 (ML), 0.53 (EIL), 0.59 (EL), and 0.94 (Me-3-keto).

Recoveries of sterols including mixtures of cholesterol, cholestanol, and coprostanone as well as methyl esters of LA, IL, 3-keto, and 5β were complete using the solvent system isooctane–chloroform–methanol (2:1:1). However, recently we have obtained poor recoveries of high specific activity [^{14}COOH]-LA on the 28-ml Sephadex LH-20 column and have found that using small amounts of mass will cause the bile acid to adhere to the gel. Therefore, we recommend diluting bile acid substrates of high specific activity with unlabeled material before purification. Using larger columns (150-ml column volume) of Sephadex LH-20, we routinely obtain 80–90% recoveries with 200 mg of commercial LA and find that evaporation of the mobile phase results in a pure crystalline product.

These improved techniques for the isolation of new bile acid metabolites will assist in the overall goal of this work, which is concerned with rapid identification of possible endogenous carcinogenic compounds and/or promoters of carcinogenesis in human large intestine or other target sites. These protocols are now being applied in studies of LA metabolism by human intestinal microflora.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Kathleen A. Fowler for her valuable technical assistance during portions of this work. Research sponsored by the National Cancer Institute under Contract No. N01-CO-25423 with Litton Bionetics, Inc.

REFERENCES

1 B. Ames, W. E. Durston, E. Yamasaki and F. D. Lee, *Proc. Nat. Acad. Sci. U.S.A.*, 70 (1973) 2281.
2 B. I. Cohen, R. F. Raicht, G. Salen and E. H. Mosbach, *Anal. Biochem.*, 64 (1975) 567.
3 E. Nystrom and J. Sjovall, *Methods Enyzmol.*, 35 (1974) 378.
4 J. E. VanLier and L. L. Smith, *Tex. Rep. Biol. Med.*, 27 (1969) 167.
5 A. R. Bourne, *J. Chromatogr.*, 92 (1974) 465.
6 V. D. Castracane, S. S. Ur Rahman and R. B. Billiar, *J. Chromatogr.*, 115 (1975) 244.
7 J. C. Touchstone and M. F. Dobbins, *J. Steroid Biochem.*, 6 (1975) 1389.
8 E. S. Haslewood and G. A. D. Haslewood, *Proc. Biochem. Soc.*, 130 (1972) 89P.
9 I. Makino, K. Shinozaki, S. Nakagawa and K. Mashimo, *J. Lipid Res.*, 15 (1974) 132.
10 M. I. Kelsey and R. J. Thompson, *J. Steroid Biochem.*, 7 (1976) 117.
11 M. I. Kelsey and S. A. Sexton, *J. Steroid Biochem.*, 7 (1976) 641.
12 L. V. Holdeman and W. E. C. Moore (Editors), *Anaerobe Laboratory Manual*, V.P.I. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va., 1972, p. 124.
13 V. C. Aries, P. Goddard and M. J. Hill, *Biochim. Biophys. Acta.* 248 (1971) 482.
14 M. I. Kelsey and S. A. Sexton, unpublished results.