Vulnerability of keto bile acids to alkaline hydrolysis

G. Lepage, A. Fontaine, and C. C. Roy

Department of Pediatrics, Hôpital Ste-Justine and the University of Montreal, Montreal, Canada

Summary Rigorous alkaline hydrolysis of the two primary (cholic and chenodeoxycholic) and of the two preponderant secondary (deoxycholic and lithocholic) bile acids found in bile led to excellent recoveries. Such was not the case with 11 different keto bile acid standards. Recoveries for a number of standards were unacceptably low and a variety of artefactual products were tentatively identified by gas–liquid chromatography. Keto bile acids bearing a keto group on C-3 were particularly vulnerable. In view of these findings, quantitative and qualitative data reported on biological specimens submitted to saponification in ethanol, methanol, or even in water are of questionable significance.

Supplementary key words primary bile acids · secondary bile acids · gas–liquid chromatography · saponification · enzymatic hydrolysis

Keto bile acids are a major constituent of human feces (1–3). These products of anaerobic bacterial dehydrogenases (4) are found in the bile of a number of mammalian species (5) but have not been reported in man except after cholecystectomy (6, 7).

Hydrolysis is a necessary step for the analysis of bile acids. Freeing the amino acid residues from the carboxyl end of the molecule can be accomplished by either alkaline or enzymatic hydrolysis. Attention has been drawn to the formation of artefactual bile acid metabolites by rigorous alkaline hydrolysis (8, 9). Although the recovery of bile acids after alkaline hydrolysis has been reported to be particularly low for keto bile acids (8, 10), the extent of structural changes and the particular vulnerability of various keto bile acids have not been studied.

Materials and Methods

A mixture of bile acid standards consisting of 2.5 mg each of lithocholic, 23 nordeoxycholic (NDC), deoxycholic, chenodeoxycholic, cholic, and tri-keto cholanic acid was saponified in open glass tubes with 40 ml of 2 N NaOH in 95% ethanol for a period of 90 min in an autoclave, at 12 psi and 120°C. After acidification to pH 1 (in ice to prevent the formation of ethyl esters[1]), bile acids were extracted thrice with ether and twice with ethyl acetate.[2] The samples were then methylated with freshly prepared diazomethane (11) and one drop of acetic acid was added to remove the excess. After adding water, bile acid methyl esters were extracted with ether and ethyl acetate. The dry residue was acetylated (12). Five μl of the sample dissolved in acetone was analyzed on a Hewlett-Packard 7610A gas chromatograph with a flame ionization detector. Each sample was chromatographed on two different liquid phase columns. A 6-ft U-shaped glass column was packed with 2% QF-1 on Chromosorb W (HP) 100–120 mesh, previously reported (13) to ensure best resolution of keto forms. The other column was U-shaped and measured 3 ft. It was packed with 3% OV-225 using the same support. Temperature programming was necessary in order to allow better separation and identification of bile acid species with nearly identical polarities. Reproductibility was checked on QF-1 from 200 to 235°C as well as on OV-225 from 240 to 250°C. Linear programming was used. Peak area measurements were carried out during GLC using an Autolab 6300 automatic digital integrator.

Similar experiments were carried out with 2.5 mg of each of the following 5β-cholanic keto acid derivatives: 3-monoketo; 3-keto,7α-hydroxy; 3-keto,7α,12α-dihydroxy; 3α-hydroxy,7-keto; 3α,12α-dihydroxy,7-keto; 3α-hydroxy,12-keto; 3α,7α-dihydroxy,12-keto; 3,7-diketo; 3,12-diketo; 3α-hydroxy,7,12-diketo; 3,7,12-triketo. These standards (Steraloids Inc. Wilton, NH) were estimated to be more than 95% pure by GLC. Ethanol (95%), methanol (95%), and water were tested as saponification solvents. Each step of the pro-

Abbreviations: Trivial names of bile acids in the text refer to hydroxy-substituted 5β-cholanic acids as follows: lithocholic, 3α; NDC, 23-nordeoxycholic, 3α,12α; deoxycholic, 3α,12α; chenodeoxycholic, 3α,7α; cholic, 3α,7α,12α. Derivatives with keto or mixed hydroxyl and keto functions are designated by positions of the functions: tri-keto cholanic acid, dehydrocholic, 3,7,12-triketo. GLC, gas–liquid chromatography.

[1] Lepage, G., A. Fontaine, and C. C. Roy, unpublished observations.

[2] All organic solvents were high purity grade.

TABLE 1. Effect of alkaline hydrolysis in ethanol 95% on a mixture of free bile acid standards

Methyl 5β-Cholanoate Acetate Derivatives[a]	Recovery of Standard	Artefactual Products	
		Identification[b]	%
Lithocholic	99.4		
23-Nordeoxycholic	94.1		
Deoxycholic	102.2		
Chenodeoxycholic	103.5		
Cholic	96.8		
3,7,12-Triketo	7	7-ketodeoxycholic	13
		12-ketochenodeoxycholic	16
		7,12-diketolithocholic	64

[a] Mixture of 2.5 mg of each standard.
[b] Tentative identification based on relative retention time as compared to internal standard (NDC) on 2% QF-1 and 3% OV-225.

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

JOURNAL OF LIPID RESEARCH

ASBMB

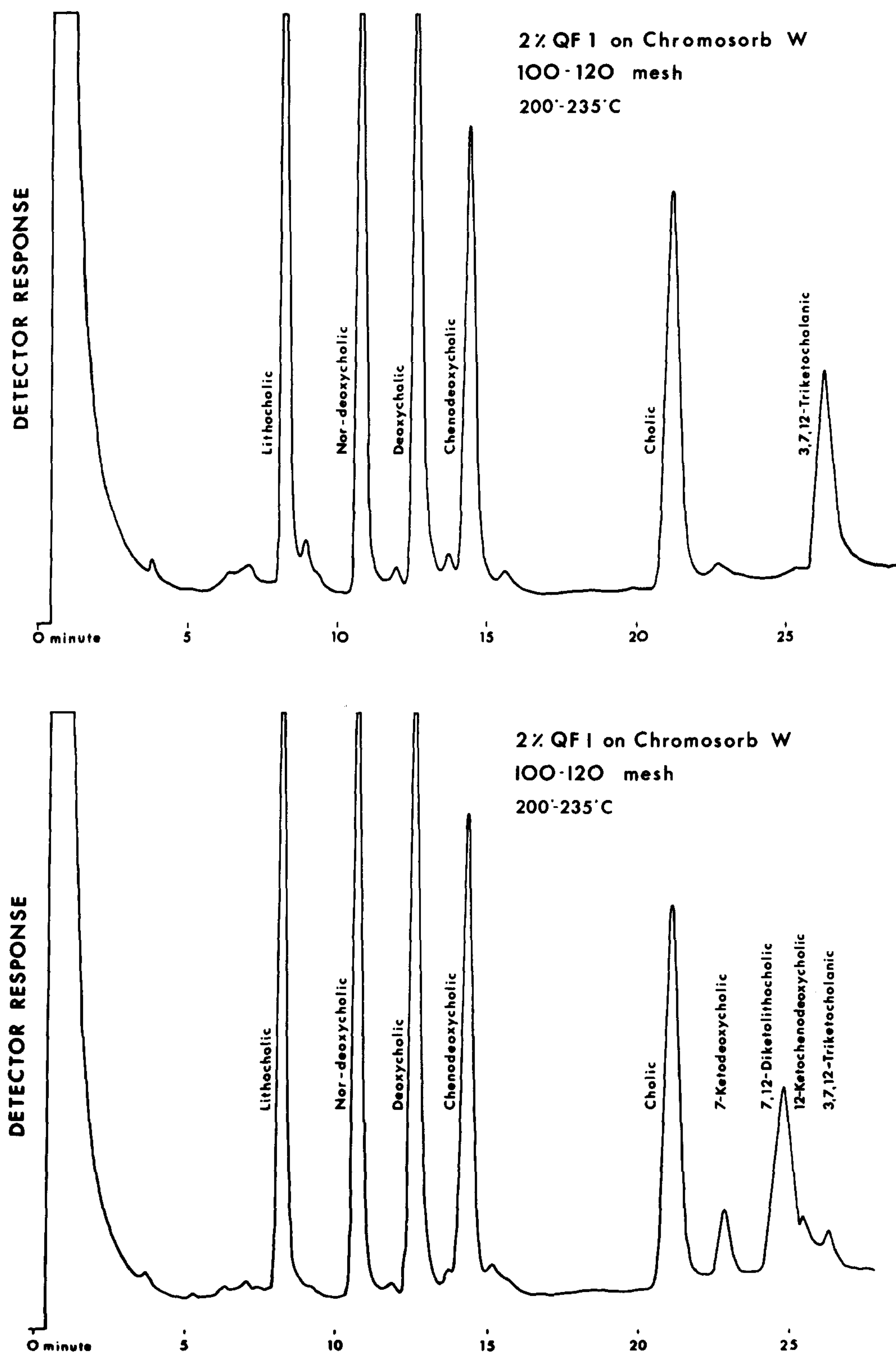


Fig. 1. Representative GLC patterns of free bile acid standards. The bile acids derivatives were obtained without prior saponification (upper chromatogram) and after saponification (lower chromatogram). The 3,7,12-triketo derivative was transformed into 7-ketodeoxycholic, 12-ketochenodeoxycholic, and 7,12-diketolithocholic by ethanolic alkaline hydrolysis.

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

TABLE 2. Effect of alkaline hydrolysis on keto bile acid standards

Methyl 5β-Cholanoate Acetate Derivative[a]	Ethanol 95%			Methanol 95%			Water		
	% Re-covered of Original Standard	Artefactual Products		% Re-covered of Original Standard	Artefactual Products		% Re-covered of Original Standard	Artefactual Products	
		Identification[b]	%		Identification	%		Identification	%
3 keto	2	3α hydroxy	98	4	3α hydroxy	96	95	3α hydroxy	5
3 keto,7α hydroxy	0	3α,7α dihydroxy U.P.[c]	95 5	3	3α,7α dihydroxy	97	95	U.P.	5
3 keto,7α,12α dihydroxy	2	3α,7α,12α tri-hydroxy U.P.	91 7	5	3α,7α,12α tri-hydroxy U.P.	88 7	54	3α,7α,12α tri-hydroxy U.P.	1 19
3α hydroxy,7 keto	93	3α,7α dihydroxy	7	98	3α,7α dihydroxy	2	100	N.D.P.[d]	
3α,12α, dihydroxy, 7 keto	80	3α,7α,12α tri-hydroxy U.P.	10 10	84	U.P.	16	90	U.P.	10
3α hydroxy,12 keto	90	3α,12α dihydroxy U.P.	4 6	95	3α,12α dihydroxy	5	89	N.D.P.	
3α,7α dihydroxy 12 keto	71	U.P.	29	70	U.P.	30	93	N.D.P.	
3,7 diketo	5	3α, hydroxy,7 keto 3α,7α dihydroxy	80 4	10	3α hydroxy,7 keto 3α,7α dihydroxy	67 3	72	3α hydroxy,7 keto U.P. U.P.	2 2 3
3,12 diketo	1	3α,12 keto 3α,12α dihydroxy U.P.	88 5 6	1	3α hydroxy,12 keto 3α,12α dihydroxy	97 2	45	N.D.P.	
3α hydroxy, 7,12 diketo	53	3α,12α, dihydroxy, 7 keto 3α,7α,dihydroxy, 12 keto	11 10	58	3α,12α,dihydroxy, 7 keto 3α,7α dihydroxy, 12 keto	9 10	75	N.D.P.	
3,7,12 triketo	7	3α,12α dihydroxy, 7 keto 3α,7α dihydroxy, 12 keto 3α hydroxy,7,12 diketo	13 16 64	0	3α,7α dihydroxy, 12 keto 3α hydroxy,7,12 diketo U.P.	5 12 10	38	3α,7α,12α tri-hydroxy 3α,12α dihydroxy, 7 keto 3α,7α,12 keto U.P.	3 7 1 17

[a] 2.5 mg of each standard.
[b] Tentative identification based on relative retention time as compared to internal standard (NDC) on 2% QF-1 and 3% OV-225.
[c] U.P., unidentified peak.
[d] N.D.P., no detectable peak.

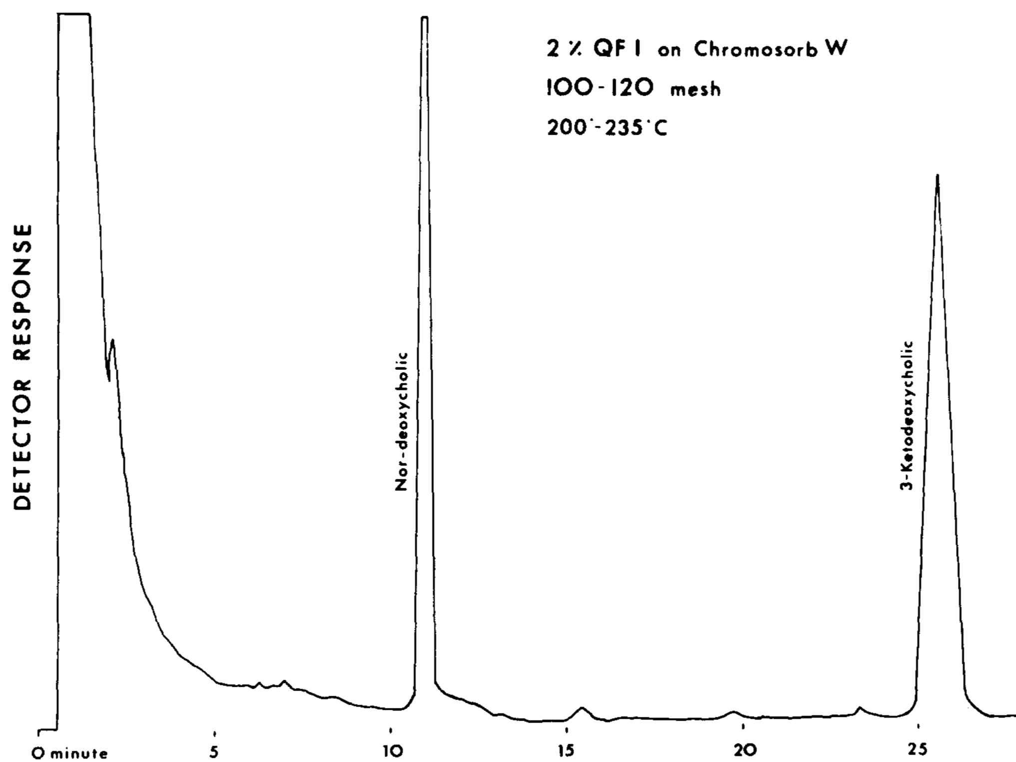
Downloaded from www.jlr.org by guest, on June 19, 2012

cedure was controlled for the presence of solvent contamination by running solvent blanks. The relative retention times of standards and of artefactual products were measured by comparison of area response with that of NDC. The possibility that alkaline hydrolysis of the internal standard could contribute to the production of artefactual peaks was ruled out since each GLC run was made with and without NDC (1 μg).

Results and discussion

Table 1 summarizes the effect of alkaline hydrolysis on a mixture of free bile acid standards. Rigorous alkaline hydrolysis of the four bile acids normally found in bile and of the internal standard (NDC) did not lead to the formation of any artefactual products and recoveries were excellent. However, this was not the case for the 3,7,12-triketo compound. The chromatogram (**Fig. 1**) shows nearly complete disappearance of the 3,7,12-triketo-5β-cholanic acid that had been added to the bile acid mixture. The artefactual products were tentatively identified as 7-ketodeoxycholic, 12-ketochenodeoxycholic, and 7,12-diketolithocholic acid.

Repeated experiments carried out with each of 11 keto bile acid standards are shown in **Table 2**. Ethanolic hydrolysis caused severe transformations. None of the keto bile acids bearing a keto group at C-3 could be recovered after saponification (**Fig. 2**). The five standards with keto groups at C-7 or C-12 were



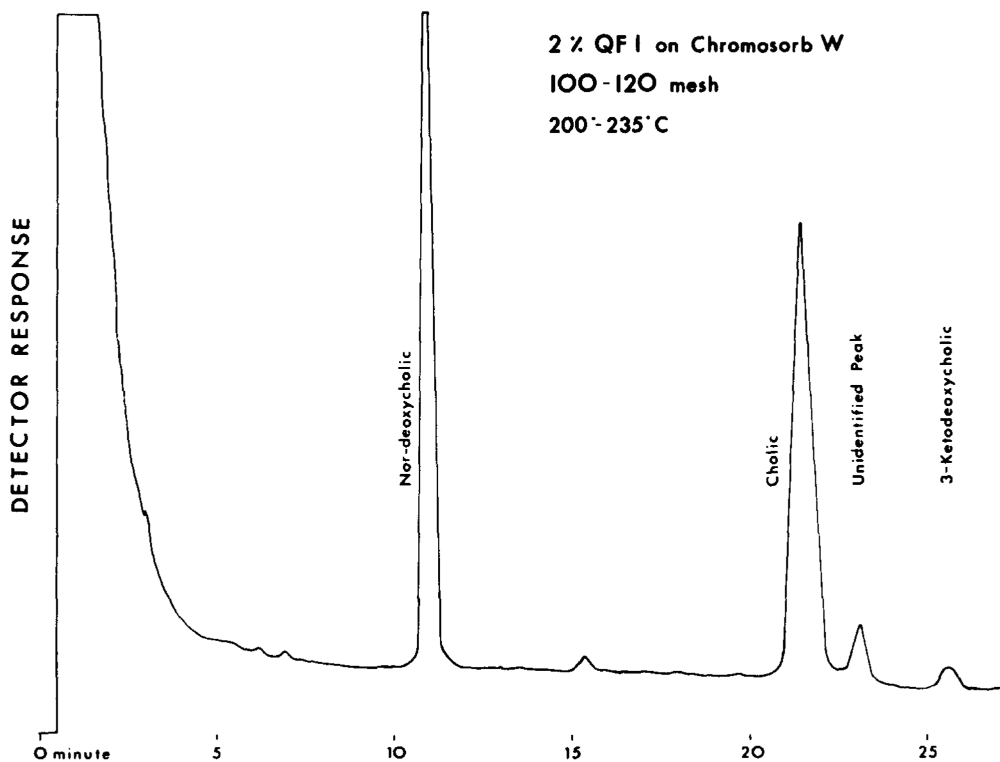


Fig. 2. These two chromatograms (QF-1) illustrate the extensive degradation of 3-keto-deoxycholic acid after ethanolic saponification. Only 2% of the original standard was recovered (Table 2) (lower chromatogram).

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

more stable; recoveries ranged from 71 to 93%. It should be noted that our recoveries of the 3-keto, 7-keto-deoxycholic, 12-keto-chenodeoxycholic, and 3,12 diketo compounds were lower than those reported previously under similar conditions of ethanolic saponification (14). Substitution of ethanol for methanol did not modify the recoveries.

The data confirm the previously mentioned (8, 10) drastic structural changes that keto bile acids undergo during alkaline hydrolysis.

Water hydrolysis appeared to effect fewer changes on keto standards; recoveries were low for compounds with two or three keto groups. This could relate to the extraction procedure after hydrolysis since the loss is not made up by artefactual products. Of interest is the fact that the recovery for the 3,12-diketo compound by Grundy, Ahrens, and Miettinen (14) was also lower than for the monoketo standards, even though a different extraction procedure was used.

In view of our findings, attention is drawn to the likelihood that keto bile acids in bile (7, 15) and in feces (2, 3), where they are a major constituent, are quantitatively and qualitatively unreliable. In the study by Soloway et al. (15) the biotransformation of 3,7,12-triketo-5β-cholanic acid (dehydrocholic acid) in man could also be explained by the effects of alkaline hydrolysis rather than by hepatic metabolism. The same stereospecificity was noted in the present study since only α epimers were recovered among the artefactual products.

Keto bile acids are particularly vulnerable to alkaline hydrolysis when it is carried out in ethanol or methanol. There is a significant advantage to the use of water as a saponification medium. Since a prerequisite for enzymatic hydrolysis is the presence of free hydroxyl groups on the steroid skeleton (16, 17), it is apparent that enzymatic cleavage of the carbon–nitrogen bond does not provide a solution to the methodological problem underlined in the present report.

We thank Doctor A. Kuksis of the University of Toronto for his invaluable help and encouragement. This paper was supported by Grant MT 4433 of the Medical Research Council of Canada and by the Canadian Cystic Fibrosis Foundation.

Manuscript received 7 January 1977 and in revised form 18 October 1977; accepted 30 November 1977.

REFERENCES

1. Danielson, H., P. Einarsson, K. Hellström, S. Lindstedt, and J. Sjövall. 1963. On the turnover and excretory products of cholic and chenodeoxycholic acid in man. *J. Biol. Chem.* **238:** 2299–2304.
2. Eneroth, P., K. Hellström, and J. Sjövall. 1968. A method for quantitative determination of bile acids in human feces. *Acta Chem. Scand.* **22:** 1729–1744.
3. Hill, M. J., and V. C. Aries. 1971. Faecal steroid composition and its relationship to cancer of the large bowel. *J. Pathol.* **104:** 129–139.
4. Drasar, B. S., and M. J. Hill. 1974. Human Intestinal Flora. Academic Press, New York. 111–116.
5. Haslewood, G. A. D. 1962. Comparative Biochemistry. Academic Press, New York. 205.
6. Malagelada, J. R., V. L. W. Go, W. H. J. Summerskill, et al. 1973. Bile acid secretion and biliary bile acid composition are altered by cholecystectomy. *Amer. J. Dig. Dis.* **18:** 455–459.
7. Hepner, G. W., A. F. Hofmann, J. R. Malagelada, A. P. Szczepanik, and P. D. Klein. 1974. Increased bacterial degradation of bile acids in cholecystectomized patients. *Gastroenterology.* **66:** 556–564.
8. Eneroth, P., and J. Sjövall. 1971. Extraction, purification and chromatographic analysis of bile acids in biological materials. *In* The Bile Acids. P. Nair and D. Kritchevsky, editors. Plenum Press, N.Y. 133–134.
9. Roseleur, O. J., and C. M. Van Gent. 1976. Alkaline and enzymatic hydrolysis of conjugated bile acids. *Clin. Chim. Acta.* **66:** 269–272.
10. Norman, A., and R. H. Palmer. 1964. Metabolites of lithocholic-24-C^{14} in human bile and feces. *J. Lab. Clin. Med.* **63:** 986–1001.
11. Evrard, E., and G. Janssen. 1968. Gas–liquid chromatographic determination of human fecal bile acids. *J. Lipid Res.* **9:** 226–237.
12. Roovers, J., E. Evrard, and H. Vanderhaege. 1968. An improved method for measuring human blood bile acids. *Clin. Chim. Acta.* **19:** 449–457.
13. Eneroth, P., and J. Sjövall. 1971. Extraction, purification and chromatographic analysis of bile acids in biological materials. *In* The Bile Acids. P. Nair and D. Kritchevsky, editors. Plenum Press, N.Y. 160.
14. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen. 1965. Quantitative isolation and gas–liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **6:** 397–409.
15. Soloway, R. D., A. F. Hofmann, P. J. Thomas, J. L. Schoenfield, and P. D. Klein. 1973. Triketocholanic (dehydrocholic) acid: hepatic metabolism and effect on bile flow and biliary lipid secretion in man. *J. Clin. Invest.* **52:** 715–724.
16. Nair, P. P., M. Gordon, and J. Reback. 1967. The enzymatic cleavage of the carbon–nitrogen bond in 3α,7α,12α-trihydroxy-5β-cholan-24-oxyl glycine. *J. Biol. Chem.* **242:** 7–11.
17. Henegouwen, G. P., A. Ruben, and K. H. Brandt. 1974. Quantitative analysis of bile acids in serum and bile using gas–liquid chromatography. *Clin. Chim. Acta.* **54:** 249–261.

ASBMB JOURNAL OF LIPID RESEARCH

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