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STEROL METABOLISM—XXX.* CHOLESTEROL 25-HYDROPEROXIDE METABOLISM

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SUMMARY

The metabolism of cholesterol 25-hydroperoxide by subcellular fractions of rat and calf liver and of rat kidney was examined. The sole metabolic transformation observed was the reduction of the 25-hydroperoxide to the corresponding 25-alcohol 5-cholestene-3β,25-diol. No evidence of enzymic rearrangement of the 25-hydroperoxide to vicinal diol products was adduced. Neither cholesterol 25-hydroperoxide nor 5-cholestene-3β,25-diol was converted by rat liver homogenates to bile acids. Attempts to demonstrate cholesterol 25-hydroperoxide as a putative intermediate in the biosynthesis of bile acids from cholesterol in rat liver homogenates and calf liver microsomes by trapping techniques were unsuccessful. It was concluded that cholesterol 25-hydroperoxide is not implicated in cholesterol metabolism in liver or kidney tissues.

INTRODUCTION

The metabolism of steroid hydroperoxides by mammalian [1–12], higher plant [13–14] and microbial [15–17] enzymes has received recent attention. With the one exception of the metabolism of cholesterol 20α-hydroperoxide by adrenal cortex mitochondria to form 5-cholestene-3β,20α,21-triol and 5-cholestene-3β,20α,22R-triol [2–4] the preponderant mode of metabolic alteration of steroid hydroperoxides is reduction to the corresponding alcohol [6–17]. Other accompanying reactions, including formal dehydration of secondary hydroperoxides to the corresponding ketone [5, 12, 14], carbon–carbon bond scission [6,7,10], and deoxygenation [7], mimic thermal decomposition reactions of the hydroperoxide substrates [18–22] and thereby may not be enzyme transformations.

The indicated rearrangement of cholesterol 20α-hydroperoxide to vicinal diol products suggested to us that the vicinal diol feature of other steroid metabolites might derive in similar fashion from a precursor hydroperoxide. Among such vicinal diol metabolites are the several ecdysterols bearing the 20α,22R- and 25,26-diol features [23, 24], the 24,25- and 25,26-diol metabolites of cholecalciferol [25, 26] and 25,26-dihydroxylated bile alcohols [27, 28]. Furthermore, the reported generation of [^{14}C]-acetone from [26-^{14}C]-cholesterol by rat liver mitochondria in the formation of bile acids [29] suggested the additional possibility of 24,25-diol intermediates in bile acid biosynthesis in liver. The present report deals with our examination of this thesis using the relatively readily accessible cholesterol 25-hydroperoxide as a model substrate and liver and kidney tissue as model sources of enzymes which might catalyze hydroperoxide-diol rearrangements associated with bile acid biosynthesis and cholecalciferol metabolism respectively.

EXPERIMENTAL

Cholesterol 25-hydroperoxide was prepared by autoxidation of cholesterol [30]. 5-Cholestene-3β,25-diol, cholic acid, and chenodeoxycholic acid were commercial samples recrystallized before use. [1,2-^{3}H]-Cholesterol obtained from New England Nuclear, Boston, Mass., was purified extensively by t.l.c. prior to use. 5-Cholestene-3β,25,26-triol was a gift of Dr. M. J. Thompson, U.S. Department of Agriculture, Beltsville, Md.

Thin-layer, gas and liquid column (Sephadex LH-20) chromatographic procedures for sterol analyses were those previously described by us in detail [31–34]. Sterols were analyzed on 0·25 mm thick chromatoplates of Silica Gel HF_{254} in the systems benzene–ethyl acetate (2:1 and 3:2, v/v), toluene–ethyl acetate (3:2, v/v), chloroform–acetone (24:1, v/v), or iso-octane–ethyl

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acetate acetic acid (5:2:1, by vol.). 5-Cholestene-3β,25-diol, 5-cholestene-3β,25,26-triol, and cholesterol 25-hydroperoxide were run as reference sterols. Sterols were detected with 50% aqueous sulfuric acid [31], sterol hydroperoxides with N,N-dimethyl-*p*-phenylenediamine [34]. Bile acids were analyzed on 0·25 mm thick chromatoplates in the system iso-octane–ethyl acetate–acetic acid–butanol (20:10:3:3, by vol.) using manganous chloride (50 mg $MnCl_2 . 4H_2O$ in 15 ml water and 0·5 ml conc sulfuric acid) for detection [35].

Cholesterol 25-hydroperoxide metabolism studies

Livers of male Sprague–Dawley rats (350–370 g) were perfused *in situ* with 0·25 M sucrose to remove blood, then excised and homogenized at 0–5°C with 0·15 M Tris–HCl buffer (pH 7·4) (4 ml buffer/g wet liver) in a loosely-fitted glass homogenizer. The homogenate was centrifuged at 3000 *g* for 10 min to remove cell debris, and the decanted supernate was used for incubations. To 4 ml of the enzyme preparation was added 1 mg of cholesterol 25-hydroperoxide in 25 μl of ethanol, and the total volume was adjusted to 10 ml with 0·15 M Tris–HCl buffer. The sample was incubated at 37°C together with similarly prepared samples containing no enzyme and boiled enzyme. Analyses of the incubation mixtures were made at 30, 60, 120 and 180 min. Results are recorded in Table 1 as Experiments No. 1–3.

In other experiments, male Sprague–Dawley rats (250–300 g) were pretreated daily for 6 days with a 2 mg/ml aqueous solution of phenobarbital administered intraperitoneally at 40 mg/kg doses. Control animals received daily injections of saline. Livers of sacrificed animals were perfused *in situ* with ice-cold 1·15% KCl solution to remove blood and then were homogenized at 0–5°C in 10% sucrose using a loosely fitted glass homogenizer and 15 strokes of the pestle. The homogenate was centrifuged at 1000 *g* for 10 min to remove cell debris, and the decanted supernate was used in ensuing incubations. Incubation media contained 12·5 mg ATP (disodium salt), 5 mg NAD, 15 mg glutathione, 20 mg sodium citrate, 0·25 mg Coenzyme A, 5 mg EDTA, 1 mg FAD (disodium salt), 1·25 mg NADP, and 8 mg $MgCl_2$, each dissolved in 0·20 ml of 0·15 M Tris–HCl buffer (pH 8·5). Incubations of these cofactors with 3·25 ml of enzyme preparation (equivalent to 2 g of wet liver) and additional Tris–HCl buffer to bring the total volume to 6·60 ml were conducted with shaking in a water bath at 37°C in air for 90 min with 600 μg of cholesterol 25-hydroperoxide dissolved in 0·1 ml of acetone. Similar incubations were conducted using boiled enzyme preparation as control. Results are recorded in Table 1 as Experiments No. 4–7.

In order to establish that these rat liver enzyme preparations were active in bile acid biosynthesis, incu-

Table 1. Cholesterol 25-hydroperoxide metabolism by rat and calf tissues

No.	Incubation conditions	Incubation			Products found by thin-layer chromatography			
		Temp	pH	Duration min	Cholesterol 25-hydroperoxide	5-Cholestene-3β,25-diol	Bile acids	Putative diols
Rat liver								
1.	3000 *g* Supernate, as is	37	7·4	180	+	+		–
2.	3000 *g* Supernate, boiled	37	7·4	180	+	+		–
3.	No tissue control	37	7·4	180	+	+		–
4.	1000 *g* Supernate, phenobarbital pretreatment, cofactors added	37	8·5	90	+	+	–	–
5.	1000 *g* Supernate, boiled, phenobarbital pretreatment, cofactors added	37	8·5	90	+	+	–	–
6.	1000 *g* Supernate, saline pretreatment, cofactors added	37	8·5	90	+	+	–	–
7.	1000 *g* Supernate, boiled, saline pretreatment, cofactors added	37	8·5	90	+	+	–	–
Calf liver								
8.	20,000 *g* Supernate, as is	30	7·0	30	–	+		–
9.	20,000 *g* Supernate, NADPH-generating system	30	7·0	30	–	+		–
Rat kidney								
10.	Whole homogenate, as is	37	7·4	180	+	+		–

bations were made using exactly the same enzyme preparations and cofactors, but using 75 μg of [1,2-^{3}H]-cholesterol (1.2×10^6 d.p.m.) dissolved in 50 μl of acetone–ethanol (1:1, v/v) as substrate. After incubating for 90 min at 37°C, 20 ml of 95% ethanol was added to stop the reactions, and the reaction mixture was saponified by warming for 1 h with 5 ml of 6 N NaOH. Precipitated material was washed with ethanol, and the pooled liquids were evaporated under vacuum. The residue was dissolved in 10 ml of water, the solution acidified to pH 1·0 with 0·6 ml of 2 N HCl, and extracted 4 times with 20 ml of petroleum ether to remove fatty acids and sterols. To the aqueous layer were added 4 mg each of cholic acid and chenodeoxycholic acid. The aqueous layer was extracted 4 times with 20 ml vol. of diethyl-ether. The ether extracts were washed with water until neutral, dried over anhydrous sodium sulfate, and evaporated under vacuum, and the bile acids were recovered by t.l.c. After further carrier dilution with 12 mg cholic acid and 12 mg chenodeoxycholic acid the sample was recrystallized from benzene–diethyl ether to constant specific radioactivity.

Calf liver (150 g) transported within 1 h from a local abbatoir to the laboratory was rinsed with cold saline and homogenized at 0–5°C with a Waring blendor in 200 ml of 0·02 M phosphate buffer (pH 7·0) also containing 10^{-4} M EDTA. The slurry was squeezed through cheese cloth, and the filtrate was homogenized in a glass homogenizer with Teflon pestle using six strokes of the pestle. The homogenate was centrifuged at 1000 *g* for 15 min to remove cell debris, then at 20,000 *g* for 15 min. The supernate was decanted and diluted as necessary with buffer to bring the total protein content to 2 mg/ml. This solution was used as the microsomal enzyme preparation. Incubations of 1 mg of cholesterol 25-hydroperoxide dissolved in 100 μl of ethanol were conducted at 30°C for 30 min with 5 ml of the enzyme preparation. A similar incubation was conducted to which 5 mg NADPH, 20 mg glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, and 10 mmol $MgCl_2$ had been added as a NADPH-generating system. Sterols were recovered by extraction with 25 ml portions of methylene chloride, filtration of the extract through phase-separating filter paper, and evaporation under vacuum. Analysis of products by t.l.c. is recorded in Table 1, Experiments No. 8–9.

Kidney tissue (8 g) from male Sprague–Dawley rats (350–370 g) was homogenized at 0–5°C in a loosely fitted glass homogenizer with 0·1 M Tris–HCl buffer (pH 7·4). Whole homogenate equivalent to 1 g of tissue was diluted with buffer to 10 ml and incubated with 1 mg of cholesterol 25-hydroperoxide dissolved in 40 μl of ethanol at 37°C. Samples were withdrawn at 30, 60, 120 and 180 min for t.l.c. analyses. Results are given in Table 1, Experiment No. 10.

Cholesterol 25-hydroperoxide trapping attempts

Homogenates of liver from male Sprague–Dawley rats prepared in 10% sucrose as previously described were centrifuged at 0–5°C at 1000 *g* for 10 min to remove cell debris, and the supernate was used as enzyme preparation. All of the cofactors previously used in other incubations of rat liver homogenates dissolved in 3·4 ml of 0·125 M Tris–HCl buffer (pH 8·5) together with 4 ml of the enzyme preparation (equivalent to 2 g wet liver) were incubated with 75 μg of [1,2-^{3}H]-cholesterol (1.2×10^6 d.p.m.). A similar incubation was conducted with boiled enzyme preparation. After incubation at 37°C for 120 min, 20 ml of ethanol was added to terminate the reaction, followed by 600 μg of cholesterol 25-hydroperoxide dissolved in ethanol. The mixture was acidified to pH 1·0 (no saponification was conducted in order not to decompose the cholesterol 25-hydroperoxide added as trapping agent), and the mixture was extracted several times with petroleum ether (b.p. 60–80°C). The petroleum ether extracts were evaporated under vacuum, and the residue was thin-layer chromatographed using iso-octane–ethyl acetate–acetic acid (5:2:1, by vol.). The cholesterol 25-hydroperoxide was recovered and reduced in methanol solution with an excess of sodium borohydride, and the sterol rechromatographed in benzene–ethyl acetate (3:2). The 5-cholestene-3β,25-diol zone was excised, the sterol eluted, and 10 mg of inactive carrier 5-cholestene-3β,25-diol was added. The sterol was then recrystallized several times from benzene–diethyl ether to constant specific radioactivity.

Homogenates of calf liver prepared in phosphate buffer as previously described were centrifuged at 1000 *g* for 15 min to remove cell debris and then at 20,000 *g* for 15 min to remove mitochondria. The total protein of the 20,000 *g* supernate was adjusted with buffer to 2 mg/ml, and 5 ml of enzyme preparation was incubated with 1·5 mg of [1,2-^{3}H]-cholesterol in 300 μl of ethanol for 30 min at 30°C. A similar incubation was performed in which 5 mg NADPH, 20 mg glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, and 10 mmol of $MgCl_2$ were added. Products were recovered by extraction several times with 25 ml portions of methylene chloride. The extracts were evaporated under vacuum, and 1 mg of cholesterol 25-hydroperoxide (as trapping agent) and 1 mg of 4-pregnene-3,20-dione (as internal reference steroid) were added. The sample was applied to a 0·5 mm thick chromatoplate of Silica Gel HF_{254} and chromatographed in toluene–ethyl acetate (3:2, v/v). In this system cholesterol 25-hydroperoxide and 4-pregnene-3,20-dione are

not resolved, thus permitting the cholesterol 25-hydroperoxide zone to be located via the strong u.v. light absorbing properties of the reference steroid 4-pregnene-3,20-dione. The cholesterol 25-hydroperoxide zone was excised from the chromatoplate and extracted with methylene chloride–methanol (9:1, v/v) and the extract evaporated under vacuum. To the thus recovered cholesterol 25-hydroperoxide there was added 5 mg each of inactive carrier sterols cholesterol, cholesterol 25-hydroperoxide, and 5-cholestene-3β,25-diol, and the sterol mixture was chromatographed on a 0·5 × 60 cm column of Sephadex LH-20 [33] developed with methylene chloride, 2 ml fractions being collected. In this system the relative retention volumes of cholesterol, 5-cholestene-3β,25-diol, and cholesterol 25-hydroperoxide were 1·0, 1·5 and 2·9 respectively, resulting in a baseline separation of the cholesterol 25-hydroperoxide from other sterols. Fractions were analyzed by gas chromatography on 3% QF-1 columns [32] and assayed for radioactivity.

RESULTS

The several model experiments involving cholesterol 25-hydroperoxide metabolism thought likely to provide interest are summarized in Table 1. Cholesterol 25-hydroperoxide metabolism was examined from two viewpoints: (1) formation of more polar sterols, the putative diols 5-cholestene-3β,24,25-triol or 5-cholestene-3β,25,26-triol, with chromatographic properties similar to those of 5-cholestene-3β,25,26-triol used as a reference sterol; and (2) formation of the recognized murine bile acids cholic and chenodeoxycholic acids. As shown in Table 1 there was no evidence adduced for either metabolic transformation of cholesterol 25-hydroperoxide.

Cholesterol 25-hydroperoxide was readily reduced to the corresponding 25-alcohol by calf liver microsomal enzymes, the substrate being totally consumed within 30 min. Addition of a NADPH-generating system did not alter these results. By contrast, incubations of cholesterol 25-hydroperoxide by rat liver or kidney, while yielding the corresponding 25-alcohol, proceeded at slower rates, and residual substrate cholesterol 25-hydroperoxide was still present in detectable amounts after 180 min at the close of the experiment. No vicinal diols or bile acids were found. Control incubations with no tissue or with boiled enzyme transformed the substrate to the corresponding 25-alcohol but at even slower rates as judged from t.l.c. The 25-alcohol is a major product of thermal degradation of cholesterol 25-hydroperoxide [18, 30], but true enzymic reduction of cholesterol 25-hydroperoxide by rat liver microsomes has been previously demonstrated [9]. The presently observed reduction of substrate to the 25-alcohol therefore includes both enzyme and thermal degradation reactions. We did not attempt to measure quantitatively the enzymic reduction.

The ready reduction of substrate by the calf liver enzyme system potentially compromises observation of any hydroperoxide–vicinal diol rearrangement. Were reduction of substrate so rapid as to preempt the rearrangement, no polar sterol derivatives would be detected. However, the sterol hydroperoxide rearrangement reaction is itself probably a very rapid reaction [2–4, 6], and our failure to find more polar sterols or bile acids in the case of the rat tissue enzyme incubations is not compromised by this objection.

Our attempts to trap radioactivity from [1,2-^{3}H]-cholesterol in cholesterol 25-hydroperoxide in rat and calf liver systems are presented in Table 2. In rat liver homogenates which were active in the conversion of substrate [1,2-^{3}H]-cholesterol to cholic and chenodeoxycholic acids there was no evidence for the participation of cholesterol 25-hydroperoxide as interme-

Table 2. Bioconversions of [1,2-^{3}H]-cholesterol by rat and calf liver

Incubation conditions	Steroids isolated	Radioactivity incorporated d.p.m.	%‡
1000 *g* Supernate of rat liver phenobarbital-treated rats, cofactors added	5-Cholestene-3β,25-diol*	0	0·000
	Cholic acid†	4460	0·371
	Chenodeoxycholic acid†	4060	0·338
1000 *g* Supernate of rat liver, saline-treated control rats, cofactors added	Cholic acid†	5440	0·453
	Chenodeoxycholic acid†	2070	0·170
20,000 *g* Supernate of calf liver	Cholesterol 25-hydroperoxide†	0	0·000
20,000 *g* Supernate of calf liver, NADPH-generating system added	Cholesterol 25-hydroperoxide†	30	0·001

* Recovered following chromatographic reisolation of cholesterol 25-hydroperoxide added as carrier and sodium borohydride reduction to the 3β,25-diol.

† Recovered by chromographic reisolation of steroid added as carrier.

‡ Percentage of [1,2-^{3}H]-cholesterol added as substrate.

diate. Similarly, no significant amounts of radioactivity were incorporated into either cholesterol 25-hydroperoxide or 5-cholestene-3β,25-diol in incubations of calf liver microsomal enzymes.

DISCUSSION

Our results establish that no hydroperoxide–diol rearrangement of cholesterol 25-hydroperoxide occurred in rat and calf liver or in rat kidney tissues but that hydroperoxide reduction to the corresponding 25-alcohol was the sole metabolic transformation. Our observations thus complement previous reports of enzymic reduction of cholesterol 25-hydroperoxide by bovine adrenal cortex mitochondria [2] and microsomes [9] and by rat liver microsomes [9]. Cholesterol 25-hydroperoxide interestingly was not altered by pig erythrocyte glutathione reductase [8]. In distinction, our prior studies of cholesterol 20α-hydroperoxide metabolism by bovine adrenal cortex mitochondria established that the rearrangement of the 20α-hydroperoxide to vicinal diols was rapidly achieved in a matter of minutes and that reduction to the corresponding 20α-alcohol 5-cholestene-3β,20α-diol was not prominent [2,6]. Thereby our present findings mitigate against involvement of hydroperoxide–diol rearrangement in the formation of 24,25- or 25,26-diol metabolites in liver or kidney. Accordingly, the uniqueness of the metabolic rearrangement of cholesterol 20α-hydroperoxide by adrenal cortex mitochondria is more apparent.

Our results also demonstrate that cholesterol 25-hydroperoxide and its metabolite 5-cholestene-3β,25-diol are not implicated in hepatic biosynthesis of bile acids from cholesterol. Neither transformation of either sterol to bile acids nor trapping of radioactivity from substrate [1,2-^{3}H]-cholesterol in either sterol was observed. Neither 25-hydroperoxide nor 3β,25-diol is generally considered an intermediate in bile acid biosynthesis, and both sterols may be formed by autoxidation of cholesterol [18, 30]. However, the 3β,25-diol has been detected in some [36–40] but not all [41] studies of the initial step of cholesterol oxidation in bile acid biosynthesis, and these items taken with the reported formation of [^{14}C]-acetone from [26-^{14}C]-cholesterol by rat liver mitochondria [29] have left the point unsettled. Our failure to trap radioactivity from substrate [1,2-^{3}H]-cholesterol in either the 25-hydroperoxide or the 3β,25-diol establishes that neither autoxidation nor enzymic 25-hydroxylation of cholesterol occurred. Although enzymic 25-hydroxylation of cholesterol by liver remains undemonstrated, enzymic 25-hydroxylation of other sterols, including cholecalciferol [42, 43] and 5β-cholestane-3α,7α,12α-triol [44, 45], has been demonstrated.

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REFERENCES

1. Björkhem I., Einarsson K. and Johansson G.: *Acta chem. scand.* **22** (1968) 1595–1605.
2. van Lier J. E. and Smith L. L.: *Biochim. biophys. Acta* **210** (1970) 153–163.
3. van Lier J. E. and Smith L. L.: *Biochim. biophys. Acta* **218** (1970) 320–326.
4. van Lier J. E. and Smith L. L.: *Biochem. biophys. Res. Commun.* **40** (1970) 510–516.
5. Mitton J. R., Scholan N. A. and Boyd G. S.: *Eur. J. Biochem.* **20** (1971) 569–579.
6. van Lier J. E., Kan G., Langlois R. and Smith L. L.: In *Biological Hydroxylation Mechanisms*, Biochemical Society Symposium No. 34 (Edited by G. S. Boyd and R. M. S. Smellie). Academic Press, New York (1972), pp. 21–43.
7. Tan L., Wang H. M. and Falardeau P.: *Biochim. biophys. Acta* **260** (1972) 731–740.
8. Little C.: *Biochim. biophys. Acta* **284** (1972) 375–381.
9. Hrycay E. G. and O'Brien P. J.: *Archs Biochem. Biophys.* **153** (1972) 480–494.
10. Hrycay E. G., O'Brien P. J., van Lier J. E. and Kan G.: *Archs Biochem. Biophys.* **153** (1972) 495–501.
11. Hrycay E. G. and O'Brien P. J.: *Archs Biochem. Biophys.* **157** (1973) 7–22.
12. van Lier J. E., Kan G. and Langlois R.: *Steroids* **21** (1973) 521–535.
13. Kirdani R. Y. and Layne D. S.: *Biochemistry* **4** (1965) 330–334.
14. Teng J. I. and Smith L. L.: *J. Am. chem. Soc.* **95** (1973) 4060–4061.
15. Tan L. and Falardeau P.: *Biochem. biophys. Res. Commun.* **41** (1970) 894–900.
16. Tan L. and Falardeau P.: *Steroidologia* **2** (1971) 65–76.
17. Kulig M. J. and Smith L. L.: *J. steroid Biochem* **5** (1974) (in press).
18. van Lier J. E. and Smith L. L.: *Steroids* **15** (1970) 485–503.
19. Teng J. I., Kulig M. J., Smith L. L., Kan G. and van Lier J. E.: *J. org. Chem.* **38** (1973) 119–123.
20. Teng J. I., Kulig M. J. and Smith L. L.: *J. Chromatog.* **75** (1973) 108–113.
21. Smith L. L., Kulig M. J. and Teng J. I.: *Steroids* **22** (1973) 627–635.
22. Tan L., Wang H. M. and Falardeau P.: *Can. J. Biochem.* **50** (1972) 706–709.
23. Thompson M. J., Kaplanis J. N., Robbins W. E. and Yamamoto R. T.: *Chem. Commun.* (1967) 650–653.
24. Kaplanis J. N., Thompson M. J., Dutky S. R., Robbins W. E. and Lindquist E. L.: *Steroids* **20** (1972) 105–120.
25. Suda T., DeLuca H. F., Schnoes H. K., Tanaka Y. and Horlick M. F.: *Biochemistry* **9** (1970) 4776–4780.
26. Horlick M. F., Schnoes H. K., DeLuca H. F., Gray R. W., Boyle I. T. and Suda T.: *Biochemistry* **11** (1972) 4251–4255.
27. Okuda K., Hoshita T. and Kazuno T.: *J. Biochem.* **51** (1962) 48–55.
28. Hoshita T., Hirofuji S., Nakagawa T. and Kazuno T.: *J. Biochem.* **62** (1967) 62–66.

29. Whitehouse M. W., Rabinowitz J. L., Staple E. and Gurin S.: *Biochim. biophys. Acta* **37** (1960) 382–385.
30. van Lier J. E. and Smith L. L.: *J. org. Chem.* **35** (1970) 2627–2632.
31. Smith L. L., Matthews W. S., Price J. C., Bachmann R. C. and Reynolds B.: *J. Chromatog.* **27** (1967) 187–205.
32. van Lier J. E. and Smith L. L.: *Analyt. Biochem.* **24** (1968) 419–430.
33. van Lier J. E. and Smith L. L.: *J. Chromatog.* **41** (1969) 37–42.
34. Smith L. L. and Hill F. L.: *J. Chromatog.* **66** (1972) 101–109.
35. Goswami S. K. and Frey C. F.: *J. Chromatog.* **53** (1970) 389–390.
36. Fredrickson D. S.: *J. biol. Chem.* **222** (1956) 109–120.
37. Fredrickson D. S. and Ono K.: *Biochim. biophys. Acta* **22** (1956) 183–184.
38. Kritchevsky D. and Tepper S. A.: *Experientia* **22** (1966) 84.
39. Mitropoulos K. A., Avery M. D., Myant N. B. and Gibbons G. F.: *Biochem. J.* **130** (1972) 363–371.
40. Mendelsohn D. and Mendelsohn L.: *Biochemistry* **7** (1968) 4167–4172.
41. Danielsson H.: *Arkiv Kemi* **17** (1961) 373–379.
42. Ponchon G., Kennan A. K. and DeLuca H. F.: *J. clin. Invest.* **48** (1969) 2032–2037.
43. Horsting M. and DeLuca H. F.: *Biochem. biophys. Res. Commun.* **36** (1969) 251–256.
44. Cronholm T. and Johansson G.: *Eur. J. Biochem.* **16** (1970) 373–381.
45. Johansson G.: *Eur. J. Biochem.* **17** (1970) 292–295.