Biochimica et Biophysica Acta, 640 (1981) 121—130

BBA 79095

EFFECTS OF CHRONIC ETHANOL INGESTION ON THE ACTIVITY OF RAT LIVER MITOCHONDRIAL 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHOHYDROLASE

CHARLES E. DREILING, ROGER J. SCHILLING and RONALD C. REITZ

Division of Biochemistry, School of Medicine, University of Nevada, Reno, NV 89557 (U.S.A.)

(Received March 17th, 1980)
(Revised manuscript received July 14th, 1980)

Key words: Ethanol effect; Cyclic nucleotide; Phosphohydrolase (Rat liver mitochondria)

Summary

Chronic ethanol ingestion induced a 47% increase in the specific activity of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (nucleoside-2′:3′-cyclic-phosphate 2′-nucleotidohydrolase, EC 3.1.4.37) in whole mitochondria. Both inner and outer mitochondrial membranes showed increased (cyclic nucleotide)phosphohydrolase activity, but the inner was increased 94% compared to 67% for the outer. Techniques which disrupt membrane structure increased (cyclic nucleotide)phosphohydrolase activity. After these treatments, whole mitochondria from ethanol-treated animals still showed a 50% increase in activity. This increase may be related either to an inherent increase in the resistance of (cyclic nucleotide)phosphohydrolase to protein degradation or turnover, or to ethanol-induced membrane changes. An increase in (cyclic nucelotide)phosphohydrolase reaction medium pH was observed when freshly isolated, highly-coupled mitochondria were used. The total increase in pH was about 2-fold greater in the controls compared to the ethanol-treated mitochondria. It is suggested that the smaller initial increase in pH and the greater activity of (cyclic nucleotide)phosphohydrolase in the mitochondria from the ethanol-treated animals relate to previously observed changes in the lipid and protein composition of the mitochondrial membranes. In addition, (cyclic nucleotide)phosphohydrolase may represent an excellent marker for membrane integrity.

Introduction

The enzyme 2′,3′-cyclic nucleotide 3′-phosphohydrolase (nucleoside-2′:3′-cyclic-phosphate 2′-nucleotidohydrolase, EC 3.1.4.37) is generally regarded as a

myelin marker [1,2], however, considerable (cyclic nucleotide)phosphohydrolase activity has been reported in the plasma membranes of the spleen, adrenal gland, liver, heart muscle and human erythrocyte membrane [3—6]. Moreover, we have demonstrated the presence of (cyclic nucleotide)phosphohydrolase activity in both the inner and outer membranes of the rat liver mitochondrion [7]. Because (cyclic nucleotide)phosphohydrolase is present in membranes other than myelin, it has been suggested that the enzyme may play a fundamental role in biomembranes [4,8]. In spite of this, virtually nothing is known regarding the physiological significance of (cyclic nucleotide)phosphohydrolase.

One approach to investigating the function of (cyclic nucleotide)phosphohydrolase is to determine the enzyme's activity during diseases affecting cellular membranes. To date, the only studies along this line have been reports of diminished (cyclic nucleotide)phosphohydrolase activity during demyelination (i.e., multiple sclerosis and subacute sclerosing panencephalitis [9]) and during defective myelin formation (i.e., jimpy and quaking mutant mice [4,10]). Because (cyclic nucleotide)phosphohydrolase is present in non-myelin membranes, studies on the enzyme during disease states or conditions which affect these membrane may assist in determining the significance of (cyclic nucleotide)-phosphohydrolase.

Chronic ethanol treatment leads to alterations in the lipid and protein composition of mitochondrial membranes [11—13], and it interferes with the conformational (orthodox-contracted) transitions of these membranes during substrate oxidation [14]. In view of these effects of ethanol, we propose that ethanol treatment may also affect the activity of the membrane-bound (cyclic nucleotide)phosphohydrolase enzyme. In this study, we found the (cyclic nucleotide)phosphohydrolase of rat liver mitochondria not only increased as a result of chronic ethanol ingestion, but also the (cyclic nucleotide)phosphohydrolase assay may be useful in estimating the degree to which liver mitochondria are damaged by ethanol.

Methods and Materials

Animals. Long-Evans male rats (140—160 g body wt.) were purchased from Simonsen Laboratories (Gilroy, CA) and maintained for 35—45 days on a low-fat liquid diet containing 36% of calories as ethanol [11]. The pair-fed rats were decapitated and their livers removed.

Mitochondria. Whole liver mitochondria were prepared by the method of Chappell and Hansford [15] as modified by Thompson and Reitz [16]. The final mitochondrial pellet was suspended in 0.25 M sucrose and 3.4 mM Tris-HCl (pH 7.4) to give 100 mg protein/ml. Outer and inner mitochondrial membranes were prepared from this pellet according to the procedure of Greenawalt [17]. The purity of the inner and outer membrane subfractions was determined by the use of the marker enzymes, monoamine oxidase for the outer [18] and cytochrome oxidase for the inner membrane [19]. The intermembrane space and matrix were assayed by adenylate kinase [20] and glutamate dehydrogenase [21], respectively.

(Cyclic nucleotide)phosphohydrolase determinations. (Cyclic nucleotide)-

phosphohydrolase activity was determined using 2',3'-cyclic adenosine monophosphate as substrate. In order to identify 2'-AMP as the reaction product, initial (cyclic nucleotide)phosphohydrolase assays were performed by the chromatographic method of Kurihara and Tsukada [1]. All subsequent assays were performed by the spectrophotometric assay recently described [22]. 1 unit of enzyme activity (U) is defined as that amount of enzyme producing 1 μmol 2'-AMP/min. Specific enzyme activity is expressed as U/mg protein.

In the studies shown in Figs. 1 and 2, an increase in absorbance was observed immediately upon adding the mitochondrial protein to the complete reaction mixture. This change in absorbance is reported as pH units/mg protein and referred to as the Δh parameter. Protein concentrations were determined by the procedure of Lowry et al. [23].

Results

Intact rat liver mitochondria were prepared as described and examined polarographically for substrate oxidation during state 3 and state 4 respiration [16]. Respiratory control rates [24] were 9.7 ± 1.6 and 5.0 ± 0.5 for β-hydroxybutyrate and succinate in controls, respectively, and 5.4 ± 1.2 and 4.4 ± 0.3, respectively, in mitochondrial from the ethanol-treated animals. After fractionation, the outer and inner membranes, the intermembrane space and the matrix were assayed for purity. The outer membrane from the ethanol-treated group contained 9.3 ± 4.3% contamination by inner membrane. The inner membrane contained 17.6 ± 2.3% contamination by outer membrane. Membranes from controls had similar contamination [7]. Although the matrix and intermembrane space contained somewhat more contamination, they did not contain (cyclic nucleotide)phosphohydrolase activity and were not examined further.

Representative tracings of the enzyme activity in whole control and ethanol-treated mitochondria are shown in Fig. 1. The addition of 0.5 mg protein to the assay mixture resulted in an initial increase in the pH of the solution (noted by Δh) and a subsequent decrease caused by substrate hydrolysis. The results of the assays from control and ethanol-treated mitochondria, however, differed in that the ethanol-treated mitochondria contained 47% more (cyclic nucleotide)phosphohydrolase activity than controls and exhibited a 62% lower Δh value. These differences were observed for each of five pairs of animals and are summarized in Table I.

Experiments were conducted to determine if membrane integrity played a role in increasing (cyclic nucleotide)phosphohydrolase activity (Table I). After exposure to Triton X-100, a 53% increase in activity was observed in mitochondria from ethanol-treated animals and a 50% increase was observed in controls. After freezing and thawing, the specific activity increased 4% and 17% in the control and the ethanol-treated groups, respectively. After either treatment, the ethanol-treated mitochondria were still (51—60% more active than their respective controls.

To define more clearly the effects of ethanol on mitochondrial (cyclic nucleotide)phosphohydrolase, the inner and outer mitochondrial membranes were separated and the activity of (cyclic nucleotide)phosphohydrolase was deter-

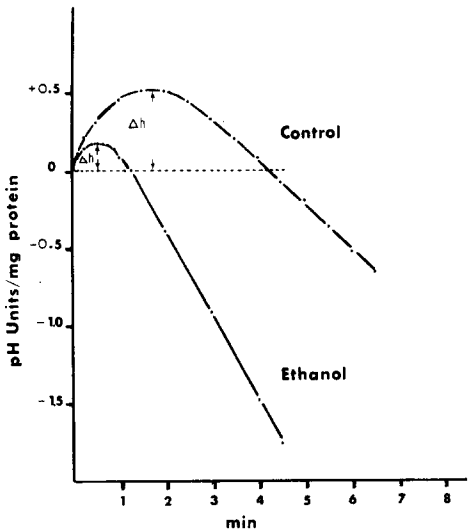

Fig. 1. Representative tracings of 2′,3′-cyclic nucleotide 3′-phosphohydrolase activity in fresh intact liver mitochondria from control and ethanol-treated rats. (Cyclic nucleotide)phosphohydrolase was assayed by the phenol red method described previously [22]. Enzyme velocity was determined from the slope of the linear portion of the tracing. Because the increase in pH does not relate to enzyme activity, pH units/mg was used rather than specific activity as enzyme units/mg. The initial rate of increase in pH change for the control was 0.148 pH units/min per mg, and the rate of decrease in pH was 0.113 pH units/min per mg. The initial rate of increase in pH change for the ethanol-treated mitochondria was 0.248 pH units/min per mg, and the rate of decrease was 0.173 pH units/min per mg. The total increase in pH is defined as the Δh parameter.

mined. A 67 and 94% increase was observed in the specific activity, respectively, of outer and inner membranes from ethanol-treated animals (Table II). There was a 97% increase in the total activity of the outer membrane and a 130% increase in the inner membrane.

The initial rise in pH (i.e., Δh; Fig. 1) was observed only with freshly pre-

TABLE I

EFFECT OF CHRONIC ETHANOL INGESTION ON (CYCLIC NUCLEOTIDE)PHOSPHOHYDROLASE ACTIVITY IN INTACT RAT LIVER MITOCHONDRIA

U = μmol 2′-AMP formed/min. (Cyclic nucleotide)phosphohydrolase was measured as previously described [22]. (Cyclic nucleotide)phosphohydrolase activity is measured as U/mg protein.

Treatment group	(Cyclic nucleotide)phosphohydrolase			
	Fresh mitochondria	After freeze-thaw	After 0.25% Triton	pH/mg protein (Δh) **
Control	0.133 ± 0.005 *	0.139	0.199 ± 0.012	0.450 ± 0.062
Ethanol	0.196 ± 0.007	0.230	0.300 ± 0.024	0.172 ± 0.027
Ethanol/control ratio	1.47	1.67	1.51	0.38
% Change due to ethanol	+47%	+65%	+51%	−62%
P value	<0.001	—	<0.01	<0.01
Number of animals	5	2	3	3

* Each value represents the mean ± S.E.

** pH/mg protein represents the maximal pH increase in fresh mitochondria and is noted as Δh in Fig. 1.

TABLE II

EFFECT OF CHRONIC ETHANOL INGESTION ON (CYCLIC NUCLEOTIDE)PHOSPHOHYDROLASE ACTIVITY IN THE INNER AND OUTER MEMBRANES OF RAT LIVER MITOCHONDRIA

The numbers in parenthesis represent the number of membrane preparations examined. Livers from two animals were pooled for each preparation. (Cyclic nucleotide)phosphohydrolase was measured as described [22].

	Inner membranes		Outer membranes	
	Specific activity	Total activity	Specific activity	Total activity
	(6)	(6)	(8)	(6)
Control	0.208 ± 0.029 *	3.75 ± 0.68 **	0.284 ± 0.031 ***	6.43 ± 0.71
	(5)	(5)	(4)	(4)
Ethanol	0.403 ± 0.047	8.64 ± 1.48	0.475 ± 0.052 ***	12.66 ± 1.71
Ethanol/control ratio	1.94	2.30	1.67	1.97
% Change due to ethanol	+94%	+130%	+67%	+97%
P value	<0.01	<0.02	<0.01	<0.01

* Specific activity = U/mg protein.

** Total activity = U (μmol 2′-AMP formed/min).

*** Compared to specific activity of inner membranes, *P* is not significant.

pared mitochondria. When fresh mitochondria were treated with detergents, freeze-thawed (Fig. 2c) or merely allowed to stand at 0°C for 4—5 h, no increase in pH was seen. Studies were undertaken to determine the cause of this increase in fresh intact mitochondria. That the initial increase in absorbance (Δh) represented an increase in the pH of the assay medium was confirmed by omitting phenol red from the mixture and following the pH change directly with a pH meter. In addition, when the phenol red was omitted, we failed to observe an increase in absorbance of the solution at 560 nm even after 10 min.

When 2′,3′-cyclic AMP was omitted from the reaction, the pH of control mitochondria increased at a rate of 0.305 pH units/min per mg (see legend to Fig. 2a) compared to 0.148 pH units/min per mg observed when substrate was present (see legend to Fig. 1 control). Similar observations were made using mitochondria from ethanol-treated animals; however, the rate of pH change was 0.473 pH units/min per mg in the absence of substrate compared to 0.248 pH units/min per mg in the presence of substrate. The total increase in pH in the absence of substrate (Fig. 2a) was 2-times greater in control mitochondria compared to the ethanol-treated mitochondria.

Figs. 1 and 2 suggest that the slower rate of pH increase in the presence of substrate was due to competing reactions. This was confirmed by adding the rate of increase (0.148 pH units/min per mg) to the rate of decrease (0.113 pH units/min per mg). The resulting rate (0.261 pH units/min per mg) was almost identical to the rate of increase in the absence of substrate (0.305 pH units/min per mg). Similar data can be calculated for the mitochondria from the ethanol-treated animals.

In order to further elucidate the significance of the initial pH increase, we examined the effect of isotonic sucrose and Triton X-100 on both the pH rise and the activity of (cyclic nucleotide)phosphohydrolase (Figs. 2b and 3). The standard (cyclic nucleotide)phosphohydrolase assay is hypotonic, and after

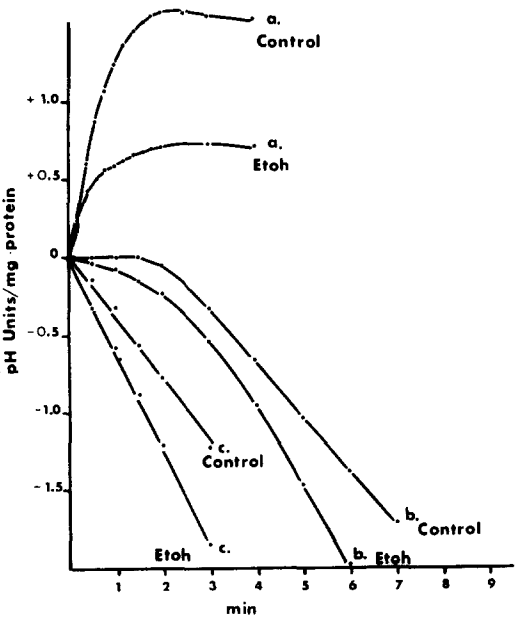

Fig. 2. Effects of substrate omission, 0.25 M sucrose and freeze-thawing on (cyclic nucleotide)phosphohydrolase activity. Curves a. These are representative curves for the pH change in the absence of substrate. The rate of increase in pH was 0.305 pH units/min per mg for the controls and 0.473 pH units/min per mg for the ethanol-treated mitochondria. Curves b. These represent the pH change in the presence of substrate after the reaction mixture was made up to 0.25 M with sucrose. The rate of pH change was 0.117 pH units/min per mg for control and 0.188 pH units/min per mg for the ethanol-treated mitochondria. Curves c. These are representative curves for the change in pH after the mitochondria had been freeze-thawed once. The rate of pH change was 0.122 pH units/min per mg for the control and 0.202 pH units/min per mg for the ethanol-treated mitochondria. The change in pH was measured as described [22].

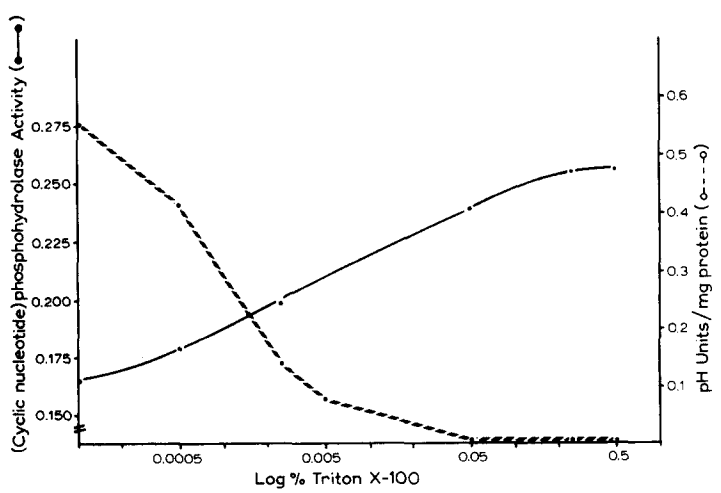

Fig. 3. Effect of Triton X-100 on (cyclic nucleotide)phosphohydrolase activity and on the Δh parameter. Because a large difference in amount of Triton was used, the abscissa is plotted as the log of Triton concentration. The Δh parameter is reported as pH units/mg protein and was measured at the maximal pH change as shown in Fig. 1. (Cyclic nucleotide)phosphohydrolase was measured as described [22].

exposure to this medium, mitochondria should expand to such a point that they burst. When fresh control and ethanol-treated mitochondria were assayed in the presence of isotonic (0.25 M) sucrose the pH decreased, after a short lag period, as the substrate was hydrolyzed; however, there was no initial increase in the pH of the medium (Fig. 2b). Thus, the pH rise seen earlier may have resulted from osmotic disruption of the mitochondria. When the mitochondria were freeze-thawed, the pH rise was obliterated supporting the idea of osmotic disruption (Fig. 2c). Neither osmotic shock nor freeze-thawing changed the fact that ethanol had induced about a 61—66% increase in the specific activity (see legend for Fig. 2).

Additional studies indicated the pH rise was dependent upon both the physical integrity and the metabolic activity of the mitochondria. Fig. 3 shows that, when control mitochondria were assayed in the presence of increasing concentrations of Triton X-100, there was a progressive decrease in the Δh parameter until no pH increase was observed at concentrations above 0.05% Triton. Fig. 3 also shows that (cyclic nucleotide)phosphohydrolase increased with increasing concentrations of Triton X-100 up to a plateau at about 0.05% Triton.

Because the increase in pH was not seen in aged mitochondria, we hypothesized that the increase in pH may be related to the coupling of oxidative phosphorylation. To test this, the uncoupler, dinitrophenol, was added to mitochondria in increasing concentrations. There was a progressive decrease in the Δh parameter (Fig. 4) accompanied by a progressive increase in dinitrophenol uncoupling of oxidative phosphorylation. These data imply the increase in pH

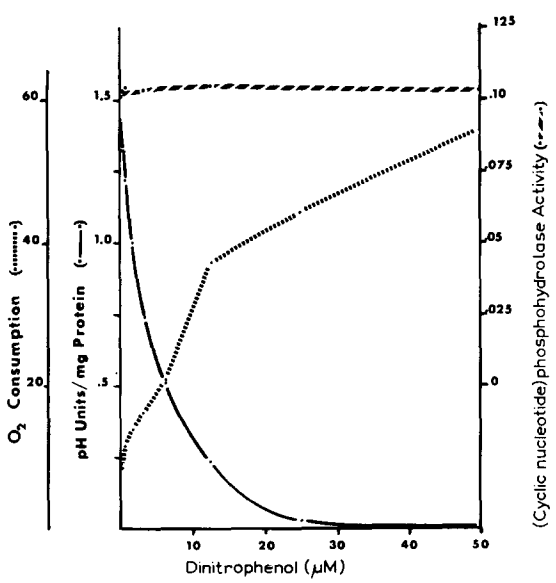

Fig. 4. Effect of dinitrophenol on mitochondrial uncoupling, the Δh parameter and (cyclic nucleotide)-phosphohydrolase activity. The Δh parameter was measured as noted in Fig. 1. Oxygen consumption was measured with freshly isolated mitochondria, and the values represent μmol O_2 consumed/min per mg protein. Dinitrophenol was added to state 4 reaction conditions [24]. (Cyclic nucleotide)phosphohydrolase was measured as described [22].

to be an energy linked event. No change in (cyclic nucleotide)phosphohydrolase was observed at any concentration of dinitrophenol.

Discussion

The data presented in this report clearly show that chronic ethanol ingestion results in a marked increase in the activity of (cyclic nucleotide)phosphohydrolase in liver mitochondria. This increase was somewhat selective to the inner mitochondrial membrane. Previous studies [1] have shown myelin (cyclic nucleotide)phosphohydrolase activity to be markedly increased by hypotonic disruption of the membrane structure, and Kurihara et al. [25] and Banik and Davison [26] have shown that full activity of the myelin enzyme could be obtained after detergent disruption of the membrane structure. This suggests membrane integrity is inversely related to (cyclic nucleotide)phosphohydrolase activity. Konings and Pierce [5] observed marked increases in (cyclic nucleotide)phosphohydrolase activity in tumor tissue membranes when compared to normal spleen tissue membranes. It is well documented that tumor membranes have marked alterations in lipid composition [27,28]; thus, the relationship of (cyclic nucleotide)phosphohydrolase to membrane structure seems to depend, to a large extent, on the lipid composition. Our findings that ethanol markedly altered the fatty acid and phospholipid composition of whole mitochondria [11] as well as the inner and outer membranes [39] suggest that the increased activity of (cyclic nucelotide)phosphohydrolase simply reflects an alteration in the integrity of each mitochondrial membrane.

Other evidence (Tables I and II) suggests this may only be partially true. Because the total activity was increased by ethanol much more than the specific activity and because this increase persisted after freeze-thawing or treatment with detergent, the ethanol-induced increase may also represent an increase in enzyme content. The effects of ethanol on mitochondrial protein synthesis would argue against this idea [13,29,30]. Further, one report [31] has shown that ethanol (40% of calories) decreased the total amount of mitochondrial protein, while another report [32] showed no change when ethanol was fed at 36% of calories. We observed a 16% decrease in the amount of protein in the mitochondrial suspensions from the ethanol-treated animals (data not shown). The 47% increase in specific activity of (cyclic nucleotide)phosphohydrolase noted in Table I would require a 32% decrease in total protein in order for the decreased protein synthesis to be the sole cause for the increased activity of the enzyme. It is well known that membrane proteins do not turn over at the same rates. Further, these proteins are removed and degraded asynchronously [33]. Previous data [34,35] suggest that (cyclic nucleotide)phosphohydrolase may be more stable than a variety of enzymes; thus, the mitochondrial (cyclic nucleotide)phosphohydrolase simply may not be degraded as rapidly as others. Its activity would appear to increase as a result of decreased synthesis of other mitochondrial proteins accounting for the increase in specific activity observed in our study.

The basis for the initial increase in pH when fresh mitochondria were assayed for (cyclic nucleotide)phosphohydrolase activity is unclear. The dinitrophenol

and ageing experiments suggest the increase may be energy-linked; the membrane disruption studies indicate the increase may be related to mitochondrial integrity. These two possibilities are not mutually exclusive, and both might account for the altered pH changes exhibited by the mitochondria from the ethanol-treated animals. Chronic ethanol ingestion has been shown to activate mitochondrial ATPase [36]. This would increase H^+ production and alter the rise in pH which accompanies mitochondrial disruption. Ethanol has also been shown to change the structural integrity of mitochondria [14,37,38]. Thus, the mitochondrial integrity or intactness may be altered by ethanol treatment, such that more H^+ binding anions become exposed to the assay medium. These anions, presumably proteins, would tend to buffer the initial increase in pH.

In conclusion, ethanol not only seems to increase the amount of (cyclic nucleotide)phosphohydrolase, but it may activate it by altering the mitochondrial membrane structure via lipid changes. (Cyclic nucleotide)phosphohydrolase may also serve as a useful marker for structural changes in membranes.

Acknowledgements

This project was funded in part by grants from the Research Advisory Board, UNR, NIH grants No. RR-09035 and No. AA 02739, and by NSF grant No. SER 76-18113.

References

1 Kurihara, T. and Tsukada, Y. (1967) J. Neurochem. 14, 1167–1174
2 Olafson, R.W., Drummond, G.I. and Lee, J.F. (1969) Can. J. Biochem. 47, 961–966
3 Drummond, G.I., Iyer, N.T. and Keith, J. (1962) J. Biol. Chem. 237, 3535–3539
4 Kurihara, T., Nussbaum, J.L. and Mandel, P. (1971) Life Sci. 10, 421–429
5 Konings, A.W.T. and Pierce, D.A. (1974) Life Sci. 15, 491–499
6 Sudo, T., Kikuno, M. and Kurihara, T. (1972) Biochim. Biophys. Acta 225, 640–646
7 Dreiling, C.E., Schilling, R.J. and Reitz, R.C. (1980) Biochim. Biophys. Acta 640, 114–120
8 Matthieu, J.-M. and Waehneldt, T.V. (1978) Brain Res. 150, 307–318
9 Riekkinen, P.J. Rinne, U.K., Arstila, A.U., Kurihara, T. and Pelliniemi, T.T. (1973) The Nature of Multiple Sclerosis, pp. 26–32, MSS Information Service, New York
10 Kurihara, T., Nussbaum, J.L. and Mandel, P. (1969) Brain Res. 13, 401–403
11 Thompson, J.A. and Reitz, R.C. (1978) Lipids 13, 540–550
12 French, S.W., Ihrig, T.J., Shaw, G.P., Tanaka, T.T. and Norum, M.L. (1971) Res. Commun. Chem. Pathol. Pharmacol. 2, 567–585
13 Rubin, E., Beattie, D.S. and Lieber, C.S. (1970) Lab. Invest. 23, 620–627
14 Schilling, R.J. and Reitz, R.C. (1979) Fed. Proc. 38, 377
15 Chappel, J.B. and Hansford, R.G. (1972) Subcellular Components: Preparation and Purification (Birnie, G., ed.), pp. 77–92, University Park Press, Baltimore
16 Thompson, J.A. and Reitz, R.C. (1976) Ann. N.Y. Acad. Sci. 273, 194–204
17 Greenawalt, J.W. (1974) Methods Enzymol. 31, 310–323
18 Tabor, C.W., Tabor, H. and Rosenthal, S.M. (1954) J. Biol. Chem. 208, 645–661
19 Smith, L. (1955) Methods Enzymol. 2, 732–740
20 Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158–175
21 Raijam, J. (1974) Biochem. J. 138, 225–232
22 Dreiling, C.E. and Mattson, C. (1980) Anal. Biochem. 102, 304–309
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–47
25 Kurihara, T., Nussbaum, J.L. and Mandel, P. (1970) J. Neurochem. 17, 993–997
26 Banik, N.L. and Davison, A.N. (1969) Biochem. J. 115, 1051–1061
27 Reitz, R.C., Thompson, J.A. and Morris, H.P. (1977) Cancer Res. 37, 561–567
28 Wood, R. (1973) Tumor Lipids: Biochemistry and Metabolism, Am. Oil Chem. Soc. Press, Champaign, IL

29 Burke, J.P., Tumbleson, M.E., Hicklin, K.W. and Wilson, R.B. (1975) Proc. Soc. Exp. Biol. Med. 148, 1051—1056
30 Bernstein, J.D. and Penniall, R. (1978) Alchim. Clin. Exp. Res. 2, 301—310
31 Banks, W.L., Kline, E.S. and Higgins, E.S. (1974) J. Nutr. 100, 581—594
32 Cederbaum, A.I., Lieber, C.S. and Toth, A. (1973) J. Biol. Chem. 248, 4977—4986
33 Schimke, R.T. (1977) Neurosciences Research Symposium Summaries (Schmitt, F.O., Adelman, G. and Worden, F.G., eds.), Vol. 8, pp. 54, MIT Press, Cambridge, MA
34 Roytta, M., Frey, H., Riekkinen, P. and Rinne, U.K. (1978) Adv. Exp. Med. Biol. 100, 569—583
35 Drummond, R.J., Hamill, E.B. and Guha, A. (1978) J. Neurochem. 34, 871—878
36 Thompson, J.A. and Reitz, R.C. (1979) Biochim. Biophys. Acta 545, 381—397
37 Porta, E.A., Bergman, B.J. and Stein, A.A. (1965) Am. J. Pathol. 46, 657—689
38 French, S.W. (1968) Gastroenterology 54, 1106—1114
39 Schilling, R.J. and Reitz, R.C. (1980) Biochim. Biophys. Acta, in the press