

BBA 72229

LIPID COMPOSITION OF LIVER PLASMA MEMBRANES FROM RATS INTOXICATED WITH CARBON TETRACHLORIDE

JOSE CAMACHO ^a and BOANERGES RUBALCAVA ^{a,b,*}

^a Departamento de Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, apartado Postal 14-740, 07000 Mexico D.F. (Mexico) and ^b Department of Chemistry, Cancer Research Center, Brigham Young University, Provo, UT 84602 (U.S.A.)

(Received February 6th, 1984)

Key words: Membrane fluidity; Liver regeneration; CCl₄ intoxication; Lipid composition

Rat liver plasma membranes were isolated from rats intoxicated acutely and chronically with carbon tetrachloride and a quantitative analysis of lipids was performed. Membranes from regenerating liver (acute intoxication) are characterized by a 60% drop in total phospholipids with a diminished phosphatidylcholine/phosphatidylethanolamine ratio (PC/PE) and a markedly decreased cholesterol level during the first 3 days after the intoxication, leading to a drastically decreased cholesterol/phospholipid (PL) ratio. In the chronically intoxicated rats (non-regenerating liver), although the phospholipids are diminished, phosphatidylcholine and phosphatidylethanolamine are equally decreased, and therefore the PC/PE ratio is not changed. Cholesterol is not diminished and, since the phospholipids are very low, the ratio cholesterol/PL is increased. These data could be correlated with the membrane fluidity. A decrease in the cholesterol/PL ratio results in a more fluid lipid matrix in the proliferative state of the cell. Treatment with colchicine during chronic intoxication prevented the increase in the cholesterol/PL ratio and improved the clinical conditions of the rats. The modulation of the cholesterol content could be a mechanism to control membrane fluidity during the different physiological states of the cell.

Introduction

For many years, liver regeneration in adult rats has been investigated in an attempt to understand the processes and factors that control cell proliferation. It is well documented that liver proliferation is regulated by several factors, including the pancreatic hormones glucagon and insulin [1]. For several years we have been studying the changes brought about by these hormones in the hepatotrophic function during the proliferative process.

This has been done in the fetal liver [2], after partial hepatectomy [3], after acute intoxication with carbon tetrachloride (CCl₄) [4] and in hepatomas [5].

In all the proliferative states cited above there exists a diminished glucagon binding which results in functional losses of glucagon-activated adenylate cyclase [6]. We found a positive correlation between the appearance of glucagon-stimulated adenylate cyclase activity and the cessation of hepatocyte proliferation over the prenatal and postnatal development [2]. There is evidence that cyclic AMP (cAMP) is an inhibitor of the transition G₁/S in the cellular cycle; therefore, if its levels are increased, the cells apparently are in G₀ [7]. It has been shown that accumulation of cAMP

* To whom correspondence should be addressed, at Brigham Young University.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid.

inhibits both in vivo and in vitro growth of normal and neoplastic cells [8] and that transformed cells contain less cAMP in vitro than cells which have not been transformed [9–11].

An experimental model of liver cirrhosis (chronic intoxication with CCl_4), showed a 2-fold elevation of adenylate cyclase basal activity, which in turn maintained very high cAMP levels. The receptors of the pancreatic hormones were not changed, and we postulated that in this particular case, maybe due to the high levels of cAMP, the liver was not regenerating [12]. Interestingly, colchicine prevented the alterations in the adenylate cyclase activity and cAMP liver content, and improved the clinical conditions of the rats.

It is apparent that liver participates in the regulation of circulating blood levels of hormones that are implicated in controlling hepatic proliferation, and that the proliferative liver suffers specific alterations at the level of the plasma membrane. The primary action of the pancreatic hormones resides in their interaction with specific receptors localized as integral proteins of the hepatocyte plasma membrane. For the glucagon receptors it has been shown that acidic phospholipids affect the hormone response and the interaction of the receptor with the nucleotide regulatory protein [13]. Also, the microviscosity (lipid membrane fluidity) has been implicated in the hormone response [14–16] and the importance of surface charge and hydrophobic interactions has been stressed as a significant regulatory mechanism of the glucagon adenylate cyclase system [17].

These factors led us to undertake the present study aimed at obtaining knowledge about the possible alterations in the lipid composition of the liver plasma membrane during both the acute (regenerating liver) and the chronic (non-regenerating liver) intoxication with carbon tetrachloride.

Materials and Methods

Wistar male rats weighing 200–250 g, fed a Purina Chow diet ad libitum, were used for the acute intoxication with CCl_4 . The treated animals received a single dose of 4 $\mu\text{l/g}$ body weight of CCl_4 in corn oil (1:1, v/v) through an intragastric tube. The control animals received only the same volume of corn oil. The rats were killed (at around

7–8 a.m.) on the days shown in the Results section.

For chronic intoxication we used Wistar male rats weighing 50–75 g initially and fed ad libitum a Purina Chow diet. The CCl_4 was administered intraperitoneally with the following scheme: 0.15 ml of CCl_4 in mineral oil three times a week for 7 weeks. The ratio CCl_4 /oil used was 1:7 in the 1st week, 1:6 in the 2nd week, 1:5 in the 3rd week and 1:4 in the remaining 4 weeks; therefore the dose of CCl_4 was always between 0.03 and 0.06 g/animal, and at the end of the treatment the rats weighed 200–250 g. The animals were divided into four groups. The first group received the CCl_4 treatment, and the second (control) received mineral oil alone. The third group consisted of rats which, in addition to the CCl_4 , received a daily dose of 10 μg of colchicine for 5 days per week for 7 weeks. The drug was dissolved in water and delivered into the stomach by an intragastric tube. The 4th group of animals received only colchicine as described for animals in the 3rd group. The rats were killed 4 or 5 days after the end of the treatment.

The livers from both acute and chronically intoxicated rats were excised to prepare plasma membranes.

Membrane preparation. Partially or highly purified plasma membranes were prepared according to the method of Neville [18] with the modification described by Pohl et al. [6]. Both preparations were used immediately or frozen in liquid nitrogen, since no changes were apparent with this treatment.

Extraction of lipids. The plasma membranes were suspended and washed twice with saline and centrifuged at 15 000 rpm (rotor SS-34). The pellet was suspended in the minimum volume of saline, 10% trichloroacetic acid was added and after 1 h at 4°C it was centrifuged at 700 \times g. The supernatant was discarded and the pellet resuspended in 1 vol. of methanol, heated to 55°C for 15 min, and, once it was cold, 2 vol of chloroform were added and left in the cold-room (4°C) overnight. The extract was then filtered through Whatman No. 1 filter paper. The filtrate was washed twice with 2 M KCl, the aqueous phase was discarded, and the solvent evaporated to dryness under a stream of nitrogen. The dried sample was im-

mediately dissolved in chloroform and frozen in liquid nitrogen.

Lipid analysis. Lipids were separated by thin-layer chromatography and quantitatively analyzed [19]. 10 μ l for phospholipids and 15 μ l for neutral lipids of the total extract from the plasma membranes were applied to plates (20 \times 20 cm) coated with 0.5 mm of silica gel and, for the quantitative analysis, also with ammonium sulphate. The chromatograms were developed with a solvent system of petroleum ether/diethyl ether/acetic acid, 90:10:1 (v/v), for neutral lipids and chloroform/methanol/acetic acid/water, 170:40:16:8 (v/v), for the phospholipids. The different lipids were identified using pure standards and well-known specific detection tests. For quantitative analysis, the thin-layer chromatography plates were heated immediately after being developed to 180°C for 30 min and read at 435 nm in a Schoeffler spectrodensitometer. Standard curves with different amounts of pure lipids were run and the values of the different lipids in the extract were interpolated to the standard curves. Free and esterified cholesterol were determined colorimetrically [20] and phospholipids in accord with the method by Bartlett [21].

Other measurements. Protein was measured by the procedure of Lowry et al. [22] using bovine serum albumin as standard.

The pure lipid standards were supplied by British Drug Houses Company and all gave only one spot by chromatography. [3 H]Glyceryltriolate was purchased from New England Nuclear. All other reagents were of analytical grade.

Results

Densitometric analysis

To test the densitometric analysis of the lipids we ran standard curves with different amounts of pure lipids. Fig. 1 shows one of these curves and it is clear that the response of the instrument is linear with the amount of lipids used in this study.

Lipid composition of liver membranes after acute intoxication with CCl_4

The phospholipid content of plasma liver membranes isolated from rats intoxicated acutely with CCl_4 is shown in Table I. The membranes, 24 and

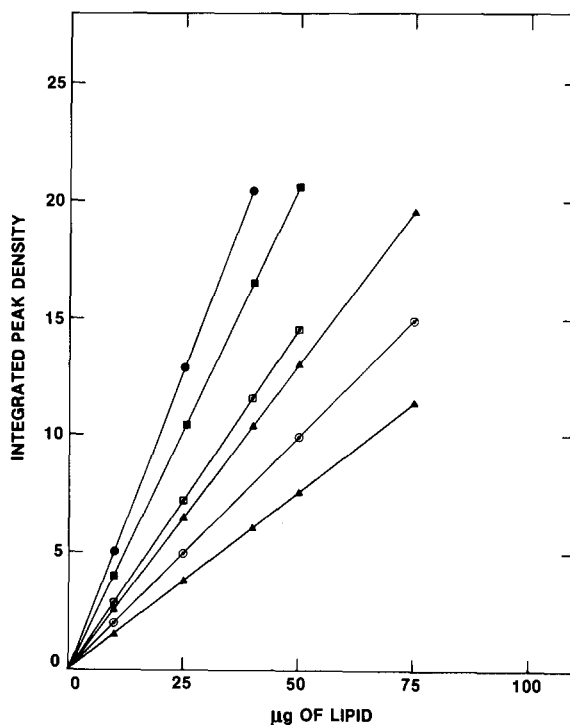


Fig. 1. Densitometric analysis of pure lipids. The amounts indicated were applied to a thin-layer chromatography plate, and after incubation at 180°C for 30 min, they were analyzed in a Schoeffler spectrodensitometer at 435 nm. The sensitivity was such that full scale was 1 unit of absorbance. ●, glyceryl trioleate; ■, phosphatidylinositol; □, phosphatidylcholine; Δ, phosphatidylethanolamine; ○, cholesterol; ▲, phosphatidylserine.

48 h after the administration of the toxic agent, were characterized by a 60% drop in total phospholipids, due primarily to a very significant decrease in the amount of phosphatidylcholine and sphingomyelin, although all the phospholipids were diminished. The ratio phosphatidylcholine/phosphatidylethanolamine (PC/PE) during the first 2 days of the intoxication was diminished (statistically significant) from the normal of 1.2 to 0.4. On the 3rd day of the treatment, the ratio was 3.5, since the phosphatidylethanolamine required one more day to recuperate to normal values.

Quantitative analysis of the neutral lipids in these membranes shows that cholesterol was severely diminished to 25% of its normal value during the first 3 days after intoxication (Table II). There was an apparent increase in the total lipids since the mono-, di- and triacylglycerols all increased; how-

TABLE I

COMPOSITION OF PHOSPHOLIPIDS OF LIVER PLASMA MEMBRANES FROM RATS AFTER ACUTE INTOXICATION WITH CARBON TETRACHLORIDE

The results ($\mu\text{g}/\mu\text{g}$ membrane protein) are expressed as the mean of at least six different experiments \pm S.D. and were obtained by densitometric analysis of lipid extracts from plasma liver membranes as described in Methods. Phosphorus determination in membranes and extracts showed over 95% recovery. The abbreviations used here and in Table IV are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine.

Days after CCl_4 intoxication	PC	PE	SPM	PI	PS	Total	PC/PE
0	0.75 ± 0.13	0.62 ± 0.08	0.34 ± 0.08	0.025 ± 0.007	0.018 ± 0.003	1.76	1.2
1	0.25 ± 0.02	0.35 ± 0.08	0.083 ± 0.008	0.022 ± 0.008	0.007 ± 0.002	0.71	0.7
2	0.22 ± 0.02	0.44 ± 0.11	0.10 ± 0.01	0.017 ± 0.006	0.006 ± 0.002	0.78	0.5
3	1.0 ± 0.25	0.28 ± 0.07	0.53 ± 0.12	0.016 ± 0.004	0.012 ± 0.003	1.83	3.5
4	0.82 ± 0.10	0.69 ± 0.03	0.42 ± 0.06	0.013 ± 0.004	0.014 ± 0.003	1.96	1.2
6	0.86 ± 0.11	0.64 ± 0.03	0.31 ± 0.07	0.022 ± 0.005	0.017 ± 0.004	1.83	1.3
10	0.86 ± 0.10	0.63 ± 0.03	0.31 ± 0.09	0.025 ± 0.008	0.018 ± 0.004	1.86	1.3
14	0.75 ± 0.17	0.58 ± 0.09	0.32 ± 0.04	0.020 ± 0.006	0.015 ± 0.003	1.69	1.2

ever, it is very difficult to accommodate this amount of triacylglycerols in the membrane. It is well known that in this type of intoxication steatosis in the liver exists [23] and, since the partially purified membranes used in the analysis depicted in Table II were obtained as a floated material, we thought this apparent increase in the acylglycerols might be due to a contamination. To disprove this, we went to the whole procedure of Neville [18] to obtain highly purified membranes. The membranes in this procedure are located at the interphase of the 50% sucrose cushion and the continuous gradient (1–24% sucrose). [^3H]Glyceroltrio-

leate was added to the homogenate and the radioactivity was followed through all steps of the purification. As shown in Table III, the highly purified plasma membranes obtained 1 and 2 days after the acute intoxication with CCl_4 contained approximately the same amounts of triacylglycerols as the normal membranes. Furthermore, the cholesterol amount was the same as in the partially purified membranes. Similar results were obtained for phospholipids (data not shown). These results indicate that the increase in the triacylglycerols shown in Table II is due to contamination. The small amount of triacylglycerols in the normal

TABLE II

COMPOSITION OF NEUTRAL LIPIDS OF LIVER PLASMA MEMBRANES FROM RATS AFTER ACUTE INTOXICATION WITH CARBON TETRACHLORIDE

The results ($\mu\text{g}/\mu\text{g}$ membrane protein) are expressed as the mean of at least six different experiments \pm S.D. and were obtained by densitometric analysis of lipid extracts from plasma liver membranes as described in Methods. Colorimetric determination of cholesterol in membranes and extracts showed over 90% recovery. The abbreviations used here and in Table V are: MG, monoacylglycerols; DG, diacylglycerols; FA, fatty acids; TRI, triacylglycerols; C, cholesterol; PL, total phospholipids.

Days after CCl_4 intoxication	MG	DG + FA	TRI	C	Total	C/PL
0	0.04 ± 0.008	0.14 ± 0.04	0.26 ± 0.06	0.96 ± 0.15	1.4	0.54
1	0.81 ± 0.01	0.18 ± 0.06	1.28 ± 0.16	0.24 ± 0.01	1.78	0.33
2	0.81 ± 0.01	0.17 ± 0.07	1.11 ± 0.18	0.25 ± 0.01	1.61	0.31
3	0.06 ± 0.01	0.16 ± 0.07	0.75 ± 0.14	0.26 ± 0.08	1.23	0.14
4	0.06 ± 0.01	0.15 ± 0.08	0.66 ± 0.11	0.72 ± 0.12	1.60	0.36
6	0.05 ± 0.01	0.13 ± 0.05	0.34 ± 0.01	0.84 ± 0.07	1.36	0.46
10	0.03 ± 0.01	0.12 ± 0.06	0.24 ± 0.09	0.95 ± 0.18	1.35	0.52
14	0.03 ± 0.01	0.09 ± 0.04	0.23 ± 0.06	0.98 ± 0.16	1.33	0.57

TABLE III

[³H]GLYCERYLTRIOLEATE RECUPERATION ON LIVER PLASMA MEMBRANE PREPARATION FROM RATS AFTER ACUTE INTOXICATION WITH CCl₄

[³H]Glyceryltriolate was added to the homogenate and the radioactivity was followed in the different fractions. A lipid extraction was made from the membrane preparations as described in Methods, and cholesterol (C) and triacylglycerol (TRI) were quantitatively analyzed by densitometry. PPM stands for partially purified plasma liver membranes, and HPM for highly purified membranes.

Days after CCl ₄ intoxication	Fractions	cpm ($\times 10^{-3}$)	Recuperation (%)	$\mu\text{g}/\mu\text{g}$ membrane protein	
				C	TRI
1	Homogenate	771	100		
	Particulate	381	49		
	PPM	330	42	0.24	1.26
	HPM	62	8	0.22	0.35
2	Homogenate	1751	100		
	Particulate	838	47		
	PPM	672	38	0.25	1.15
	HPM	152	8	0.23	0.26

TABLE IV

COMPOSITION OF PHOSPHOLIPIDS OF LIVER PLASMA MEMBRANES FROM RATS CHRONICALLY INTOXICATED WITH CARBON TETRACHLORIDE

The results ($\mu\text{g}/\mu\text{g}$ membrane protein) are expressed as the mean of at least four different experiments \pm S.D. and were obtained by densitometric analysis of lipid extracts from plasma liver membranes as described in Methods. The phosphorus determination performed in membranes and extracts showed over 95% recovery. See Table I for abbreviations.

Group	PC	PE	SPM	PI	PS	Total	PC/PE
Control	0.75 ± 0.13	0.62 ± 0.08	0.34 ± 0.08	0.025 ± 0.007	0.018 ± 0.003	1.7	1.2
CCl ₄	0.30 ± 0.16	0.23 ± 0.1	0.039 ± 0.01	0.015 ± 0.003	0.007 ± 0.002	0.58	1.3
CCl ₄ + colchicine	0.64 ± 0.09	0.38 ± 0.04	0.091 ± 0.008	0.033 ± 0.005	0.012 ± 0.002	1.15	1.7
Colchicine	1.28 ± 0.24	0.76 ± 0.07	0.13 ± 0.01	0.061 ± 0.009	0.028 ± 0.005	2.25	1.7

TABLE V

COMPOSITION OF NEUTRAL LIPIDS OF LIVER PLASMA MEMBRANES FROM RATS CHRONICALLY INTOXICATED WITH CARBON TETRACHLORIDE

The results ($\mu\text{g}/\mu\text{g}$ membrane protein) are expressed as the mean of at least four different experiments \pm S.D. and were obtained by densitometric analysis of lipid extracts from plasma liver membranes as described in Methods. Colorimetric determination of cholesterol in membranes and extracts showed over 90% recovery. See Table II for abbreviations.

Group	MG	DG + FA	TRI	C	Total	C/PL
Control	0.042 ± 0.007	0.15 ± 0.06	0.23 ± 0.08	0.98 ± 0.13	1.4	0.55
CCl ₄	0.039 ± 0.008	0.11 ± 0.03	0.27 ± 0.05	0.98 ± 0.14	1.4	1.6
CCl ₄ + colchicine	0.032 ± 0.009	0.11 ± 0.04	0.20 ± 0.05	0.65 ± 0.10	1.0	0.56
Colchicine	0.061 ± 0.009	0.15 ± 0.05	0.27 ± 0.06	0.45 ± 0.10	0.9	0.19

membranes and in the highly purified membranes is consistently found.

A very important characteristic of these membranes is that the ratio cholesterol/phospholipids (C/PL) was drastically decreased in the first 4 days after intoxication with CCl_4 (Table II) due to the low cholesterol values (statistically significant). This has been related to an increase in membrane fluidity [24]. It is important to emphasize that these changes occur precisely when the pancreatic hormone receptors and the adenylate cyclase activity are changed [4].

Lipid composition of the liver membranes after chronic intoxication with CCl_4

Analysis of plasma liver membrane phospholipids in the chronically intoxicated rats is shown in Table IV. The total phospholipids were diminished by 67% and both phosphatidylcholine and phosphatidylethanolamine were equally decreased. Therefore, the PC/PE ratio did not change. On the other hand, sphingomyelin was diminished by 89% and the acidic phospholipids also decreased. Colchicine treatment diminished the effect of CCl_4 on the phospholipids in the membranes. The total phospholipids did not return to normal values. This is due primarily to sphingomyelin, which remained 74% under control values, but it should be noted that colchicine alone decreased this phospholipid. With the colchicine treatment of intoxicated rats, phosphatidylcholine returned to its normal value (the small decrease is not statistically significant) while phosphatidylethanolamine was still 39% below. Therefore, the PC/PE ratio increased.

A striking difference in the neutral lipid analysis between the acute and the chronic intoxication was that in this latter case cholesterol was not diminished and, since the phospholipids were very low, the ratio C/PL increased to 1.6 from the normal of 0.55 or from the acute intoxication values of 0.14–0.3 (Table V). All of these changes were statistically significant. In the chronic intoxication the membrane fluidity should be drastically diminished [24] and, in a previous study, we reported a 2-fold increase in the adenylate cyclase basal activity in these membranes [12].

Discussion

Perhaps the most important change we observed in the lipid pattern during regeneration of the liver after acute intoxication with CCl_4 is the decrease in the ratio cholesterol/phospholipids (C/PL). This ratio decreases in the first 3 days after the intoxication, and returns to normal values on the 6th day. The decreased C/PL ratio has been observed also in the regeneration of the liver after partial hepatectomy and in hepatomas, and a correlation with an increase in the membrane fluidity was found [25]. In those proliferative states of the liver, as well as in this study, the decrease in the C/PL ratio is due to the low content of cholesterol in the membranes. Cholesterol is a regulator of membrane fluidity. If the membrane has a high content of unsaturated fatty acids, cholesterol diminishes the fluidity. If the saturated fatty acids are high, then cholesterol increases the membrane fluidity. In hepatocyte proliferation after *trans*-stilbeneoxide treatment, the C/PL ratio is diminished in the reticuloendoplasmic lipids, with an increase in the polyenoic fatty acids which could mean an increase in membrane fluidity [26].

Different processes in the cell, such as cell cycle [27], differentiation [28], growth [29] and transformation [30], are apparently accompanied by changes in membrane fluidity. Our study, as well as those cited, stresses the importance of an increase in the fluidity of the lipid bilayer in order for the proliferative state to be established. Therefore, modulation of the content of cholesterol could be a mechanism to control membrane fluidity during the different physiologic states of the cell. ($\text{Na}^+ + \text{K}^+$)-ATPase and adenylate cyclase, which are very important in regulation of cell growth by regulating ionic metabolism and cyclic nucleotides, are affected by the lipid composition and the membrane fluidity [13,31,32].

Furthermore, during chronic intoxication with CCl_4 , the C/PL ratio increases, which could be correlated with a decrease in the membrane fluidity; and we have shown [12] a 2-fold increase in the adenylate cyclase activity which, in turn, produces a permanent increase in the cAMP levels. The treatment with colchicine, which prevented these changes, also prevented the increase in the C/PL ratio.

By histological and chemical analysis, we have shown [12] the presence of liver cirrhosis in the chronically treated animals. Thick bands of collagen were apparent and liver collagen content was statistically higher in both the CCl_4 and CCl_4 -plus-colchicine groups. Since colchicine prevented the increase in adenylate cyclase activity and in C/PL ratio without preventing the increase in fibrous tissue, this suggests that we are dealing in our study with hepatocyte plasma membrane.

An increase of this ratio above 1.0 has been reported for dogs with a cholesterol-enriched diet and for men with liver disease [33]. The physical form of these membranes is not well understood. It seems that the excessive cholesterol is adsorbed in the lipid bilayer as crystals. This increased C/PL ratio could be further evidence that there is no regeneration in the cirrhotic rat. It would be important to study the cell cycle in hepatocytes coming from these cirrhotic rats, and also to measure the production of very-low-density lipoproteins (VLDL) which have been suggested as a hepatic chalone [34].

Differences in the synthesis of sphingolipids have been shown during the cell cycle [35] and an increase in the synthesis of these lipids apparently stops the cell in G_0 . Our data indicate that in the proliferative state there is a pronounced decrease in sphingomyelin, although this is also the case in the chronic intoxication.

Another distinctive change observed during the regeneration of the liver is the decreased PC/PE ratio. This change could be explained by a decreased production of choline and, in turn, the enzyme choline kinase would not work for lack of substrate. Sphingomyelin is also diminished and its synthesis requires CDPcholine. Choline-deficiency states promote hepatic DNA synthesis initiation concomitant to altered hormone and VLDL changes similar to those observed in the CCL_4 acute intoxicated rats [1,4]. An alternative explanation for the decreased PC/PE ratio could be a decreased activity of phosphatidylethanolamine methyltransferase. This enzyme is stimulated by glucagon, and the PC/PE ratio decreases at the same time that the binding of this hormone is practically abolished, and consequently the glucagon-stimulated adenylate cyclase activity would also be diminished [4]. Axelrod and coworkers [24]

proposed a mechanism for glucagon-stimulated adenylate cyclase activity based on the transformation of phosphatidylcholethanolamine to phosphatidylcholine by the methyltransferase enzyme. We are presently studying this ratio in connection with other proliferative states of the hepatocyte in order to gain a better understanding of its possible importance, but in this study we can see that during the chronic intoxication with CCl_4 , where some other parameters present during the regeneration of the liver do not exist, the PC/PE ratio does not change.

Acknowledgments

The authors wish to thank Drs. Roland K. Robins and Leo P. Vernon for critically reading the manuscript, Miss. Eunice Zavala and Mr. Agustin Martinez for excellent technical assistance, and Mrs. Merle Tanner-White for preparing the manuscript. This work was supported in part with Grant PCCBBNA 005072 to B.R. from CONACYT, Mexico.

References

- 1 Leffert, H.L., Kock, K.S. and Rubalcava, B. (1976) *Cancer Res.* 36, 4250–4255
- 2 Blazquez, E., Rubalcava, B., Montesano, R., Orci, L. and Unger, R.H. (1976) *Endocrinology* 98, 1014–1023
- 3 Leffert, H.L., Alexander, N.M., Faloona, G., Rubalcava, B. and Unger, R. (1975) *Proc. Natl. Acad. Sci. USA* 72, 4033–4036
- 4 Mourelle, M. and Rubalcava, B. (1981) *J. Biol. Chem.* 256, 1656–1660
- 5 Mourelle, M., Morris, H.P. and Rubalcava, B. (1980) *Toxicology* 15, 173–180
- 6 Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971) *J. Biol. Chem.* 256, 1849–1856
- 7 Pastan, I.H., Johnson, S. and Anderson, W.B. (1975) *Annu. Rev. Biochem.* 44, 491–522.
- 8 Cho-Chung, Y.S. (1979) in *Influence of Hormones on Tumor Development* (Kallen, J.A. and Hilf, R., eds.), pp. 55–93, CRC Press, Boca Raton.
- 9 Heidrick, M.L. and Ryan, W.L. (1971) *Cancer Res.* 31, 1313–1315
- 10 Otten, J., Johnson, G.S. and Pastan, I. (1971) *Biochem. Biophys. Res. Commun.* 44, 1192–1198.
- 11 Sheppard, J.R. (1972) *Nature, New Biol.* 236, 14–16
- 12 Mourelle, M., Rojkind, M. and Rubalcava, B. (1981) *Toxicology* 21, 213–222
- 13 Rubalcava, B. and Rodbell, M. (1973) *J. Biol. Chem.* 248, 3831–3837

- 14 Hanski, E., Rimon, G. and Levitzki, A. (1979) *Biochemistry* 18, 846–853
- 15 Houslay, M.D., Dipple, I., Rawal, S., Sauerheber, R.D., Esgate, J.A. and Gordon, L.M. (1980) *Biochem. J.* 190, 131–137.
- 16 Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) *J. Biol. Chem.* 255, 4519–4527
- 17 Rubalcava, B., Grajales, M.O., Cerbon, J. and Pliego, J.A. (1983) *Biochim. Biophys. Acta* 759, 243–249
- 18 Neville, D.F. (1968) *Biochim. Biophys. Acta* 154, 540–552
- 19 Skipski, V.P. and Barclay, M. (1969) *Methods Enzymol.* 14, 530–598
- 20 Abell, L.L., Levy, B.B., Brody, B.B. and Kendell, F.E. (1952) *J. Biol. Chem.* 195, 357–366
- 21 Bartlett, G.R. (1959) *J. Biol. Chem.* 234, 466–468
- 22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- 23 Stetten, D., Jr. and Salcedo, J. (1944) *J. Biol. Chem.* 156, 27–32
- 24 Hirata, F., Strittmatter, W.J. and Axelrod, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 368–372
- 25 Cheng, S. and Levy, D. (1979) *Arch. Biochem. Biophys.* 196, 424–429
- 26 Susuki, Y., Depierre, J.W. and Ernster, L. (1980) *Biochim. Biophys. Acta* 601, 532–543
- 27 De Laat, S.W., Van der Saag, P.T. and Shinitzki, M. (1977) *Proc. natl. Acad. Sci. USA* 74, 4458–4461
- 28 Kawasaki, Y., Wakayama, N., Koike, T., Kawai, M. and Amano, T. (1978) *Biochim. Biophys. Acta* 509, 440–449
- 29 Collard, J.G., DeWildt, A., Oomen-Meuelmans, E.P.M., Smeekens, J., Emmelot, P. and Inbar, M. (1977) *FEBS Lett.* 77, 173–178
- 30 Borochoy, H., Zahler, P., Wilbrandt, W. and Shinitzki, M. (1977) *Biochim. Biophys. Acta* 470, 382–388
- 31 Kemelberg, H.K. (1975) *Biochim. Biophys. Acta* 413, 143–156
- 32 Orly, J. and Schranem, M. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3433–3437
- 33 Collins, J.J. and Phillips, M.C. (1982) *J. Lipid Res.* 23, 291–298
- 34 Leffert, H.L., Koch, K.S., Moran, T. and Rubalcava, B. (1979) *Gastroenterology* 76, 1470–482
- 35 Hakomori, S. (1975) *Biochim. Biophys. Acta* 417, 55–89