Molecular and Cellular Biochemistry **154**: 77–82, 1996.
© 1996 *Kluwer Academic Publishers. Printed in the Netherlands.*

Vitamin A supplementation inhibits chemiluminescence and lipid peroxidation in isolated rat liver microsomes and mitochondria

A. Palacios, V.A. Piergiacomi and A. Catalá*

Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296, 1900, La Plata, Argentina

Received 29 April 1995; accepted 11 August 1995

Abstract

In the present study we investigated if administration of vitamin A could protect rat liver microsomes and mitochondria from *in vitro* peroxidation. Appreciable decrease of chemiluminescence and lipid peroxidation was measured in microsomal membranes from rats receiving vitamin A, with respect to control animals. In membranes derived from control animals, the fatty acid composition was profoundly modified when subjected to *in vitro* peroxidation mediated by ascorbate-Fe^{++}, with a considerable decrease of 20:4 n6 and 22:6 n3 in mitochondria and 18:2 n6 and 20:4 n6 in microsomes. As a consequence the peroxidizability index, a parameter based on the maximal rate of oxidation of specific fatty acids was higher in supplemented animals than in control group when both kind of membranes were analyzed. These changes were less pronounced in membranes derived from rats receiving vitamin A. These results are in agreement with previous results that indicated that vitamin A may act as an antioxidant protecting membranes from deleterious effects. (Mol Cell Biochem **154**: 77–82, 1996)

Key words: Vitamin A, rat liver, microsomes, mitochondria, peroxidation chemiluminescence

Abbreviations: BHT – butylated hydroxytoluene; BSA – bovine serum albumin; CL – chemiluminescence; PI – peroxidizability index

Introduction

Lipid peroxidation is one of the main events induced by oxidative stress [1] and is particularly active in those tissues whose membranes are rich in polyunsaturated fatty acids [2, 3]. As retinoids have been found to act effectively *in vitro* as antioxidants and radical scavengers [4], and considering that mammalian liver play a major role in long chain fatty acids and vitamin A metabolism [5, 6] and assay was carried out to see whether a vitamin A supplemented diet could modify the *in vitro* susceptibility of rat liver membranes to non-enzymatic lipid peroxidation. Vitamin A has been shown by other investigators to act as an antioxidant in many clinical situations, in our specific subject we have shown that vitamin A inhibits chemiluminescence and lipid peroxidation in rat liver microsomes and mitochondria. Thus to avoid artifacts and misinterpretations, we followed the degradation process by determining chemiluminescence [7] and evaluating the loss of specific fatty acids by the peroxidizability index [8] calculated from fatty acid composition determined by gas liquid chromatography. The results obtained in the present study show that rat liver microsomal and mitochondrial membranes are protected by vitamin A when subjected to non enzymatic lipoperoxidation.

*Member of Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas de la Republica Argentina
Address for offprints: A. Catalá, Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296, (1900) La Plata, Argentina

Materials and methods

All trans retinol palmitate, type IV and butylated hydroxy-toluene were from the Sigma Chemical Co. (St. Louis, MO). BSA (fraction V) were obtained from Wako Pure Chemicals Industries Ltd., Japan. Standards of fatty acids methyl esters were generously supplied by NU Chek Prep, Inc, Elysian, MN, USA. All other reagents and chemicals were of analytical grade from Sigma.

Animals and membrane preparation

Female Wistar AH/HOK rats 7 weeks old, weighing 120–137 g were used. Two groups of three rats were considered, and designated A (vitamin A-supplemented), and B (control). All rats were fed commercial rat chow and water *ad libitum*. Group A received one daily 0.39 g/kg body wt intraperitoneal injection of retinol palmitate, dissolved in 0.15 M NaCl, for 7 days. The eighth day all the rats were sacrificed by cervical dislocation and the liver was rapidly recovered, cut into small pieces and washed extensively with 0.15 M NaCl. A homogenate 30% (w/v) was prepared in 0.25 M sucrose solution, 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, using a potter-Elvejhem homogenizer. The homogenate was spun at 10,000 g for 10 min, 3 ml of the resultant supernatant was applied to a Sepharose 4B column (1,6 × 12 cm) equilibrated and eluted with 10 mM Tris-HCl (pH 7.4), 0.01% NaN_3. The microsomal fraction appearing in the void volume (10–16 ml) was brought to 0.25 M sucrose by addition of solid sucrose. All operations were performed at 4°C and under dim light. The quality of this microsomal preparation is similar in composition as regards concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [9]. Mitochondria was prepared as already described [10].

Peroxidation of microsomes and mitochondria

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to microsomes or mitochondria [7, 11–13]. Membranes at a concentration of 1–6 mg of protein were incubated at 37°C with 0.01 M phosphate buffer (pH 7.4), 0.4 mM ascorbate, final volume 2 ml. Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron for lipoperoxidation [13]. Membrane preparations which lacked ascorbate were carried out simultaneously. Chemiluminescence was measured as counts per min in a liquid scintillation analyzer Packard 1900 TR.

Measurement of fatty acid composition

Microsomal or mitochondrial lipids were extracted with chloroform/methanol (2:1 v/v) [14] from native or peroxidated membranes. Fatty acids were transmethylated with 5 % HCl in methanol at 80°C for 60 min. Fatty acid methyl esters were analyzed with a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a DB-225 megabore column (30 m × 0.53 mm i.d., J & V Scientific, Folson, CA, USA). Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250°C, the column temperature was held at 90°C for 1 min, 90–180°C at 15°C/min, 180–200°C at 3°C/min, 200–220°C at 3°C/min, 220°C for 7 min. Fatty acid methyl esters peaks were identified by comparison of retention times with those of standards.

Peroxidizability Index

Peroxidizability Index (PI) was calculating according to the formula [8, 15], PI = (per cent of monoenoic acids × 0.025) + (per cent of dienoic acids × 1) + (per cent of trienoic acids × 2) + (per cent of tetraenoic acids × 4) + (per cent of pentaenoic acids × 6) + (per cent of hexaenoic acids × 8).

Protein determination

Proteins were determined by the method of Lowry *et al.* [16] using BSA as standard.

Estimation of retinoids

For extraction of vitamin A compounds, fractions were gently mixed with 2 vol ethanol-BHT, followed by successive additions of hexane-BHT. After brief centrifugation the upper phase was withdrawn and concentrated under nitrogen and then dissolved in a final volume of 3 ml of hexane-BHT. Retinoid concentration was measured with a spectrophotometer Shimadzu at 330 nm using retinyl palmitate as standard [17].

Results

Vitamin A content in total homogenate and microsomal preparations were appreciably higher in group A rats than in control rats (Table 1). Microsomes showed a higher concentration of vitamin A than mitochondria in both group A and control animals. The total cpm/mg protein originated from chemiluminescence {measured after 60 min of incubation) resulting from the addition of ascorbic acid to rat liver

Table 1. Vitamin A concentration in homogenate, mitochondria and microsomes from vitamin A-supplemented and control rats

	μg Vitamin A/mg protein	
Fraction	Vitamin A-supplemented group	Control group
Homogenate	0.203 ± 0.0430	0.030 ± 0.0026*
Mitochondria	0.052 ± 0.0047	0.017 ± 0.0032*
Microsomes	0.241 ± 0.0300	0.070 ± 0.0041*

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between control and supplemented group are indicated by *. $p < 0.001$ using Student's *t* test.

Table 2. Total chemiluminescence (after 60 min incubation) of rat liver microsomes and mitochondria induced by ascorbate-Fe^{++}.

	cpm/mg protein	
Fraction	Vitamin A-supplemented group	Control group
Mitochondria	1,013,521.6 ± 275,879	629,690 ± 220,693
Microsomes	489,752.0 ± 14,598	712,524 ± 50,680*

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between control and supplemented group are indicated by *. $p < 0.01$ using Student's *t* test.

microsomes or mitochondria is shown in Table 2. Chemiluminescence of rat liver microsomes and mitochondria induced by ascorbate-Fe^{++} showed different results when the vitamin A-supplemented and the control group were compared. This is consistent with a considerable increase in maximal or peak induced CL of microsomal membranes isolated from control rats (Fig. 1A). No significant differences in maximal CL was observed when mitochondrial membranes from both groups were assayed (Fig. 1B). The fatty acid composition of total lipids in liver mitochondria of rats supplemented with vitamin A and control group is presented in Table 3. As compared with native mitochondria, in the peroxidized mitochondria, the levels of 20:4 n6 and 22:6 n3 were lower. The fatty acid composition of total lipids in liver microsomes of rats supplemented with vitamin A and control group is presented in Table 4. As compared with native microsomes, in the peroxidized microsomes, the levels of 20:4 n6 and 18:2 n6 were lower. In the vitamin A-supplemented group only the level of 20:4 n6 decreased significantly when compared with native microsomes. There were marked differences when the PI of native and peroxidized microsomes and mitochondria was compared. These changes were less pronounced in membranes derived from rats receiving vitamin A. Although vitamin A has no influence on the percentage distribution of fatty acid species with different amounts of double bonds isolated from mitochondrial or microsomal membranes from vitamin A and control group (Tables 3 and 4), lipoperoxidation in the presence of ascorbate-Fe^{++} evokes an important decrease in the relative content of

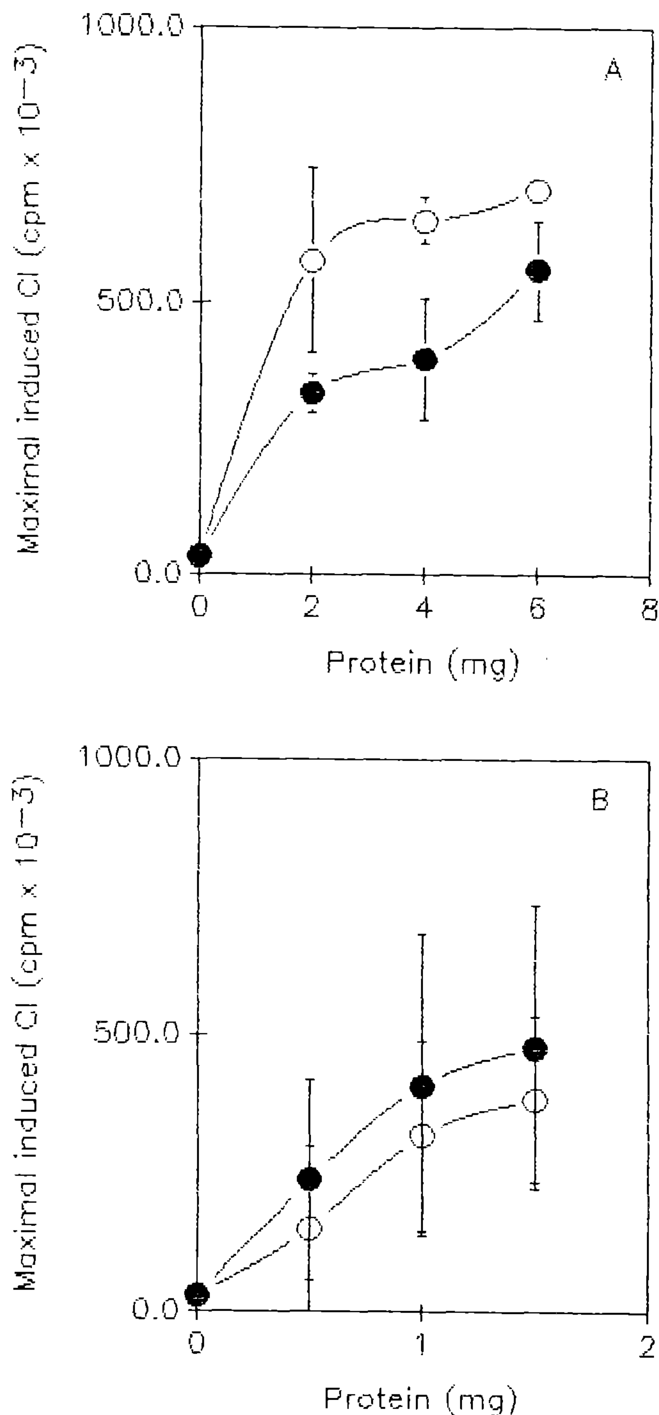

Fig. 1. Effect of treatment with vitamin A on ascorbate-Fe^{++} lipoperoxidation induced *in vitro*. Microsomal and mitochondrial membranes from control O---O and vitamin A supplemented group ●---●. A: microsomes, B: mitochondria. Each point is the mean value ± S.D. of three experiments.

the more polyunsaturated fatty acids. As a result the peroxidizability index of peroxidized membranes in the vitamin A group is significantly higher than in control group (Table 5).

Table 3. Fatty acid composition (Area %) of rat liver mitochondria native and peroxidized from vitamin A-supplemented and control group

	Vitamin A -supplemented group		Control group	
Fatty acid	Native	Peroxidized	Native	Peroxidized
16:0	14.977 ± 2.894	16.323 ± 3.692	12.983 ± 0.985	10.403 ± 3.110
16:1 (n–7)	tr	tr	tr	tr
18:0	27.173 ± 0.729	25.160 ± 0.679	27.190 ± 2.257	25.857 ± 2.851
18:1 (n–9)	5.223 ± 0.362	14.923 ± 5.167*	6.017 ± 2.298	19.413 ± 4.032*
18:2 (n–6)	14.937 ± 3.450	13.090 ± 0.335	14.527 ± 1.330	13.640 ± 2.750
18:3 (n–3)	1.017 ± 0.478	0.440 ± 0.762	0.393 ± 0.423	tr*
20:4 (n–6)	24.007 ± 3.136	15.607 ± 1.389*	23.593 ± 1.273	12.405 ± 3.758*
22:5 (n–3)	0.960 ± 0.125	1.090 ± 1.888	0.957 ± 0.261	tr*
22:6 (n–3)	7.453 ± 1.389	2.530 ± 2.207*	8.703 ± 0.456	tr*

Data are given as the mean ± S.D. of three experiments. Statistically significant differences in fatty acid concentration between native and peroxidized mitochondria are indicated by *. $p < 0.05$ using Student's *t* test.

Table 4. Fatty acid composition (Area %) of rat liver microsomes native and peroxidized from vitamin A-supplemented and control group

	Vitamin A-supplemented group		Control group	
Fatty acid	Native	Peroxidized	Native	Peroxidized
16:0	16.197 ± 5.638	20.970 ± 2.682	17.320 ± 2.548	20.183 ± 2.380
16:1 (n–7)	1.230 ± 2.130	tr	tr	tr
18:0	22.697 ± 2.786	20.033 ± 4.324	24.460 ± 5.365	16.640 ± 5.689
18:1 (n–9)	9.673 ± 2.803	15.933 ± 3.821*	8.730 ± 6.535	13.300 ± 5.352
18:2 (n–6)	8.900 ± 1.775	5.500 ± 3.688	11.773 ± 0.662	2.567 ± 1.476*
18:3 (n–3)	1.440 ± 0.292	0.643 ± 1.114	0.823 ± 0.446	tr
20:4 (n–6)	23.990 ± 4.791	7.703 ± 1.775*	19.490 ± 3.301	4.793 ± 1.472*
22:5 (n–3)	tr	tr	2.647 ± 2.231	tr
22:6 (n–3)	tr	tr	tr	tr

Data are given as the mean ± S.D. of three experiments. Statistically significant differences in fatty acid concentration between native and peroxidized microsomes are indicated by *. $p < 0.05$ using Student's *t* test.

Table 5. Peroxidizability index of native and peroxidized mitochondria and microsomes from vitamin A supplemented and control group

	Vitamin A-supplemented group		Control group	
Fraction	Native	Peroxidized	Native	Peroxidized
Mitochondria	192.09 ± 5.15	103.55 ± 12.56*	185.45 ± 8.06	58.41 ± 8.68*
Microsomes	121.44 ± 8.54	38.00 ± 11.70*	107.50 ± 2.26	22.07 ± 6.33*

Data are given as the mean ± S.D. of three experiments. Statistically significant differences in PI between native and peroxidized microsomes are indicated by *. $p < 0.001$ using Student's *t* test.

Discussion

Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation [18–21], it is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects of reactive oxygen species and other free radicals.

By virtue of its hydrophobicity, vitamin A can partition into lipid membranes, where it plays an antioxidant role [22, 23]. The possible mechanism of protection by vitamin A against peroxidation and the function of retinoids as antioxidants and effective radical scavengers when assayed in membrane preparations *in vivo* has been already described [24–27). To further investigate this effect we analyzed if administration of vitamin A could protect rat liver microsomes or mitochondria from *in vivo* peroxidation. Several studies have shown that membranes enriched with vitamin A are protected against oxidative stress occurring *in vivo*, and exhibit resistance to lipid peroxidation induced *in vitro* [28, 29]. Previous investigations from our laboratory have shown that the fatty acid composition of rat

liver microsomes is modified after non-enzymatic peroxidation in the presence of ascorbate-Fe^{++} [30]. As a result of the relative decrease in fatty acid unsaturation of peroxidized microsomes, the PI, a parameter based on the maximal rate of oxidation *in vitro* of specific fatty acids, decreased a 60% compared to native microsomes. A higher chemiluminescence in mitochondria than in microsomes could be due to the concentration of vitamin A in both membranes; thus the content of vitamin A was 4.6 and 4.1 times higher in microsomes than in mitochondria when the supplemented and control group were compared.

In the present studies we have determined that either rat liver mitochondria or microsomes obtained from supplemented vitamin A rats are protected against lipoperoxidation when compared with similar membranes obtained from control rats. In our experimental conditions we did not find any changes in fatty acid composition of rat liver mitochondria and microsomes of rats treated with vitamin A when compared with control group. In a previous study it has been demonstrated that the fatty acid composition, both of liver retinyl esters and of the total liver lipids can be significantly altered by high intake of vitamin A [31], with an increase of the ratios of more unsaturated to less unsaturated fatty acids while those of elongated to unelongated fatty acids fell. One possible explanation for this discrepancy between fatty acid composition in our study and those of Furr and Olson may be the time and amount of vitamin provided to the animals as explained by Baker *et al.* [32].

Ciaccio *et al.* [29] have recently demonstrated that brain and heart membrane preparations from rats receiving vitamin A, assayed *in vitro* in the presence of an ascorbate-Fe^{++} induction system showed a delay at the beginning of the lipid peroxidation and generated lesser amounts of TBARS, with respect to membranes from control rats. Our results and those reported previously [29] are consistent with the hypothesis that vitamin A may act as a physiological antioxidant in cell membranes where it is localized. However, further studies are needed to more adequately evaluate these observations.

Acknowledgements

We wish to thank Med. Vet. Miguel Ayala for providing the animals used in this study and Med. Vet. Santiago Corva for performing statistical analysis. This research was supported in part by the Japan International Cooperation Agency (JICA).

References

1. Di Mascio P, Murphy ME, Sies H: Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. Ann J Clin Nutr 53: 194S–200S, 1991
2. Dix TA, Aikens J: Mechanisms and biological relevance of lipid peroxidation initiation. Chem Res Toxicol 6: 2–18, 1993
3. Monahan FJ, Gray JI, Asghar A, Haug A, Strasburg GM, Buckley DJ, Morrissey PA: Influence of diet on lipid oxidation and membrane structure in porcine muscle microsomes. J Agric Food Chem 42: 59–63,1994
4. Halevy O, Sklan D: Inhibition of arachidonic acid oxidation by β-carotene, retinol and α tocopherol. Biochim Biophys Acta 918: 304–307, 1987
5. Catalá A: Interaction of fatty acids, acyl CoA derivatives and retinoids with microsomal membranes: effect of cytosolic proteins. Mol Cell Biochem 120: 89–94, 1993
6. Blomhoff R, Green MH, Green JB, Berg T, Norum KR: Vitamin A metabolism: New perspectives on absorption, transport, and storage. Physiol Rev 71: 951–990, 1991
7. Puntarulo S, Cederbaum AI: Stimulation of microsomal chemiluminescence by ferritin. Biochim Biophys Acta 1157: 1–8, 1993
8. Witing LA, Horwitt MK: Effect of degree of fatty acid unsaturation in tocopherol deficiency-induced creatinuria. J Nutr 82: 19–25, 1964
9. Tangen O, Jonsson J, Orrenius, S: Isolation of rat liver microsomes by gel filtration chromatography. Anal Biochem 54: 597–603, 1973
10. Schneider WC, Hogeboom HG: Intracellular distribution of enzymes. Further studies and distribution of cytochrome c in rat liver homogenates. J Biol Chem 178: 123–128, 1950
11. Cervato G, Viani P, Gatti P, Cestero B: Studies on lipid peroxidation in different model membranes: role of cysteamine. Chem Phys Lipids 62: 31–38, 1992
12. Ohyashiki T, Sakata N, Mohri T, Matsui K: Fluorescence characteristics of peroxidation products in porcine intestinal brush-border membranes. Arch Biochem Biophys 284: 375–380, 1991
13. Wright JR, Rumbaugh RC, Colby HD, Miles PR: The relation between chemiluminescence and lipid peroxidation in rat hepatic microsomes Arch Biochem Biophys 192: 344–351, 1979
14. Folch J, Lees M, Sloane Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497–509, 1957
15. Pirozhkov SV, Eskelson CD, Watson RR, Hunter GC, Piotrowski JJ, Bernhard V: Effect of chronic consumption of ethanol and vitamin E on fatty acid composition and lipid peroxidation in rat heart tissue. Alcohol 9: 329–334, 1992
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–275, 1951
17. Goodman DS, Huang HS, Shiratori T: Tissue distribution and metabolism of newly absorbed vitamin A in the rat. J Lipid Res 6: 390–396, 1965
18. Rice-Evans C, Hochstein P: Alterations in erythrocyte membrane fluidity by phenylhydrazine-induced peroxidation of lipids. Biochem Biophys Res Commun 100: 1537–1542, 1981
19. Vladimirov YA, Olenev VI, Suslova TB, Cheremisina ZP: Lipid peroxidation in mitochondrial membrane. Adv Lipid Res 17: 173–249, 1980
20. Richter C: Biophysical consequences of lipid peroxidation in membranes. Chem Phys Lipids 44: 175–189, 1987
21. Kagan VE: Lipid Peroxidation in Biomembranes. CRC Press, Boca Raton, FL, 1988, pp. 55–146
22. Blomhoff R, Green MH, Norum KR: Vitamin A: Physiological and biochemical processing. Ann Rev Nutr 12: 37–57, 1992
23. Biesalsky HK: Comparative assessment of the toxicology of vitamin A and retinoids in man. Toxicology 57: 117–161, 1989
24. Frei B: Reactive oxygen species and antioxidant vitamins: mechanisms of action. Amer J Med 97: 5–10, 1994
25. Bonorden WR, Pariza MW: Antioxidant nutrients and protection from free radicals. In: Frank N. Kotsonis (ed.) Nutritional Toxicology.

Raven Press, 1994, pp. 19–45
26. Palmer S: Antioxidant vitamins and cancer risk. Nutr 10: 433–434,1994
27. Olson JA: Carotenoids and vitamin A: an overview. In: Ong and Packer (eds) Biochemical and Clinical Applications, 1992, pp. 178–192
28. Alam SQ, Alam BS: Lipid peroxide α-tocopherol and retinoid levels in plasma and liver of rats fed diets containing β carotene and 1-3-cis-retinoic acid. J Nutr 113: 2608–2614, 1983
29. Ciaccio M, Valenza M, Tesoriere L, Bongiorno A, Albiero R, Livrea MA: Vitamin A inhibits doxorubicin-induced membrane lipid peroxidation in rat tissues *in vivo*. Arch Biochem Biophys 302: 103–108, 1993
30. Catalá A, Arcemis C, Cerruti C: Interaction of rat liver microsomes containing saturated or unsaturated fatty acids with fatty acid binding protein: Peroxidation effect. Mol Cell Biochem 137: 135–139, 1994
31. Furr HC, Olson JA: The effect of high vitamin A intake on fatty acid composition of rat liver retinyl esters and total liver fatty acids. Nutr Res 9: 395–403, 1989
32. Baker HJ, Russell-Lindsey J, Weisbroth H: The Biology of the Laboratory Rats, Vol I. Academic Press, New York, 1979, Ch. 6