

Intraorganellar localization of CoASH-independent phytanic acid oxidation in human liver peroxisomes

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In human tissues phytanic acid is α -oxidized to pristanic acid in peroxisomes. Studies of the intraorganellar site of α -oxidation of [$1\text{-}^{14}\text{C}$]phytanic acid to pristanic acid in peroxisomes isolated from human liver demonstrate that phytanoyl-CoA ligase is present in the peroxisomal membrane and that the enzyme system for α -oxidation of phytanic acid to pristanic acid is in the peroxisomal matrix. In contrast to the β -oxidation system for fatty acids, the substrate for α -oxidation is free phytanic acid. The studies described in this manuscript report a novel fatty acid oxidation system where the substrate for the enzyme system is free fatty acid; however, phytanoyl-CoA ligase regulates the α -oxidation of phytanic acid at the organellar (peroxisomal) level.

Peroxisome; Phytanic acid; Phytanoyl-CoA ligase; Phytanic acid α -oxidation

1. INTRODUCTION

The daily human diet is estimated to contain 50–100 mg of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a highly branched fatty acid [1] and excessive amounts of phytanic acid accumulate in a number of neurological diseases [2]. The fact that only trace amounts of phytanic acid are present in normal tissues in spite of large consumption (50–100 mg/day) suggests that there must be an efficient catabolic pathway which disposes of the ingested phytanic acid. Due to the presence of a methyl group at the β -carbon of phytanic acid it first undergoes α -oxidation to pristanic acid followed by further degradation by β -oxidation [1]. Recent studies with purified subcellular organelles suggest that peroxisomes are the major site of α -oxidation of phytanic acid to pristanic acid in human tissues [3,4]. The specific activity for α -oxidation of phytanic acid in isolated peroxisomes from human cultured skin fibroblasts was 29- and 124-fold higher than in mitochondria and microsomes [3,4]. The excessive accumulation of phytanic acid, as the result of a defect in α -oxidation of phytanic acid, in patients with diseases with a defect in the biogenesis of peroxisomes (e.g. Zellweger Syndrome) also suggests that peroxisomes may be the site for α -oxidation of phytanic acid [1,2,5]. The deficient α -oxidation of phytanic acid only in peroxisomes from peroxisomal disorders, classical Refsum Disease [6] and Rhizomelic Chondrodysplasia Punctata [4], as compared to the normal activities in mitochondria and microsomes from patients with Refsum Disease, Zellweger Syndrome and Rhizomelic Chondrodysplasia Punctata support the

conclusion that in humans the peroxisome is the primary site of α -oxidation of phytanic acid to pristanic acid.

The activation of phytanic acid to phytanoyl-CoA is prerequisite for its α -oxidation [3,4,6,7]. Therefore, α -oxidation of phytanic acid to pristanic acid constitutes four steps: activation of phytanic acid to phytanoyl-CoA, α -hydroxylation of phytanic acid to α -hydroxyphytanic acid, formation of 2-oxophytanic acid, and finally decarboxylation to pristanic acid and CO_2 [1,8,9]. As peroxisomes are the major site of α -oxidation of phytanic acid in human tissues, we examined the intraperoxisomal distribution of enzyme activities for activation (phytanoyl-CoA ligase) and α -oxidation of phytanic acid. These studies demonstrate that phytanoyl-CoA ligase is localized in the peroxisomal membrane and that the enzyme system for α -oxidation of phytanic acid is present in the matrix of peroxisomes. The activation of phytanic acid to phytanoyl-CoA was required for its α -oxidation only in intact peroxisomes but not in isolated matrix or permeabilized peroxisomes. This suggests that phytanic acid is the true substrate. The activation of phytanic acid to phytanoyl-CoA may be required for the transport of phytanic acid through the peroxisomal membrane for its α -oxidation in the matrix.

2. MATERIALS AND METHODS

Nycodenz was obtained from Accurate Chemical and Scientific Corp., Westbury, NY; ATP and CoASH were purchased from P-L Biochemicals, Milwaukee, WI; Desulfo-CoA, naproxen and digitonin were purchased from Sigma Chemical Co., St. Louis, MO; Triton X-100 was purchased from Bio-Rad Laboratories, Melville, NY; [$1\text{-}^{14}\text{C}$]phytanic acid (55mCi/mmol) was purchased from Amersham

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International, Arlington Heights, IL.; and [$1\text{-}^{14}\text{C}$]phytanoyl CoA was synthesized as described [10].

Peroxisomes from human liver were isolated according to the procedures described previously [11,12]. The fractions containing the catalase peak were pooled and dialyzed against the homogenization buffer [3,4,12] for 2 h to lower the concentration of nycodenz. Peroxisomal integrity was measured by the latency of catalase activity, a matrix enzyme. Total catalase activity was measured by diluting peroxisomes 1:1 with 2% of Triton X-100. The free catalase activity was measured by suspending peroxisomes in homogenization buffer in the absence of Triton X-100. The percent total catalase activity minus percent free catalase is an index of peroxisomal integrity. To separate the membrane and matrix of peroxisomes, peroxisomes were incubated with digitonin (0.2 mg/ml) for 1 h at 4°C and then centrifuged at 50,000 rpm for 1 h in a Beckman 70 Ti rotor. Separation of membrane (residue) and matrix (soluble) was confirmed by measuring catalase activity. Catalase activity was present only in the matrix, and not in the membranes.

The oxidation of phytanic acid to pristanic acid was measured as $^{14}\text{CO}_2$ released from [$1\text{-}^{14}\text{C}$]phytanic acid according to the method described previously [3,4,6]. Briefly, the reaction volume of 0.25 ml contained 12 μM [$1\text{-}^{14}\text{C}$]phytanic acid, 30 mM KCl, 5 mM MgCl_2 , 50 μM CoASH, 10 mM ATP, 0.25 mM NADPH, 0.17 mM FAD, and 20 mM MOPS-HCl, pH 7.8 in 0.25 M sucrose. The reaction was started by the addition of 20–50 μg of protein and stopped with 50 μl of 5 N H_2SO_4 after 2 h. $^{14}\text{CO}_2$ was collected in KOH-wetted cotton by shaking over night and then radioactivity was measured.

Phytanoyl CoA ligase activity was measured as mentioned earlier [13]. Briefly, the reaction mixture of 0.5 ml contained 12 μM [$1\text{-}^{14}\text{C}$]phytanic acid, 50 mM KCl, 5 mM MgCl_2 , 50 μM CoASH, 10 mM ATP, and 30 mM MOPS-HCl buffer, pH 7.8.

The transport of phytanoyl-CoA into peroxisomes was studied as described previously [12]. Briefly, the peroxisomes were incubated with [$1\text{-}^{14}\text{C}$]phytanoyl-CoA under isotonic conditions for 10 min, they were then separated from the incubation medium by centrifuging through an organic layer of brominated hydrocarbons into an aqueous layer at the bottom of the tube containing 0.396 M sucrose, 1 mM EDTA in 3 mM imidazole buffer, pH 7.4, as cushion. The radioactivity in the bottom layer (cushion) containing the sedimented peroxisomes was measured as an index of phytanoyl-CoA transported into peroxisomes. The radioactivity that migrated through organic layer with the disrupted peroxisomes was used as the blank [12]. Protein concentrations were measured by the method of Bradford [14].

3. RESULTS AND DISCUSSION

Recently we have demonstrated that in human tissues phytanic acid is α -oxidized to pristanic acid in peroxisomes [3,4,6]. The activation of phytanic acid to phytanoyl-CoA by phytanoyl-CoA ligase is an initial and obligatory step in its α -oxidation in peroxisomes [3,4,7]. The phytanoyl-CoA ligase present in peroxisomes is a different enzyme than the other known acyl-CoA ligases such as palmitoyl-CoA ligase and lignoceroyl-CoA ligase [7]. The intraperoxisomal identification of the enzyme system for α -oxidation of phytanic acid is necessary for understanding the nature of the associated enzyme system and the molecular mechanism of the inherited defects in the degradation of phytanic acid in Refsum Disease, Rhizomelic chondrodysplasia punctata and other peroxisomal diseases.

Peroxisomes are made up of a granular matrix surrounded by a single limiting membrane. The matrix proteins are released as soluble proteins as soon as the

Table I

Activation and oxidation of phytanic acid by intact peroxisomes and membrane and matrix of peroxisomes isolated from human liver

Sets	Naproxen (50 μM)	Activation (nmol/h/mg/ protein)	Oxidation (pmol/h/mg/ protein)	
			Phytanic acid	Phytanoyl CoA
Peroxisomes	–	13.34 ± 1.77	35.53 ± 4.08	36.23 ± 3.94
	+	3.08 ± 0.21	9.31 ± 2.16	33.35 ± 2.50
Membrane	–	19.86 ± 2.55	1.36 ± 0.61	0.97 ± 0.26
	+	4.46 ± 0.75	1.07 ± 0.35	0.66 ± 0.15
Matrix	–	0	17.48 ± 2.25	16.85 ± 2.60
	+	0	16.20 ± 1.92	15.72 ± 2.15

The enzyme activities were measured as described in the text. The results are expressed as mean \pm S.D. of duplicate values from at least three different preparations.

peroxisomal limiting membrane is disrupted [15]. To understand the intraperoxisomal organization of the enzyme system for α -oxidation of phytanic acid, the enzyme activities for the activation of phytanic acid by phytanoyl-CoA ligase and α -oxidation of phytanic acid was studied in highly purified intact peroxisomes (over 95% purity) [11,12] and in the matrix and membranes isolated following digitonin treatment of the purified peroxisomes and digitonin permeabilized peroxisomes as described in Section 2. The enzyme activity for the activation of phytanic acid by phytanoyl-CoA ligase was observed in the peroxisomal membrane, but not the matrix proteins, whereas the enzyme activity for α -oxidation of phytanic acid or phytanoyl-CoA to pristanic acid was found in the peroxisomal matrix proteins (Table I). The rate of α -oxidation of phytanic acid was 13-times higher in the matrix (17.48 ± 2.25 pmol/h/mg protein) than that in the peroxisomal membranes (1.36 pmol/h/mg protein) (Table I). These results suggest that similar to the fatty acid β -oxidation system, phytanic acid is activated in peroxisomal membranes and then oxidized to pristanic acid in the matrix of peroxisomes. Consistent with previous observations, Naproxen, an inhibitor of acyl-CoA ligases [16], inhibited the α -oxidation of phytanic acid and not of phytanoyl-CoA in intact peroxisomes, again suggesting that biosynthesis of phytanoyl-CoA is a necessary and intermediary step in the α -oxidation of phytanic acid in intact peroxisomes. However, surprisingly, we observed that free phytanic acid can be oxidized in the matrix (17.48 ± 2.25 pmol/h/mg protein) as efficiently as phytanoyl-CoA (16.85 ± 2.60 pmol/h/mg protein) suggesting that phytanic acid may be the substrate for α -oxidase in the matrix (Table I). The inability of Naproxen to inhibit α -oxidation of free phytanic acid in the isolated matrix proteins also supports the conclusion that free phytanic acid may be the substrate for α -oxidation enzyme system. We observed that 90% of the phytanoyl-CoA was hydrolyzed to free phytanic acid, in

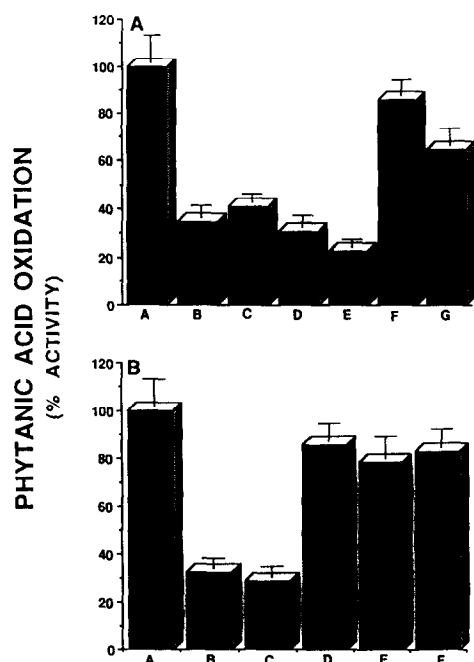


Fig. 1. (A) Effect of cofactors and metal ions on the rate of phytanic acid α -oxidation in peroxisomes (A, complete medium containing ATP, CoASH and $MgCl_2$; B, minus CoASH; C, minus $MgCl_2$; D, minus ATP; E, minus CoASH, $MgCl_2$ and ATP; F, Complete medium plus digitonin; G, minus CoASH, $MgCl_2$ and ATP but plus digitonin). Peroxisomes were incubated with digitonin (0.1 mg/ml) for 1 h at 4°C. 100% activity for phytanic acid oxidation was 35.53 ± 4.08 pmol/h/mg protein. (B) Effect of naproxen and desulfo CoA on phytanic acid α -oxidation by peroxisomes. Phytanic acid oxidation in peroxisomes isolated from human liver was measured (A, control peroxisomes; B, with naproxen; C, with desulfo CoA; D, with digitonin; E, with digitonin and naproxen; F, with digitonin and desulfo CoA) as described previously [5,6,9]. Peroxisomes suspended in homogenization buffer were preincubated with either 50 μM naproxen or 50 μM desulfo CoA and where indicated along with Digitonin (0.1 mg/ml) for 1 h at 4°C. 100% activity for phytanic acid oxidation was 35.53 ± 4.08 pmol/h/mg protein.

assay conditions when phytanoyl-CoA was incubated with matrix protein, by acyl-CoA hydrolase present in peroxisomes [12] suggesting that free phytanic acid may be the true substrate for oxidation of phytanic acid in the matrix of peroxisomes. We also examined the α -oxidation of phytanic acid in conditions in which the cofactors and metal ions required for the activation of fatty acids were deleted or substituted by a nonfunctional cofactor (e.g. desulfo-CoA) in intact peroxisomes and peroxisomes permeabilized with digitonin (Fig. 1). The α -oxidation of phytanic acid was inhibited by deletion of individual or all cofactors required for the activation of phytanic acid to phytanoyl-CoA only in intact peroxisomes but not in the digitonin permeabilized peroxisomes (Fig. 1A). Similarly, the substitution of CoASH by desulfo-CoA, an analog of CoASH which does not support activation of fatty acids, inhibits α -oxidation of phytanic acid only in intact peroxisomes but not in permeabilized peroxisomes (Fig. 1B). The observed activity (20–25%) of phytanic acid α -oxidation in

intact peroxisomal preparation in the absence of fatty acid activating cofactors or in the presence of desulfo-CoA was proportional to the percent of broken peroxisomes in these conditions. These studies also support the conclusion that phytanic acid, and not phytanoyl-CoA, is the true substrate for the phytanic acid α -oxidation system. The permeabilization of peroxisomes with digitonin treatment of peroxisomes inhibited the α -oxidation of phytanic acid by approximately 15% (Fig. 1). The loss of activity in isolated matrix (17.48 ± 2.25 pmol/h/mg protein) as compared to intact peroxisomes (35.53 ± 4.08 pmol/h/mg protein) and permeabilized peroxisomes (29.36 ± 3.87 pmol/h/mg protein) may be due to the inactivation of the enzyme during the isolation of membrane and matrix as the addition of membranes to the matrix fraction did not result in an increase in activity (data not shown here).

To further establish that free phytanic acid is a true substrate for α -oxidation, we examined the status of phytanoyl-CoA transported into intact peroxisomes and its rate of α -oxidation (Fig. 2). The transport of phytanoyl-CoA into intact peroxisomes was studied as described in Section 2, and the status of phytanoyl-CoA in peroxisomes was studied by Dole partition [17] of the radioactive fatty acid after transport assay into aqueous phase (phytanoyl-CoA) and organic phase (free phytanic acid). As shown in Fig. 2, at zero time 97% of the fatty acid transported into peroxisomes was phytanoyl-CoA. However, phytanoyl-CoA was quickly hydrolyzed to free phytanic acid in intact peroxisomes (Fig. 2). Thirty minutes following its transport into peroxisomes 68% of the phytanoyl-CoA was hydrolyzed to free phytanic acid, and 99% of phytanoyl-CoA was hydrolyzed at 60 min. These results suggest that peroxisomes have a very active phytanoyl-CoA hydrolase which quickly degrades phytanoyl-CoA to phytanic acid. In a parallel experiment, the rate of α -oxidation of phytanic acid, transported into peroxisomes, was determined at different time intervals after the transport assay. The rate of α -oxidation of phytanic acid into peroxisomes was linear up to 3 h, very much later than the time when phytanoyl-CoA is hydrolyzed to free phytanic acid (Fig. 2). The fact that phytanoyl-CoA is quickly hydrolyzed to free phytanic acid in intact peroxisomes and production of $^{14}CO_2$ continues even at times when 99% of the transported phytanoyl-CoA was hydrolyzed to free phytanic acid supports the conclusion that free phytanic acid is the true substrate for its α -oxidation in peroxisomes.

Phytanoyl-CoA ligase is an enzyme distinct from the other known acyl-CoA ligases such as palmitoyl-CoA ligase and lignoceroyl-CoA ligase which are present in the peroxisomal membrane [7,18]. The higher specific activities of phytanoyl-CoA ligase and phytanic acid α -oxidation in peroxisomes from human tissues and in mitochondria from rat tissues suggests that subcellular distribution of phytanoyl-CoA ligase controls the site of

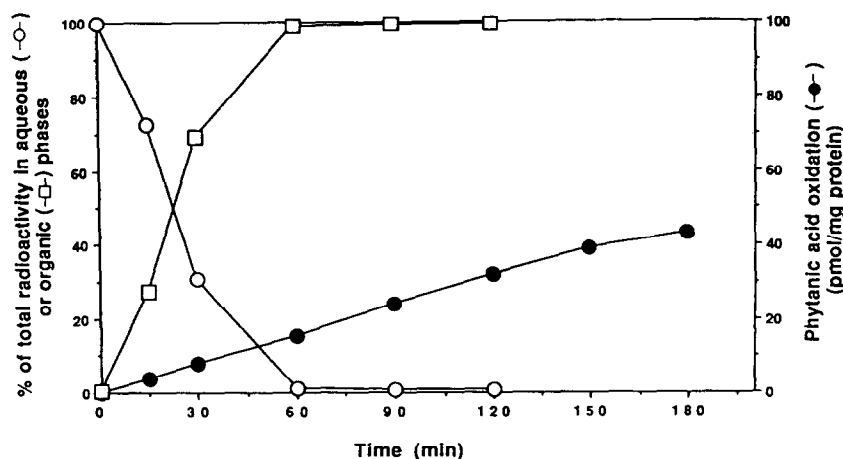


Fig. 2. The status of phytanoyl-CoA following transport into peroxisomes and its α -oxidation. Peroxisomes were incubated with [1- 14 C]phytanoyl-CoA for 10 min and transport of phytanoyl-CoA into peroxisomes was measured by separating the peroxisomes from the incubating medium by centrifuging through the organic layer into an aqueous cushion at the bottom of the tube [12]. The radioactivity in the bottom aqueous layer containing peroxisomes was measured as the phytanoyl-CoA transported into peroxisomes. The status of phytanoyl-CoA, transported into peroxisomes, was measured by Dole partition [17] of radioactivity (fatty acid) in peroxisomes into aqueous (phytanoyl-CoA, ○-○) and organic (free phytanic acid, □-□) phases after different time intervals following the transport assay. The 100% activity of phytanoyl-CoA into peroxisomes, at 0 time in the bottom aqueous phase, was 8490 dpm. In a parallel experiment the rate of formation of 14 CO $_2$ (●-●) was measured as an index of α -oxidation of phytanic acid in peroxisomes from the bottom aqueous layer at different time periods following the separation of peroxisomes from the medium by centrifuging through the organic layer in the transport assay [12].

phytanic acid oxidation by providing the phytanoyl-CoA for oxidation. The strict requirement for activation of phytanic acid to phytanoyl-CoA for α -oxidation in intact peroxisomes, but not in permeabilized peroxisomes or isolated matrix, clearly demonstrates that the function of phytanoyl-CoA ligase in peroxisomal membrane may be to synthesize phytanoyl-CoA for its transport into peroxisomes. This implies that the active site of phytanoyl-CoA ligase is on the cytoplasmic surface of the peroxisomal membrane and that the phytanoyl-CoA synthesized on the cytoplasmic surface enters the peroxisomal matrix where it is hydrolyzed to free phytanic acid and then α -oxidized to pristanic acid. The active site of palmitoyl-CoA ligase is on the cytoplasmic surface of the peroxisomal membrane and that of lignoceroyl-CoA ligase on the luminal surface [18]. Consistent with the topology of the active site of palmitoyl-CoA and lignoceroyl-CoA ligases in the peroxisomal membrane, palmitoyl-CoA is synthesized on the cytoplasmic surface and transported as palmitoyl-CoA, whereas lignoceric acid is transported into peroxisomes as such and then activated to lignoceroyl-CoA on the luminal surface of the membrane prior to its β -oxidation in the matrix [12]. Although the subcellular site for β -oxidation of pristanic acid has not been clearly established, the excessive accumulation and defective oxidation of pristanic acid in diseases which lack peroxisomes [19] suggests that the degradation of pristanic acid by β -oxidation in human tissues also takes place in peroxisomes. Except for acyl-CoA ligases, the other enzymes for β -oxidation of fatty acids are present in the peroxisomal matrix [17,21]. The actual substrates for

fatty acid β -oxidation are the acyl-CoA derivatives. The finding that phytanic acid is α -oxidized to pristanic acid in the matrix of peroxisomes suggests that for β -oxidation of pristanic acid, a product of phytanic acid α -oxidation, needs to be activated to pristanoyl-CoA prior to β -oxidation. However, at present it is not known whether pristanic acid is activated to pristanoyl-CoA by a putative pristanoyl-CoA ligase, with the active site being on the luminal surface, or by lignoceroyl-CoA ligase, the active site of which lies on the luminal surface of peroxisomes [18]; or that whether it first leaves the peroxisomes for its activation on the cytoplasmic surface and then re-enters the peroxisomes for its β -oxidation.

In summary: in peroxisomes the phytanoyl-CoA ligase is present in the peroxisomal membrane and enzyme system for α -oxidation of phytanic acid to pristanic acid is in the matrix of peroxisomes. The efficient oxidation of free phytanic acid in permeabilized peroxisomes or isolated peroxisomal matrix as compared to the absolute requirement for activation of phytanic acid to phytanoyl-CoA in intact peroxisomes suggests that phytanoyl-CoA ligase in the peroxisomal membrane regulates the α -oxidation of phytanic acid by providing phytanoyl-CoA for its entry into peroxisomes. The conclusion that the free phytanic acid is the true substrate for the enzyme system for α -oxidation of phytanic acid in the matrix of peroxisomes is supported by the following observations. (i) The α -oxidation of phytanic acid in isolated matrix or permeabilized peroxisomes with digitonin did not require fatty acid activating cofactors (e.g. ATP, CoASH). (ii) The α -oxida-

tion of phytanic acid in isolated matrix or permeabilized peroxisomes was not inhibited by naproxen, an inhibitor of acyl-CoA ligases. (iii) Phytanoyl-CoA is quickly hydrolyzed to free phytanic acid following its translocation into intact peroxisomes. (iv) The α -oxidation of phytanic acid continued at a linear rate for 2 h past the time when all of the phytanoyl-CoA was hydrolyzed to free phytanic acid.

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REFERENCES

- [1] Steinberg, D. (1989) in: *The Metabolic Basis of Inherited Disease*, 6th ed. (Scriver, C.A., Beaudet, A.L., Sly, W.S. and Valle, D. Eds.) Chapter 59, pp. 1533–1550, McGraw-Hill, New York.
- [2] Brown, F.R., Voight, R., Singh, A.K. and Singh, I. (1992) *Adv. Clin. Neurosci.* 2, 385–400.
- [3] Singh, I., Lazo, O., Pahan, K. and Singh, A.K. (1992) *Biochim. Biophys. Acta* 1180, 221–224.
- [4] Singh, I., Pahan, K., Dhaunsi, G.S., Lazo, O. and Ozand, P. (1993) *J. Biol. Chem.* 268, 72–9979.
- [5] Lazarow, P.B. and Moser, H.W. (1989) in: *The Metabolic Basis of Inherited Disease*, 6th edn. (Scriver, C.A., Beaudet, A.L., Sly, W.S. and Valle, D. Eds.) pp. 1479–1509, McGraw-Hill, New York.
- [6] Singh, I., Pahan, K., Singh, A.K. and Barbosa, E. (1993) *J. Lipid Res.* (in press).
- [7] Pahan, K., Cofer, J., Baliga, P. and Singh, I. (1993) *FEBS Lett.* 322, 101–104.
- [8] Steinberg, D., Herndon Jr. J.H., Uhlendorf, B.W. and Mize, C.E., Avigan, J. and Milne, G.W.A. (1967) *Science* 156, 1740–1742.
- [9] Draye, J.-P., Van Hoop, F., Hoffmann, E. and Vamecq, J. (1987) *Eur. J. Biochem.* 167, 573–578.
- [10] Akanuma, H. and Kishimoto, Y. (1979) *J. Biol. Chem.* 254, 1050–1056.
- [11] Dhaunsi, G.S., Gulati, S., Singh, A.K., Orak, J.K., Asayama, K. and Singh, I. (1992) *J. Biol. Chem.* 267, 6870–6873.
- [12] Singh, I., Lazo, O., Dhaunsi, G.S. and Contreras, M. (1992) *J. Biol. Chem.* 267, 13306–13313.
- [13] Singh, I., Singh, R.P., Bhushan, A. and Singh, A.K. (1985) *Arch. Biochem. Biophys.* 236, 418–426.
- [14] Bradford, M. (1976) *Anal. Biochem.* 72, 248–254.
- [15] Wolvetang, E.J., Tager, J.M. and Wanders, R.J. (1992) *Prog. Clin. Biol. Res.* 375, 223–229.
- [16] Knights, K. and Jones, M.E. (1992) *Biochem. Pharmacol.* 43, 1465–1471.
- [17] Dove, V.P. (1956) *J. Clin. Invest.* 35, 150–154.
- [18] Lazo, O., Contreras, M. and Singh, I. (1990) *Biochemistry* 29, 3981–3986.
- [19] ten Brink, H.J., Schor, D.S.M., Kok, R.M., Poll-The, B.T., Wanders, R.J.A. and Jakobs, C. (1992) *J. Lipid Res.* 33, 1449–1457.
- [20] Singh, H., Brogan, M., Johnson, D. and Poulos, A. (1992) *J. Lipid Res.* 33, 1597–1605.
- [21] Hashimoto, T. (1990) *Prog. Clin. Biol. Res.* 321, 137–152.