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Acyl-CoA synthetase and the peroxisomal enzymes of β-oxidation in human liver

Quantitative analysis of their subcellular localization

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1. The presence of acyl-CoA synthetase (EC 6.2.1.3) in peroxisomes and the subcellular distribution of β-oxidation enzymes in human liver were investigated by using a single-step fractionation method of whole liver homogenates in metrizamide continuous density gradients and a novel procedure of computer analysis of results. 2. Peroxisomes were found to contain 16% of the liver palmitoyl-CoA synthetase activity, and 21% and 60% of the enzyme activity was localized in mitochondria and microsomal fractions respectively. 3. Fatty acyl-CoA oxidase was localized exclusively in peroxisomes, confirming previous results. 4. Human liver peroxisomes were found to contribute 13%, 17% and 11% of the liver activities of crotonase, β-hydroxyacyl-CoA dehydrogenase and thiolase respectively. 5. The absolute activities found in peroxisomes for the enzymes investigated suggest that in human liver fatty acyl-CoA oxidase is the rate-limiting enzyme of the peroxisomal β-oxidation pathway, when palmitic acid is the substrate.

We have previously established (Bronfman *et al.*, 1979) the presence in human liver of fatty acyl-CoA oxidase and the peroxisomal fatty acid-oxidizing system initially reported by Lazarow & de Duve (1976) in rat liver. In this rodent, liver peroxisomes contain the complete sequence of β-oxidation enzymes (Lazarow, 1978) and a long-chain acyl-CoA synthetase (Krisans *et al.*, 1980; Mannaerts *et al.*, 1982), the enzyme that catalyses the first step in fatty acid oxidation, i.e. their activation to the corresponding acyl-CoA esters.

In the human liver it is not known if peroxisomes contain acyl-CoA synthetase, nor has the relative contribution of this organelle to the activity of other enzymes of the β-oxidation pathway [enoyl-CoA hydratase (crotonase), β-hydroxyacyl-CoA dehydrogenase and thiolase] been studied.

The activities of acyl-CoA synthetase and of the enzymes of the β-oxidation sequence are increased in the livers of rats treated with clofibrate (Lazarow, 1978; Krisans *et al.*, 1980), a drug which, like a variety of other hypolipidaemic compounds in the rat, induces a marked peroxisomal proliferation (Hess *et al.*, 1965; Leighton *et al.*, 1975: Lazarow, 1977).

This proliferative phenomenon has not been observed in humans (Staubli & Hess, 1975; Hanefeld *et al.*, 1980; de la Iglesia *et al.*, 1981, 1982), and it has been proposed that it may be limited to rodents, since neither proliferation nor increase in the peroxisomal β-oxidation activity occurs in marmosets, dogs or rhesus monkeys treated with a peroxisomal proliferator (Holloway *et al.*, 1982*a*,*b*). However, it has also been shown that, in the rat, the increase of peroxisomal β-oxidation associated with hypolipidaemic drug treatment can be dissociated from hepatomegaly and peroxisome proliferation (Lazarow *et al.*, 1982).

Human and rat liver peroxisomes have a different enzymic composition (de Duve & Baudhuin, 1966). Also, at the level of the subcellular localization of enzymes, there are marked differences: it has been shown that enzymes with mitochondrial–peroxisomal localization in the rat are exclusively peroxisomal in human liver (Noguchi & Takada, 1978, 1979).

These considerations have prompted us to investigate the presence of acyl-CoA synthetase

Abbreviation used: PFAOS, peroxisomal fatty acid-oxidizing system.

(EC 6.2.1.3; substrate, palmitoyl-CoA) in human liver and to evaluate quantitatively the subcellular distribution of this and other enzymes of the β-oxidative pathway. To overcome problems inherent to the fractionation of small amounts of tissue, a single-step method of subcellular fractionation, employing whole liver homogenates, was used (Bronfman *et al.*, 1979), and a novel procedure of computer-assisted analysis of subcellular-fractionation data was developed; it allows the computation and statistical evaluation of the apparent relative contribution of various organelles to the enzymic activity detected in subcellular fractions.

Experimental

Preparation of homogenates and subcellular fractionation of human liver

Liver biopsies (300–800mg) were obtained from patients undergoing surgery for uncomplicated gall-stone or gastroduodenal-ulcer disease. Informed consent from the patients was obtained by following the procedures approved by the Ethics Committee of the Medical School of the Universidad Catolica de Chile. Liver-function tests were normal in all cases. The biopsies obtained at the beginning of surgery were placed immediately in ice-cold 0.25M-sucrose. After decapsulation, the tissue, cut in small pieces, was placed into a Teflon/glass tissue grinder model A (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) containing 5ml of homogenization medium (0.25M-sucrose/1mM-dithiothreitol/3mM-imidazole/HCl, pH7.4) per g wet wt. of tissue. Homogenization was performed at 0°C, with two strokes of the pestle, driven at 1200rev./min.

Fractionation of total homogenates by the one-step isopycnic-gradient procedure was performed by using metrizamide linear density gradients from 1.10 to 1.28g/ml (Bronfman *et al.*, 1979). The gradients (4.5ml) were prepared with a Beckman density-gradient former. Metrizamide solutions, containing 3mM-imidazole, were neutralized to pH7.2–7.4 with 1M-NaOH. The homogenate (0.5ml) was layered on top of each gradient and centrifugation performed for 90min at 39000rev./min and 6–10°C in a Beckman SW-65 rotor.

After centrifugation, 12–14 fractions were collected by slicing the tube with a Beckman tube slicer fitted with a cooling jacket, at 0–2°C. Density measurements in each fraction were performed by the procedure described by Beaufay *et al.* (1964).

In some experiments, enough tissue was available for fractionation of the homogenate by differential centrifugation in addition to the isopycnic method. The procedure used was that of de Duve *et al.* (1955), except that the N and M fractions were sedimented together.

Enzyme assays

Established procedures were used for the determination of marker enzymes: catalase for peroxisomes and glutamate dehydrogenase for mitochondria (Leighton *et al.*, 1968), NADPH:cytochrome *c* reductase for microsomal vesicles (Beaufay *et al.*, 1974) and acid phosphatase for lysosomes (Appelmans *et al.*, 1955). Protein was measured by the method of Lowry *et al.* (1951), as modified by Wattiaux *et al.* (1978), with bovine serum albumin as standard.

Phosphoglucomutase, a soluble-compartment marker, was determined by the method of Ray & Roscelli (1964) as modified by Leighton *et al.* (1977). For the assay, 1.8ml of a mixture (pH7.4) containing 31mM-histidine, 10mM-Tris/HCl, 12mM-$MgCl_2$, 0.02mg of bovine serum albumin/ml and 0.02mM-glucose 1,6-bisphosphate, was preincubated with 0.1ml of enzyme for 7min at 30°C. Glucose 6-phosphate dehydrogenase (Sigma, type XII; 10μl; 5 units) and 0.1ml of 8mM-$NADP^+$ were then added and the incubation continued for 3min more. The reaction was started by the addition of 0.1ml of 10mM-glucose 1-phosphate, and the A_{340} was recorded in a Shimadzu UV 150-02 spectrophotometer equipped with a cuvette positioner (Shimadzu Seisakusho, Kyoto, Japan) during 3–5min at 30°C.

Fatty acyl-CoA oxidase and the activity of the PFAOS, determined as palmitoyl-CoA-dependent NAD^+ reduction, were assayed as previously described (Bronfman *et al.*, 1979).

For the determination of palmitoyl-CoA synthetase we used two procedures, a spectrophotometric assay and the radiochemical assay described by Krisans *et al.* (1980), with modifications.

In the spectrophotometric determination, palmitate-dependent NAD^+ reduction was measured in the presence of an excess of fatty acyl-CoA oxidase, enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase. As source of these enzymes, the fraction corresponding to step 4 of the purification of fatty acyl-CoA oxidase from rat liver as described by Inestrosa *et al.* (1980) was used. This enzyme fraction contains a high specific activity of PFAOS and a negligible amount of palmitoyl-CoA synthetase activity. The reaction mixture for the assay contained, in a final volume of 1ml, 0.12mM-sodium palmitate, 1mM-ATP, 1mM-$MgCl_2$, 0.32mM-NAD^+, 0.1mM-CoA, 0.02mM-FAD, 2.4mM-dithiothreitol, 40mM-nicotinamide, 1.6mM-KCN, 0.1% Triton X-100, 200mM-Tris/HCl buffer, pH8.5, and 0.04–0.06 unit of PFAOS. After 5min preincubation at 37°C, the reaction was started by the addition of 50μl of enzyme diluted in 10mM-Tris/HCl, pH8.0, containing 0.1% Triton X-100, and the A_{340} was

recorded for about 15 min at 37°C. Blanks without enzyme were run simultaneously. Omission of ATP or CoA completely suppressed the activity.

In the radiochemical assay, the reaction mixture contained 5 mM-ATP, 5 mM-$MgCl_2$, 1 mM-dithiothreitol, 1 mM-NAD^+, 0.5 mM-CoA, 180 mM-Tris/HCl, pH 8.0, 0.125% Triton X-100, 175 μM-[1-^{14}C]palmitic acid (2 Ci/mol) and 0.04–0.05 unit of PFAOS activity, in a final volume of 0.25 ml. To prepare the palmitic acid solution, palmitic acid dissolved in n-heptane was evaporated to dryness under N_2 and then resuspended by sonication in 375 mM-Tris/HCl buffer (pH 8.0) containing 0.625% Triton X-100. The reaction was started by addition of the enzyme diluted in 10 mM-Tris/HCl (pH 8.0)/1 mM-dithiothreitol. Incubation was performed for 10 min at 37°C. Palmitic acid that had not reacted was separated from the reaction products as described by Krisans *et al.* (1980), with minor modifications. Controls where the enzyme, ATP or CoA were omitted were run in each experiment. Under the conditions described, the rate of the reaction was proportional to enzyme concentration up to 150 μg of homogenate protein in the reaction mixture. In the absence of NAD^+ and PFAOS activity, both the rate of the reaction and the range within which the measured velocity is directly proportional to enzyme concentration are markedly decreased.

Crotonase and β-hydroxyacyl-CoA dehydrogenase were assayed as described by Lazarow (1978).

For the determination of thiolase, 5,5'-dithiobis-(2-nitrobenzoic acid) was used for measuring disappearance of free CoA in the presence of acetoacetyl-CoA. Methods described for the assay of this enzyme based on the absorption band of the thioester bond at 233 nm (Lazarow, 1978) were not suitable, owing to the high absorbance of metrizamide at this wavelength. For the assay, 30 μl of enzyme diluted in 0.1% Triton X-100 was incubated for 5 min at 37°C in a total volume of 0.18 ml, containing 60 nmol of CoA, 30 nmol of acetoacetyl-CoA and 55 nmol of KH_2PO_4, pH 7.5. The reaction was stopped by addition of 0.8 ml of 0.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid) in 100 mM-KH_2PO_4, pH 8.0, and the A_{413} was read. The amount of CoA that had reacted was estimated from the A_{413} of a blank from which the enzyme was omitted. Blanks without CoA were run for each assay. Under the conditions described, the time-course of CoA disappearance is linear for 10 min and the amount of CoA reacted is proportional, up to 20 nmol, to enzyme concentration.

For all enzymes, a possible effect of metrizamide on enzyme activity was tested both by including metrizamide in the enzyme assay and by exposing the homogenate to metrizamide concentrations covering the range at which enzymes could be exposed. Only palmitoyl-CoA synthetase was found to be slightly inhibited by metrizamide in the enzyme assay; 10–15% inhibition was found when 3% metrizamide was included in the test, which is the concentration in the enzyme assay for the highest-density fractions.

Results from enzymic assays are expressed in μmol of substrate utilized or of product formed per mg of protein in 1 min. For catalase, one unit of activity is defined as the amount of enzyme destroying 90% of the substrate in 1 min in a volume of 50 ml under the conditions of the assay (Leighton *et al.*, 1968).

Substrates, coenzymes and chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. Radiolabelled palmitic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Calculations and presentation of results

Distribution of enzymes in metrizamide gradients are presented in the form of normalized (de Duve, 1967) and averaged (Bowers & de Duve, 1967) histograms. The ordinate represents average frequency of the component in each fraction: $Q/\Sigma Q \cdot \Delta\rho$, where Q represents the activity found in the fraction, ΣQ the total activity recovered and $\Delta\rho$ the increment in density from top to bottom of the gradient in the centrifuge tube. Frequency is plotted against density.

For the quantitative evaluation of enzyme subcellular localization in fractionation experiments, we used a computational method based on constrained linear-regression analysis. A partial report of its application and theoretical basis has been made (Bronfman *et al.*, 1982). The main difference between this method and normal linear-regression analysis of data from cell-fractionation experiments (Leighton *et al.*, 1968; Krisans *et al.*, 1980) is that the values of the fraction of the unknown localized enzyme that is assigned by the computer program to an organelle (marker enzyme) is constrained to be greater than or equal to zero. This constraint overcomes the problem of obtaining statistically significant negative values for the fraction of a constituent present in a subcellular compartment, a result that we usually encountered when normal linear regression was employed for the analysis of cell-fractionation data. Using real and computer-simulated experiments, we have found that these negative values can only be avoided by increasing considerably the number of experiments analysed, and that many fewer experiments are needed when constrained regression is used (M. Bronfman & E. Feytmans, unpublished work).

The computer program used was that of Dixon & Brown (1979), running on a Digital DEC-10 computer. The statistical significance of the assignations was calculated by Student's t test.

Results

Absolute values of enzyme activities in human liver

The absolute activity values for the measured enzymes and proteins are listed in Table 1. The observed values for marker enzymes and protein are 1.5–3 times less than those reported by our laboratory (Leighton *et al.*, 1977) and others (Amar-Costesec *et al.*, 1974) for rat liver, except for phosphoglucomutase, which has 1.4 times more activity.

The absolute activities of fatty acyl-CoA oxidase and the enzymes of the β-oxidative sequence (crotonase, β-hydroxyacyl-CoA dehydrogenase and thiolase) were 3–5 times less in human than in rat liver. In contrast, the activity of palmitoyl-CoA synthetase, assayed radiochemically, was similar to that reported for rat liver by Krisans *et al.* (1980).

Consistently higher values for palmitoyl-CoA synthetase activity were obtained when the enzyme was assayed by the spectrophotometric method. Although this difference could be due to the different assay conditions, the possibility also exists that the substrate would cycle through the β-oxidation sequence. This would give an overestimation of palmitoyl-CoA synthetase measured at the level of NAD^+ reduction. However, as shown below, no significant differences were found between both methods for the distribution of the enzyme in isopycnic-density equilibration experiments. The median density and recovery of enzymes are shown in Table 2.

Excessive and highly variable recoveries were obtained for fatty acyl-CoA oxidase. In complete homogenates, this enzyme presents a high blank because of endogenous activity, which results in

Table 1. *Absolute values of measured enzymes and protein*

Values of activity refer to 1 g wet wt. of liver. They are given as means ± S.D. in units for enzymes and in mg for protein. PFAOS activity is measured at the level of NAD^+ reduction.

	No. of experiments	Absolute values
Catalase	16	21.4±5.4
Glutamate dehydrogenase	16	4.9±1.7
NADPH:cytochrome *c* reductase	16	2.6±0.8
Acid phosphatase	12	1.6±0.6
Phosphoglucomutase	8	212.5±69.8
Palmitoyl-CoA synthetase		
Radiochemical assay	4	2.7±0.4
Spectrophotometric assay	6	5.9±1.7
Fatty acyl-CoA oxidase	6	0.15±0.07
β-Hydroxyacyl-CoA dehydrogenase	6	11.2±5.2
Crotonase	6	462.4±130.2
Thiolase	4	53.9±14.4
PFAOS	6	0.22±0.07
Protein	16	124.2±30.1

Table 2. *Median density and recovery of enzymes and protein*

Total liver homogenates were centrifuged in metrizamide continuous density gradients as described in the Experimental section. Results are means ± S.D. The median densities of particulate catalase, particulate fatty acyl-CoA oxidase and PFAOS were computed from the enzyme activity present at densities higher than 1.175 g/cm³.

	No. of experiments	Median density (g/cm³)	Recovery (%)
Catalase	13	1.1005±0.0229	103.7±11.6
Particulate catalase	13	1.2067±0.0095	—
Glutamate dehydrogenase	13	1.1122±0.0087	103.1±12.6
NADPH: cytochrome *c* reductase	13	1.1060±0.0085	98.4±16.7
Acid phosphatase	9	1.0911±0.0079	98.1±15.8
Phosphoglucomutase	8	1.0665±0.0060	106.4±14.3
Palmitoyl-CoA synthetase			
Radiochemical assay	4	1.0980±0.0138	83.3±14.6
Spectrophotometric assay	2	1.1025±0.0002	114.1±14.7
Fatty acyl-CoA oxidase	5	1.1831±0.0304	179.1±40.3
Particulate fatty acyl-CoA oxidase	5	1.2048±0.0080	—
β-Hydroxyacyl-CoA dehydrogenase	3	1.1146±0.0088	95.7±4.3
Crotonase	3	1.1043±0.0139	84.4±16.6
Thiolase	3	1.1029±0.0043	95.8±4.3
PFAOS	5	1.1568±0.0257	83.9±17.5
Particulate PFAOS	5	1.2068±0.0155	—
Protein	13	1.0968±0.0104	106.9±15.9

variability and underestimation of the activity (Bronfman *et al.*, 1979).

Isopycnic fractionation of human liver homogenates in metrizamide density gradients

The density distribution of marker enzymes after isopycnic equilibration of total liver homogenates in metrizamide density gradients is shown in Fig. 1.

Only 25–30% of catalase, the peroxisomal marker, appears to be particle-bound. Most of the remaining activity stays in the top fractions, which, judged from the behaviour of phosphoglucomutase, correspond to soluble activity. A large soluble fraction of catalase and other peroxisomal enzymes is also observed in rat liver and has been attributed to enzyme release during homogenization and fractionation (Leighton *et al.*, 1975; Lazarow & de Duve, 1973). Some tailing is observed for the particulate catalase peak. It is not known if this reflects heterogeneity of peroxisomes or organelle disruption during sedimentation.

As previously reported (Bronfman *et al.*, 1979, 1982), the fractionation procedure gave good resolution of peroxisomes from other organelles. However, microsomal fractions and mitochondria are not well resolved, and very similar median densities are observed for their marker enzymes (Table 2). In agreement with results from Wattiaux *et al.* (1978) for rat liver, the resolution is better for lysosomes, judged from the pattern for acid phosphatase.

The density distributions of palmitoyl-CoA synthetase and the enzymes of β-oxidation are presented in Fig. 2. Similar distributions of palmitoyl-CoA synthetase were observed whether the enzyme was assayed radiochemically or spectrophotometrically. The main fraction of the

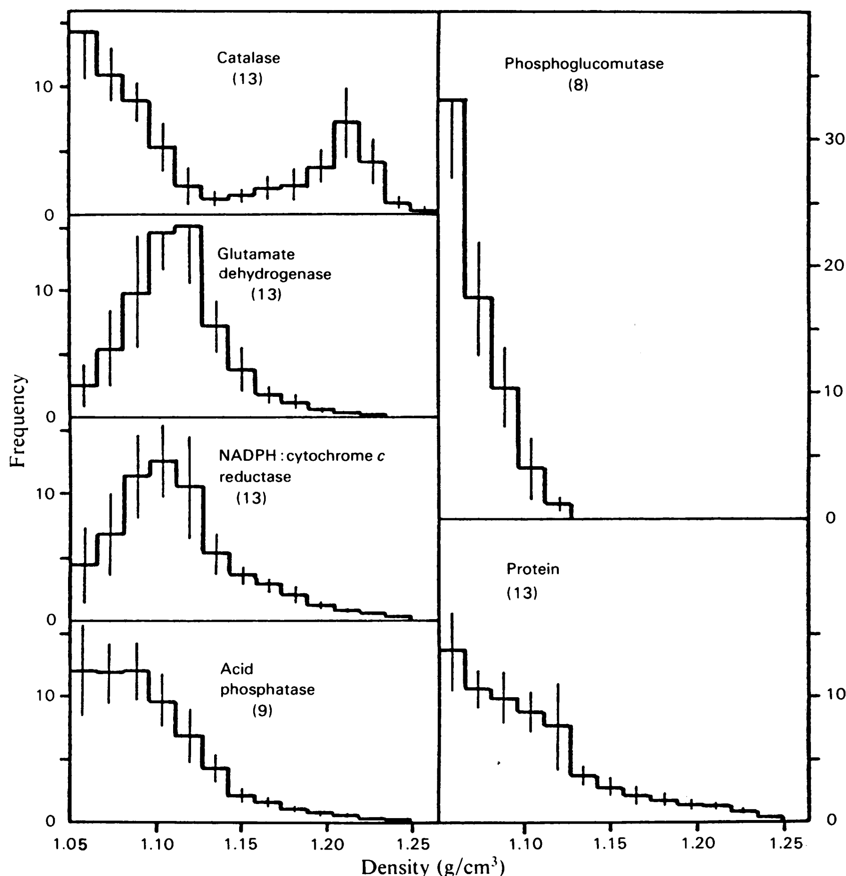

Fig. 1. *Fractionation of total human liver homogenates in metrizamide density gradients: subcellular distribution of marker enzymes*

Standardized and averaged results are shown; the numbers of experiments are given in parentheses. Bars represent the S.D. of the frequency in each fraction.

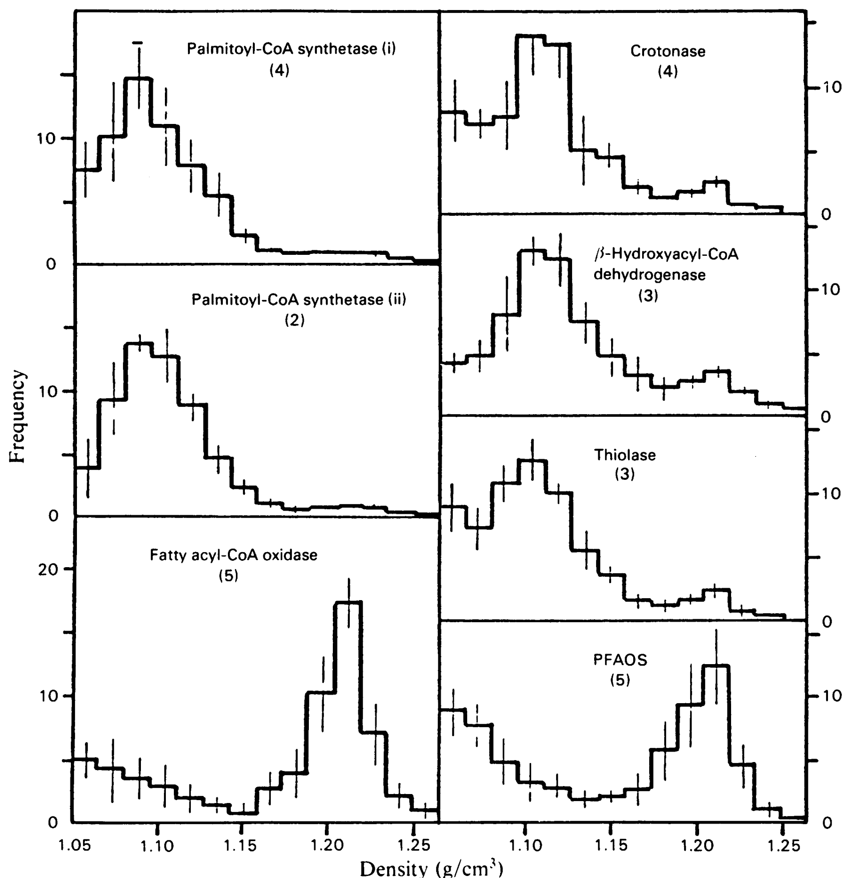

Fig. 2. *Fractionation of total human liver homogenates in metrizamide density gradients: subcellular distribution of palmitoyl-CoA synthetase and the enzymes of β-oxidation*

Standardized and averaged results are shown; the numbers of experiments are given in parentheses. Bars represent S.D. of the frequency in each fraction. Palmitoyl-CoA synthetase: (i) radiochemical assay; (ii) spectrophotometric assay.

synthetase activity is located in the microsomal–mitochondrial fractions of the gradients, and only a very small amount appears to be associated with peroxisomes.

As previously described (Bronfman *et al.*, 1979), fatty acyl-CoA oxidase and PFAOS have a peroxisomal pattern of subcellular distribution. However, the fraction of particulate oxidase activity is twice that found for catalase, suggesting that the oxidase is more tightly bound to peroxisomes than is catalase. In contrast, in rat liver equal amounts of these enzymes are particle-bound (Inestrosa *et al.*, 1980). An intermediate distribution between those of catalase and the oxidase is found for PFAOS particulate and soluble activities. PFAOS distribution pattern, in the lower-density region of the gradient, could be due to mitochondrial contribution of crotonase and β-hydroxyacyl-CoA dehydrogenase activities. As shown in Table 2, a good coincidence of the median densities of particulate catalase, fatty acyl-CoA oxidase and PFAOS is observed.

The enzymes of the β-oxidative sequence, crotonase, β-hydroxyacyl-CoA dehydrogenase and thiolase, present a clear bimodal distribution, with a peak in the peroxisomal region and most of the activity in the microsomal–mitochondrial fractions.

Quantitative analysis of enzyme distribution

In order to estimate the fraction of each enzyme activity present in the different subcellular organelles, the data presented in Fig. 2 were analysed by constrained liner regression. Straightforward results were obtained for all enzymes (Table 3), except for thiolase. Analysis of this enzyme distribution resulted in values not statistically different from zero for the assignation to mito-

Table 3. *Assignation by constrained linear-regression of palmitoyl-CoA synthetase and the enzymes of β-oxidation*
The compartments are identified by their corresponding marker enzymes. Results are given in percentages, ±S.D., and correspond to analysis of the data shown in Fig. 2: *statistically not different from zero. Analysis of enzyme distributions not including the marker enzymes for which non-significant or zero values were previously obtained did not change significantly the percentages already assigned to the other marker enzymes (see the text for details).

	Catalase (peroxisomes)	Glutamate dehydrogenase (mitochondria)	NADPH:cyto-chrome c reductase (microsomal fractions)	Phosphoglucomutase (soluble compartment)
Crotonase	13.3±2.7	61.6±4.9	0.0	18.3±2.6
β-Hydroxyacyl-CoA dehydrogenase	17.4±3.5	76.5±3.5	0.0	5.4±3.1*
Thiolase	10.5±3.2	64.0±6.0	0.0	20.1±4.2
Palmitoyl-CoA synthetase (radiochemical assay)	15.8±6.2	20.5±10.3	59.9±13.8	6.6±5.1*
Palmitoyl-CoA synthetase (spectrophotometric assay)	14.0±10.6*	24.0±11.5	60.4±19.0	4.6±5.3*

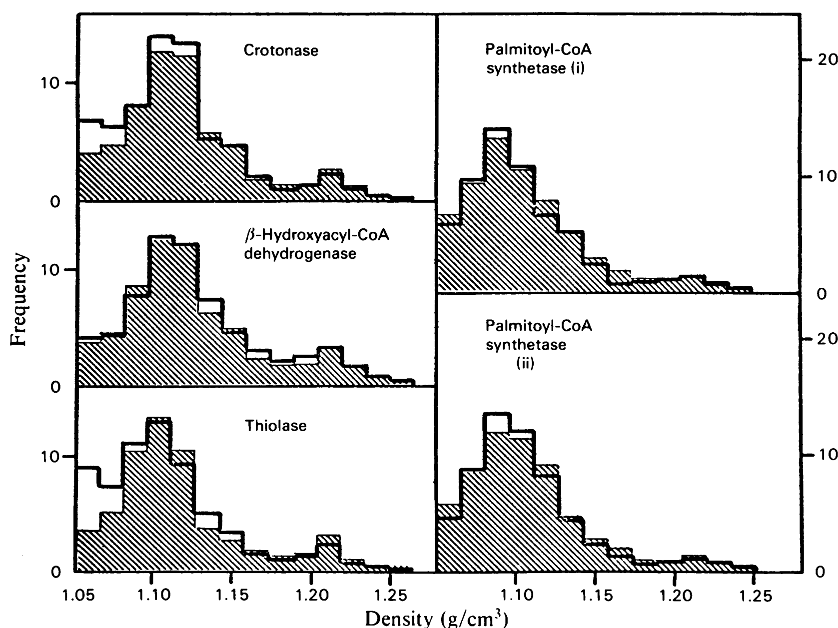

Fig. 3. *Experimental and constrained-linear-regression-predicted distributions of palmitoyl-CoA synthetase and the enzymes of β-oxidation*
The marker-enzyme frequencies from Fig. 1 were multiplied by the percentage calculated by constrained linear regression (Table 3), and their sums are represented by the shaded areas. The experimental distribution (the same as in Fig. 2) is represented by the continuous line. Palmitoyl-CoA synthetase: (i) radiochemical assay; (ii) spectrophotometric assay.

chondria and microsomal fractions (38.5±28.3 and 27.2±28.8% respectively). Values for peroxisomes and the soluble compartment were significant. The data presented in Table 3 for thiolase were obtained by distributing the enzyme between the marker enzymes from mitochondria, peroxisomes and the soluble compartment. In this case, values for the different subcellular compartments were significant, and the residual mean square of the regression was lower (better fitting) than when the microsomal and not the mitochondrial marker was considered, or when both markers were eliminated.

In Fig. 3, the distributions of palmitoyl-CoA synthetase and the enzymes of β-oxidation, which can be predicted by linear combinations of marker enzymes by using data from Table 3, are compared with the experimental distributions. A good fit is observed in all cases.

No activity of crotonase, β-hydroxyacyl-CoA

dehydrogenase and thiolase is assigned by the constrained-linear-regression method to the microsomal fraction. In all cases, a small, although significant amount of the activity is assigned to peroxisomes, whereas only for crotonase and thiolase a significant proportion of the activity appears to be present in the soluble compartment. This difference might arise because the reference marker enzyme for peroxisomes, catalase, has a soluble and a particulate component, and the total distribution of the enzyme was used as reference in the analysis. If the proportion of soluble activity of a peroxisomal enzyme is different from that of the marker, catalase, we have found, by computer simulation, that the activity assigned to peroxisomes by constrained linear regression will be overestimated if the unknown enzyme has less soluble activity than does catalase, whereas it will be underestimated, and the difference assigned to the soluble compartment, if it has more soluble activity than catalase. Differences of 15–20% between the proportion of soluble activity of a peroxisomal enzyme and catalase will result in a 10–15% over- or under-estimation of the peroxisomal activity. The possibility then exists that peroxisomal crotonase and thiolase are underestimated by the method.

Analysis of the distribution of palmitoyl-CoA synthetase assayed radiochemically or spectrophotometrically gave coincident values for the fraction of activity assigned by constrained linear regression to each subcellular compartment. However, the fraction of activity of this enzyme assigned to peroxisomes was not significantly different from zero for the distribution obtained by using the spectrophotometric assay. Although this could result from the fact that too few experiments were analysed in this case, the rather high proportion of synthetase activity assayed radiochemically assigned to peroxisomes by constrained linear regression is in apparent disagreement with the density distributions in metrizamide gradients, when compared with that assigned to the enzymes of β-oxidation. Only a small amount of the synthetase activity is detected at the level of the peroxisomal fractions, whereas the peroxisomal contribution to this enzyme as estimated by constrained linear regression is higher than that of crotonase or thiolase, for which a clear peroxisomal peak is observed. As proposed above, differences in the soluble and particulate proportions of this enzyme from those of catalase, or partial inhibition of the activity in the peroxisomal fractions by metrizamide, could explain these results. However, none of the experiments reported so far can be taken as definitive proof of a peroxisomal or non-peroxisomal localization of palmitoyl-CoA synthetase. These considerations prompted us to measure the presence of palmitoyl-CoA synthetase in purified peroxisomal fractions.

Purification of peroxisomes by differential and equilibrium density-gradient centrifugation of human liver

In three experiments, 0.4–0.5 g of human liver was fractionated by differential centrifugation (Fig. 4). The proportion of soluble catalase activity was lower in these experiments than that previously found for whole homogenates in metrizamide gradients, suggesting that some of the enzyme has leaked out from the organelle during sedimentation in that medium. The distribution of palmitoyl-CoA synthetase is in agreement with a microsomal localization of most of the activity. In two of the three experiments reported in Fig. 4, the activities of crotonase, β-hydroxyacyl-CoA dehydrogenase and thiolase were also determined. In all cases, less than 6% of the activity was present in the microsomal P fraction or in the L fraction, whereas the percentages in the NM fraction were 62.6–81.5%, 76–65% and 69.5–56.7% for crotonase, β-hydroxyacyl-CoA dehydrogenase and thiolase. The remaining activities were found in the S fraction. These results are in good agreement with the mainly mitochondrial location of these enzymes deduced by constrained linear regression (Table 3).

Results from metrizamide-density-gradient fractionation of L fractions are presented in Fig. 5. The distributions of marker enzymes and protein are similar to results reported by Wattiaux *et al.* (1978) for L fractions from rat liver. A bimodal distribution with a clear peroxisomal peak is observed for palmitoyl-CoA synthetase, demonstrating that a fraction of this enzyme is indeed localized in peroxisomes. Although all synthetase assays were performed on freshly isolated fractions, recoveries for this enzyme were low. We found that the synthetase activity in the L fraction was in fact very labile. A similar observation has been made by Krisans *et al.* (1980) for the palmitoyl-CoA synthetase activity in rat liver L fractions.

Because of the low recoveries of the synthetase, constrained linear regression cannot be applied, since one of the basic postulates of the method is that recoveries must be good. If constrained linear regression is done on gradients presented in Fig. 5, 60–70% of the synthetase from the L fraction is assigned to peroxisomes and the remaining activity is assigned to mitochondria. No activity is assigned to the microsomal fraction, suggesting that the labile synthetase activity corresponds to this fraction.

Although no clear conclusions about the proportion of palmitoyl-CoA synthetase present in

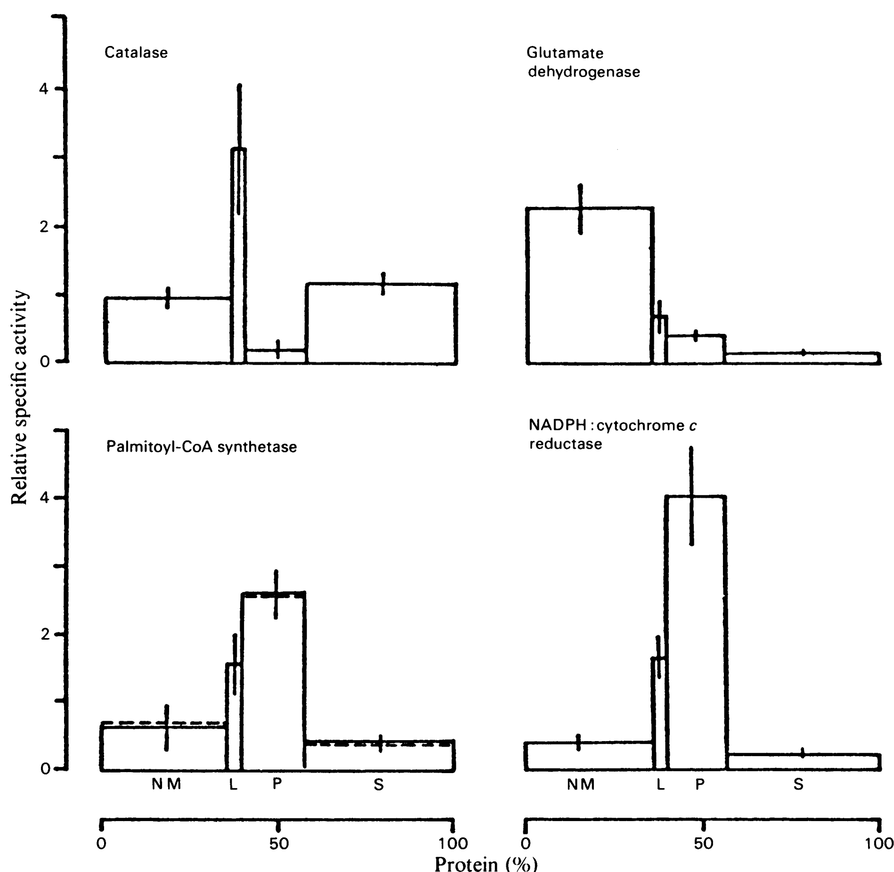

Fig. 4. *Subcellular fractionation of human liver homogenates by differential centrifugation*
Fractionation was performed as described by de Duve *et al.* (1955), except that the nuclear (N) and heavy-mitochondrial (M) fractions were sedimented together (NM). Key: L, light mitochondrial fraction; P, microsomal fraction; S, supernatant fraction. Standardized and averaged results from three experiments are presented. Recoveries were 98.2 ± 4.4, 105.9 ± 19.2, 98.0 ± 19.3, 109.0 ± 10.5 and $97.4 \pm 8.1\%$ for catalase, glutamate dehydrogenase, palmitoyl-CoA synthetase, NADPH : cytochrome *c* reductase and protein respectively. Bars represent S.D. The percentage contribution of each subcellular organelle to the activity of palmitoyl-CoA synthetase, calculated by constrained linear regression, was 14.7 ± 6.0 in peroxisomes (catalase), 12.2 ± 6.5 in mitochondria (glutamate dehydrogenase) and 62.4 ± 4.6 in microsomal fractions (NADPH : cytochrome *c* reductase). The broken line in the distribution of palmitoyl-CoA synthetase represents the predicted distribution of the enzyme calculated as a linear combination of the distributions of marker enzymes, by using the coefficients reported above.

peroxisomes can be drawn from the experiments presented in this section, they clearly show that a fraction of this enzyme indeed has a peroxisomal localization and validate the constrained-linear-regression analysis of experiments with total liver homogenates.

The absolute activities of palmitoyl-CoA synthetase and the enzymes of the β-oxidation cycle in peroxisomes are shown in Table 4. Fatty acyl-CoA oxidase appears as the rate-limiting step of the β-oxidative sequence.

Discussion

Variable amounts, ranging from 10 to 17%, of the total liver activity were found in peroxisomes for the enzymes of the β-oxidation cycle: crotonase, β-hydroxyacyl-CoA dehydrogenase and thiolase. It is noteworthy that these enzymes were assayed with short-chain substrates, whereas the human liver peroxisomal β-oxidation system is more specific for long-chain substrates (Bronfman *et al.*, 1979). It is then possible that the relative contribution of peroxisomes to these enzyme activities might be higher.

Previous reports on the subcellular localization of palmitoyl-CoA synthetase in human liver (Berge *et al.*, 1980) attributed the enzyme activity to microsomal fractions and mitochondria. However, only differential centrifugation of liver homogenates was performed in those studies, which is

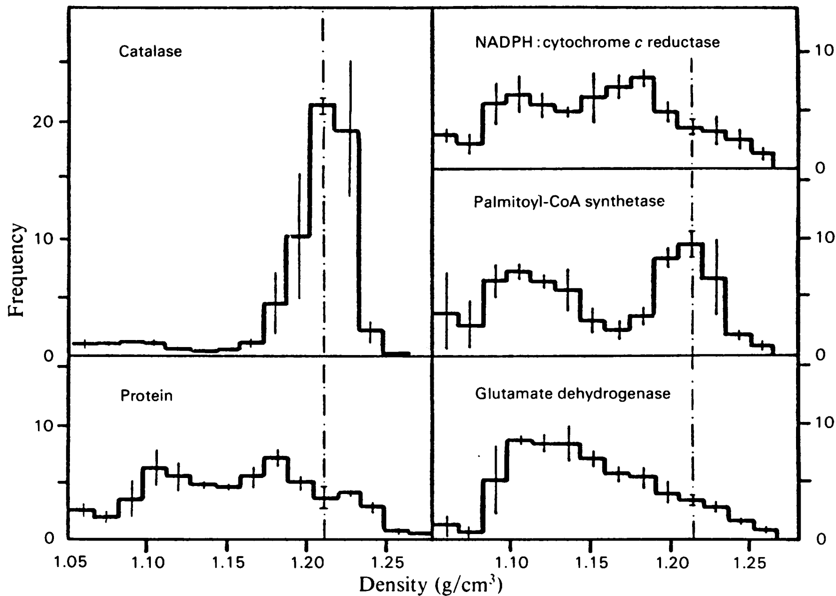

Fig. 5. *Subfractionation of L fractions in metrizamide continuous density gradients*
L fractions were obtained by differential centrifugation (Fig. 4). Standardized and averaged results of three experiments are shown. Bars represent S.D. of the frequencies in each fraction. Recoveries ranged between 80 and 110% for marker enzymes and protein. The recovery for palmitoyl-CoA synthetase was 40–60% in the three experiments considered (assayed spectrophotometrically).

Table 4. *Absolute values of enzyme activities in human liver peroxisomes*
The activities in peroxisomes were calculated by multiplying the total enzyme (units) in the liver (Table 1) by the percentage of activity in peroxisomes assigned by constrained linear regression (Table 3). For fatty acyl-CoA oxidase a 100% localization in peroxisomes was assumed.

	Enzyme in peroxisomes (units/g of liver)
Palmitoyl-CoA synthetase	
Radiochemical assay	0.43
Spectrophotometric assay	0.83
Fatty acyl-CoA oxidase	0.15
Crotonase	61.50
β-Hydroxyacyl-CoA dehydrogenase	1.96
Thiolase	5.65

clearly insufficient for detecting the amount of enzyme present in peroxisomes.

For palmitoyl-CoA synthetase, we assume that about 15% of the enzyme activity is present in peroxisomes, on the basis of the coincidence between the values obtained by constrained-linear-regression analysis of total homogenate fractionation in metrizamide gradients (radiochemical assay, with four experiments) and the differential-centrifugation experiments.

The percentage and amount of palmitoyl-CoA synthetase present in human liver peroxisomes is at least twice that reported for rat liver by Krisans *et al.* (1980). In contrast, the absolute activity of fatty acyl-CoA oxidase is only 20% of that reported in rat liver by Inestrosa *et al.* (1979). As a consequence, fatty acyl-CoA oxidase would be the rate-limiting step of the entire β-oxidation pathway, including fatty acid activation. In the rat there is proportionally less synthetase (Krisans *et al.*, 1980; Leighton *et al.*, 1982).

Substrate availability to the peroxisome could be an alternative rate-limiting step for the system (Leighton *et al.*, 1982). Nothing is known at present about the nature of the fatty acyl moiety that crosses the peroxisomal membrane. Mannaerts *et al.* (1982) presented evidence that, in rat liver, the peroxisomal acyl-CoA synthetase is localized at the cytoplasmic side of the peroxisomal membrane. They suggest that a carrier might exist in the peroxisomal membrane that exchanges external long-chain acyl-CoA for internal short-

chain acyl-CoA molecules which are formed in the terminal reaction of the peroxisomal β-oxidation.

Alternatively, on the assumptions that the peroxisomal membrane is impermeable to CoA and acyl-CoA species and is permeable to carnitine and its acyl derivatives, we have proposed that fatty acids enter the peroxisome as acylcarnitine (Leighton *et al.*, 1982). This would require an extra-peroxisomal system capable of generating acyl-carnitine, as well as carnitine acyltransferase activity inside the peroxisome. In the rat, carnitine acyltransferases are present in peroxisomes (Markwell *et al.*, 1973). However, the activity of carnitine palmitoyltransferase is absent or very low (Markwell *et al.*, 1977; Leighton *et al.*, 1982). If this mechanism of supplying fatty acids to peroxisomes exists, carnitine acyltransferase could be the rate-limiting step for the β-oxidation system in rat liver. In contrast, in humans, about 20% of the total carnitine palmitoyl- and oleoyl-transferases of the liver are present in peroxisomes (Bronfman & Leighton, 1984), suggesting that the translocation of long-chain acylcarnitine is more important in human peroxisomes and that the pattern of fatty acid utilization is different.

Very little is known about the physiological role of the peroxisomal β-oxidation or about its relative contribution to the overall process of fatty acid β-oxidation by the liver cell. Whatever its importance may be, it is apparent from the present work that there are differences between the human and rat liver systems. Further research on the subcellular localization of β-oxidation-related enzymes in human liver could help to clarify these points.

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References

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M. & Berthet, J. (1974) *J. Cell Biol.* **61**, 201–212

Appelmans, F., Wattiaux, R. & de Duve, C. (1955) *Biochem. J.* **59**, 438–445

Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964) *Biochem. J.* **92**, 184–205

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974) *J. Cell Biol.* **61**, 188–200

Berge, R. K., Vollset, S. E. & Farstad, M. (1980) *Scand. J. Clin. Invest.* **40**, 271–278

Bowers, W. E. & de Duve, C. (1967) *J. Cell Biol.* **32**, 339–348

Bronfman, M. & Leighton, F. (1984) *Biochem. J.* **224**, 721–730

Bronfman, M., Inestrosa, N. C. & Leighton, F. (1979) *Biochem. Biophys. Res. Commun.* **88**, 1030–1036

Bronfman, M., Leighton, F. & Feytmans, E. (1982) *Ann. N.Y. Acad. Sci.* **386**, 408–410

de Duve, C. (1967) in *Enzyme Cytology* (Roodyn, D. B., ed.), pp. 1–26, Academic Press, London and New York

de Duve, C. & Baudhuin, P. (1966) *Physiol. Rev.* **46**, 323–357

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617

de la Iglesia, F. A., Pinn, S. M., Lucas, J. & McGuire, E. J. (1981) *Micron* **12**, 97–98

de la Iglesia, F. A., Lewis, J. E., Buchanau, R. A., Marcus, E. L. & McMahou, G. (1982) *Atherosclerosis* **43**, 19–37

Dixon, W. J. & Brown, M. B. (eds.) (1979) *Biomedical Computer Programs*, pp. 464–482, P-series-BMDP3R, University of California Press, Berkeley

Hanefeld, M., Kemmer, C., Leonhardt, W., Kunze, K. D., Jaross, W. & Haller, H. (1980) *Atherosclerosis* **36**, 159–172

Hess, R., Staubli, W. & Reiss, W. (1965) *Nature* (*London*) **208**, 856–858

Holloway, B. R., Bentley, M. & Thorp, I. M. (1982*a*) *Ann. N.Y. Acad. Sci.* **386**, 439–442

Holloway, B. R., Thorp, I. M., Smith, G. D. & Peters, T. J. (1982*b*) *Ann. N.Y. Acad. Sci.* **386**, 453–455

Inestrosa, N., Bronfman, M. & Leighton, F. (1979) *Biochem. J.* **182**, 779–788

Inestrosa, N., Bronfman, M. & Leighton, F. (1980) *Biochem. Biophys. Res. Commun.* **95**, 7–12

Krisans, S. K., Mortensen, R. M. & Lazarow, P. B. (1980) *J. Biol. Chem.* **255**, 9599–9607

Lazarow, P. B. (1977) *Science* **197**, 580–581

Lazarow, P. B. (1978) *J. Biol. Chem.* **253**, 1522–1528

Lazarow, P. B. & de Duve, C. (1973) *J. Cell Biol.* **59**, 507–524

Lazarow, P. B. & de Duve, C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2043–2046

Lazarow, P. B., Shio, H. & Leroy-Houyet, A. M. (1982) *J. Lipid Res.* **23**, 317–326

Leighton, F., Poole, B., Baufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513

Leighton, F., Coloma, L. & Koenig, C. (1975) *J. Cell Biol.* **67**, 281–309

Leighton, F., Lopez, F., Zemelman, V., Morales, M. N. & Walsen, O. (1977) in *Membranous Elements and Movement of Molecules* (Reid, E., ed.), pp. 197–215, Ellis Horwood, Chichester

Leighton, F., Brandan, E., Lazo, O. & Bronfman, M. (1982) *Ann. N.Y. Acad. Sci.* **386**, 62–80

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mannaerts, G. P., Van Veldhoven, P., Van Broekhoven, A., Vandenbroek, G. & Debeer, L. J. (1982) *Biochem. J.* **204**, 17–23

Markwell, M. A. K., McGroarty, E. J., Bieber, L. L. & Tolbert, N. E. (1973) *J. Biol. Chem.* **248**, 3426–3432

Markwell, M. A., Bieber, L. L. & Tolbert, N. E. (1977) *Biochem. Pharmacol.* **26**, 1697–1702

Noguchi, T. & Takada, Y. (1978) *J. Biol. Chem.* **253**, 7598–7600
Noguchi, T. & Takada, Y. (1979) *Arch. Biochem. Biophys.* **196**, 645–647
Ray, W. J. & Roscelli, G. A. (1964) *J. Biol. Chem.* **239**, 1228–1236
Staubli, W. & Hess, R. (1975) in *Hypolipidemic Agents* (Kritchevsky, D., ed.), pp. 229–289, Springer Verlag, Berlin, Heidelberg and New York
Wattiaux, R., Wattiaux-de Coninck, S., Ronveaux-Dupal, M. F. & Dubois, F. (1978) *J. Cell Biol.* **78**, 349–368