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Metabolism of saturated and polyunsaturated fatty acids by normal and Zellweger syndrome skin fibroblasts

Jacqueline M. STREET, David W. JOHNSON, Harmeet SINGH and Alf POULOS
Department of Chemical Pathology, Adelaide Children's Hospital, North Adelaide, SA 5006, Australia

The metabolism of 1-^{14}C-labelled derivatives of palmitic ($C_{16:0}$), arachidonic ($C_{20:4,n-6}$) lignoceric ($C_{24:0}$) and tetracosatetraenoic ($C_{24:4,n-6}$) acids was studied in normal skin fibroblast cultures and in cultures of fibroblasts from peroxisome-deficient (Zellweger's syndrome) patients. Radiolabelled products of the fatty acids included carbon dioxide, C_{14-24} saturated and mono-unsaturated fatty acids formed from released acetate either by synthesis *de novo* or by elongation of endogenous fatty acids, fatty acids formed by 2–6-carbon elongation of added substrates, and a number of water-soluble compounds, some of which were tentatively identified as the amino acids glutamine, glutamic acid and asparagine. The labelled amino acids were found predominantly in the culture medium. Zellweger's syndrome fibroblasts showed a marked decrease in radiolabelled carbon dioxide and water-soluble-product formation from [1-^{14}C]-labelled arachidonic, tetracosatetraenoic and lignoceric acids but not from [1-^{14}C]palmitic acid, and the production of radiolabelled C_{14-18} fatty acids was also diminished. However, the elongation of individual fatty acids was either normal or above normal. Our data support the view that the oxidation of 20:4, 24:4 and 24:0 fatty acids in cultured skin fibroblasts takes place largely in peroxisomes, and further that the acetyl-CoA released by the β-oxidation process is available for the synthesis of fatty acids and amino acids. We speculate that the generation of C_2 units used for synthesis is a major peroxisomal function and that this function is absent or greatly impaired in Zellweger's syndrome cells.

INTRODUCTION

Peroxisomes were first described in 1954 (Rhodin, 1954), but their more important functions such as fatty acid β-oxidation have been characterized more recently (Lazarow & de Duve, 1976). In particular, peroxisomes probably play an important role in the oxidation of very-long-chain fatty acids (VLCFA) (greater than 22 carbon atoms in length) (Osmundsen *et al.*, 1980; Singh *et al.*, 1984*a*, 1987*a*,*b*; Singh & Poulos, 1986). VLCFA are known to accumulate in several disease states (Igarashi *et al.*, 1976; Brown *et al.*, 1982; Moser *et al.*, 1984; Sharp *et al.*, 1987; Poulos *et al.*, 1987) including Zellweger's syndrome, a fatal autosomal recessive disease with multiple biochemical and clinical abnormalities. The disease is characterized ultrastructurally by an absence or severe deficiency of peroxisomes (Goldfischer *et al.*, 1973; Arias *et al.*, 1985). The accumulation of saturated, mono-unsaturated and polyunsaturated VLCFA in Zellweger tissues suggests that peroxisomes are involved in the regulation of catabolism or biosynthesis of these fatty acids.

A number of studies have described the elongation and desaturation of long-chain (greater than 14 carbon atoms in length) and very-long-chain saturated and polyunsaturated fatty acids by human tissues. Rosenthal & Hill, working with cultured human endothelial cells, reported that a number of VLCFA, including $C_{24:4}$, $C_{24:5}$, $C_{26:4}$ and $C_{26:5}$ were synthesized from $C_{20:3,n-6}$ (Rosenthal & Hill, 1984) and subsequently demonstrated elongation of $C_{20:5,n-3}$ to $C_{22:5}$ and $C_{20:4,n-6}$ to $C_{22:4}$ (Rosenthal & Hill, 1986). Human skin fibroblasts in culture have been shown to elongate $C_{22:4,n-6}$ (Gavino *et al.*, 1981), $C_{20:3}$, $C_{20:4}$ and $C_{20:5}$ (Banerjee & Rosenthal, 1986) and the saturated fatty acids $C_{16:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{22:0}$ (Tsuji *et al.*, 1984). Recently, Christensen *et al.* (1988) have reported that normal and Zellweger's syndrome fibroblasts can elongate $C_{22:1,n-9}$ to $C_{24:1,n-9}$ and $C_{18:3,n-3}$ to a number of elongated products including C_{20} and C_{22} polyunsaturated fatty acids.

Metabolic products from long chain fatty acid (LCFA) and VLCFA oxidation in normal and peroxisomal-deficient skin fibroblasts have not been fully characterized. Traditionally, oxidation of fatty acids has been determined by measurement of $^{14}CO_2$ production from ^{14}C-labelled fatty acids, although several studies have reported, in addition, formation of water-soluble products in various tissue preparations (Van Hinsbergh *et al.*, 1978; Rosenthal & Hill, 1984; Kondrup & Lazarow, 1985; Christensen *et al.*, 1986). Singh & Poulos (1986) identified the water-soluble product from lignocerate and stearate in disrupted fibroblast cell homogenates as predominantly acetyl-CoA. In rat brain preparations glutamate was the principal water-soluble product from lignoceric (Uda *et al.*, 1981) and palmitic acids (Kawamura & Kishimoto, 1981). Tissue differences are apparent since palmitic acid oxidation by rat liver preparations gave rise to mainly organic acids, with amino acids as a minor component (Kawamura & Kishimoto, 1981).

Comparison of fatty acid metabolism in tissues from normal subjects and patients with peroxisomal disorders permits analysis of the relative roles of mitochondrial and peroxisomal β-oxidation and the interrelationship of

Abbreviations used: VLCFA, Very-long-chain fatty acids; LCFA, long-chain fatty acids; BMEM, Basal Modified Eagle's Medium; DCF, dichlorofluorescein; $C_{16:0}$, palmitic acid; $C_{20:4}$, arachidonic acid; $C_{24:4}$, tetracosatetraenoic acid; $C_{24:0}$, lignoceric acid.

β-oxidation and metabolic products from fatty acids. In the present paper we report the metabolism of the LCFA palmitic ($C_{16:0}$) and arachidonic ($C_{20:4,n-6}$) acids as well as the VLCFA lignoceric ($C_{24:0,n-6}$) and tetracosatetraenoic ($C_{24:4,n-6}$) acids in cultured human skin fibroblasts from normal subjects and patients with Zellweger's syndrome.

MATERIALS AND METHODS

Materials

All solvents used were from May and Baker Australia Pty. Ltd., VIC or from Ajax Chemicals, Sydney, NSW, Australia. Reversed-phase KC-18 t.l.c. plates were purchased from Whatman Inc., Clifton, NJ, U.S.A. and thin-layer silica gel 60 plates were from E. Merck, Darmstadt, Germany. Basal Modified Eagle's Medium (BMEM) and fetal calf serum were purchased from Flow Laboratories, North Ryde, NSW, Australia. Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) was from Commonwealth Serum Laboratories, Melbourne, VIC, Australia, and bovine serum albumin (fatty-acid free), butylated hydroxytoluene (BHT) and Dowex-50W strongly acid cation-exchange resin (12% cross-linked; dry mesh 200–400) were from Sigma Chemical Co., St. Louis, MO, USA. Bio-Rad protein assay dye reagent concentrate was from Bio-Rad Laboratories, Richmond, CA, U.S.A., LKB Ultrafilm (^{3}H-labelled) from LKB-Produkter AB, Bromma, Sweden and 10% Palladium on carbon catalyst from Riedel-de Haën AG, Hannover, Germany. NCS solubilizer for liquid scintillation counting and [1-^{14}C]arachidonic acid ($C_{20:4,n-6}$) (58.3 mCi/mmol) were purchased from Amersham Australia Pty. Ltd., North Ryde, NSW, Australia and [1-^{14}C]palmitic acid ($C_{16:0,n-6}$) (53 mCi/mmol) from New England Nuclear, Boston, MA, U.S.A. [1-^{14}C]Lignoceric acid ($C_{24:0}$) (59 mCi/mmol) was prepared as described previously (Singh & Poulos, 1986). [1-^{14}C]Tetracosatetraenoic acid ($C_{24:4,n-6}$) was prepared in the following manner. *ZZZZ*-Docosa-7,10,13,16-tetraenoic acid ($C_{22:4,n-6}$) (NU CHEK PREP, Elysian, U.S.A.) was converted to [1-^{14}C]*ZZZZ*-tetracosa-9,12,15,18-tetraenoic acid ([1-^{14}C]$C_{24:4,n-6}$) by two sequential one-carbon homologation procedures involving esterification with diazomethane, reduction, with lithium aluminium hydride, mesylation with methane sulphonyl chloride in pyridine, displacement with sodium cyanide in dimethyl sulphoxide, and hydrolysis in alkaline ethanol/water (3:2, v/v). In the second displacement step, tricosa-8,11,14,17-tetraenylmethane sulphonate (5 mg) was reacted with sodium [^{14}C]cyanide (0.5 mCi, 55 mCi/mmol) (Amersham International, Amersham, Bucks., U.K.) in dimethyl sulphoxide-d_6 (1 ml) at 70 °C for 4 h. The 1-^{14}C-labelled nitrile was extracted with hexane and hydrolysed in ethanol/10% aqueous sodium hydroxide (2:1, v/v, 1 ml) at 80 °C for 16 h to afford [1-^{14}C]*ZZZZ*-tetracosa-9,12,15,18-tetraenoic acid ([1-^{14}C]$C_{24:4,n-6}$) which was purified by chromatography on a silica gel 60 plate (10 cm × 20 cm) with hexane/ether/acetic acid (40:10:1, by vol.). G.c.m.s. of the methyl ester showed one homogenous peak, EI-MS *M/z* 362 ([^{14}C]-M^+, 3.8%), 360 ([^{12}C]-M^+, 0.7%), 150 (17%), 80 (100%).

Zellweger lines

The diagnosis of Zellweger's syndrome was based on clinical history and examination and confirmed by biochemical investigations (Polous *et al.*, 1987). Cell lines were kindly provided by Dr. A. Clague (Royal Brisbane Hospital), Dr. J. Rogers and Dr. L. Sheffield (Murdoch Institute, Melbourne, Australia), and Dr. G. Sherwood (Hospital for Sick Children, Toronto, Canada).

Fibroblasts

Cultured skin fibroblasts from normal subjects and Zellweger patients were grown in tissue culture flasks (25 cm^2) in BMEM containing 10% (v/v) fetal calf serum for 7–10 days until confluent. The culture medium was removed and the cells were incubated in BMEM without fetal calf serum for 24 h prior to addition of substrate. Between 3 and 5 different normal and Zellweger cell lines were used with each substrate.

Preparation of substrates

Radiolabelled arachidonic and tetracosatetraenoic acids (15–30 nmol) were dried under N_2 and incubated in 0.6 M-NaOH (5 μl) and fetal calf serum for 30 min at 37 °C. BMEM was added and the substrate was sterilized by passage through a Millex-GS filter (0.22 μm pore size). The final concentration of fetal calf serum was 1–2%. The substrate mixture was gassed with CO_2 prior to addition to the fibroblast cultures. Under these conditions, 70% of the substrate bound to fetal calf serum and passed through the filter.

Radiolabelled lignoceric and palmitic acids, which are not efficiently bound to fetal calf serum, were bound to bovine serum albumin (fatty-acid free) as follows. The substrates were incubated in 0.6 M-NaOH (5 μl) and ethanol (300 μl) at 75 °C for 15 min. After cooling to room temperature, 1 ml of fatty-acid free bovine serum albumin (50 mg/ml) was added slowly and the substrates incubated at 37° C for 60 min. Fetal calf serum and BMEM were added, and the substrates were sterilized and further prepared as described above.

Incubation of 1-^{14}C-labelled fatty acids with fibroblasts in culture

Prepared substrates (approx. 4 nmol; 5×10^5 d.p.m.) were added to confluent normal and Zellweger cell cultures, and the flasks were sealed with sterilized rubber stoppers. Corresponding control incubations were done in culture flasks without cells. After incubation at 37 °C for periods between 7 h and 4 days, the released $^{14}CO_2$ was quantified as described previously (Poulos, 1981). The medium was removed and the cells washed with Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) prior to harvesting in 3 ml of 0.1% (v/v) Triton X-100. The harvested cells were disrupted by ultrasonication and an aliquot (100 μl) was assayed for protein as described by Bradford (1976). All substrates and cell lines were incubated under similar conditions with similar amounts of added radioactivity.

Lipid extraction and analysis

Lipids were extracted from the media and cell suspension by the Bligh & Dyer (1959) method. BHT was added at all stages of extraction and analysis, to minimize auto-oxidation of unsaturated fatty acids. Lipid hydrolysis and methylation of the liberated fatty acids in the lower phase were carried out in methanol/H_2SO_4 (200:3, v/v) at 75 °C for 3 h. The methyl esters were extracted with 3 ml of hexane and purified by t.l.c. on a silica gel 60 t.l.c. plate in dichloromethane. The zone corresponding to the fatty acid methyl esters was revealed

under u.v. light after spraying with dichlorofluorescein (DCF) (0.1 %, w/v) in methanol/water (95:5, v/v). The scraped regions of gel were extracted with 3 ml of chloroform/methanol (2:1, v/v). The DCF was removed by partitioning with 1 drop of 2 M-NH_4OH and 600 μl of 0.1 M-KCl. The bottom layer was washed with 500 μl of synthetic upper phase (Folch *et al.*, 1957). Extracted fatty-acid methyl esters were applied to reversed-phase KC-18 plates and developed twice in the same direction in acetonitrile/tetrahydrofuran (90:10, v/v). Appropriate standards were co-chromatographed to assist identification of the products. Autoradiographs were prepared by exposing ^{3}H-labelled Ultrafilm to the plates for 5–10 days.

Argentation t.l.c.

Argentation t.l.c. was used to separate fatty acid groups on the basis of the degree of unsaturation. Silica-gel 60 plates (20 cm × 20 cm, 0.25 mm) were sprayed with $AgNO_3$ as described previously (Inomata *et al.*, 1982) and fatty-acid methyl esters were chromatographed in hexane/ether (95:5, v/v). Mixtures of C_{16-24} saturated, mono-unsaturated and polyunsaturated fatty acids were co-chromatographed to assist identification of the products. After spraying the plate with DCF, bands were revealed under u.v. light and extracted as described above. The saturated, mono-unsaturated and polyunsaturated fatty acids groups were further separated by reversed-phase t.l.c. as described above.

Hydrogenation

Cell lipid extracts were hydrogenated in order to gain information about fatty-acid chain length. The extracts were diluted to 5 ml with hexane, and palladium catalyst (0.1 g) was added. The solution was stirred vigorously under hydrogen for 7 h, filtered and the product chromatographed by reversed-phase t.l.c. as described above.

Identification of water-soluble product

Preliminary experiments characterizing the radiolabelled product observed in the aqueous phase, following partitioning of media and cell extracts, showed that the product was not volatile (> 90 %) and that no volatile product was released by alkaline hydrolysis. Only small amounts of the compound(s) were extracted from acidified aqueous solution with ethyl acetate or diethyl ether (< 10 %) and extractable products were not released by alkaline hydrolysis or methanol/H_2SO_4 trans-esterification. The compound(s) were removed by dialysis against water using dialysate tubing with a molecular mass cut-off of 20000 Da. Although partially soluble in chloroform and soluble in chloroform/methanol (2:1, v/v), the product(s) were preferentially extracted from the latter into aqueous solution (> 75 %). Using the Bligh & Dyer (1959) extraction method, some losses of these products may occur on partitioning because of incomplete extraction.

The upper aqueous phase extract resulting from the extraction of conditioned medium (5 ml of medium) was dried under N_2. The radiolabelled product was dissolved in 2 × 10 ml of aqueous solution adjusted to pH 2 with 0.1 M-HCl and applied to a Dowex 50 column (7 cm × 1 cm) prepared in water. The column was washed with 40 ml of water and eluted with 20 ml of 2 M-ammonia. In four experiments using radiolabelled product from $C_{20:4}$ and $C_{24:4}$, 41–48 % of the radioactive product was not retained by the column and 45–64 % was retained until eluted with ammonia. No radioactivity was observed in the aqueous wash. The non-retained and retained fractions were reapplied to new Dowex 50 columns under similar conditions and exhibited consistent elution patterns with that observed with initial separation. On the basis of these results the retained fractions were assayed for the presence of radiolabelled amino acids by t.l.c. and by ion-exchange chromatography. T.l.c. was carried out on cellulose plates in acetone/*n*-butanol/acetic acid/water (35:35:10:20, by vol.) Before ion-exchange chromatography, pooled samples were treated with an equivalent volume of sulphosalicylic acid solution (20 %, w/v), centrifuged (1000 ***g***, 10 min) and the supernatant so formed diluted 1:1 with 0.24 M-lithium citrate buffer, pH 2.75, containing 100 μmol of norleucine/l as the internal standard. The amino acids were separated on a cation-exchange column using lithium citrate buffers and a Dionex Amino Acid Analyser. Detection was carried out by post-column derivatization using *o*-phthalaldehyde in borate buffer, pH 10.4. Retention times of individual amino acids were determined using Pierce amino acid standard mixtures.

The non-retained fraction was analysed for volatility and solubility characteristics. As with the total water-soluble product, the product was not volatile (< 20 %) and no volatile product was released on alkaline hydrolysis. Following alkaline hydrolysis the compounds could not be extracted into hexane and only a small proportion (10 %), also non-volatile, was extracted by ethyl acetate.

RESULTS

Uptake and incorporation into lipids

In 3 days, 37–66 % of the radioactivity from [1-^{14}C]-arachidonic acid added to normal cell cultures was recovered in the lipid extracts of the cells. In contrast, 90–95 % was recovered in the lipid extract from cultured Zellweger fibroblasts using the same substrate. With [1-^{14}C]tetracosatetraenoic acid, 11–24 % of the label was recovered from normal cells and 71–95 % from Zellweger cell lipid extracts. Neither of these results reflects a true uptake of the labelled substrates by the cells, since in all cases only a small proportion (2–4 %) of the substrates remained unchanged in the media. Metabolism of the label by the cells into CO_2 and water-soluble products, incorporation into synthesis of fatty acids and elongation and desaturation of the substrate accounted for the remaining radioactivity.

With the saturated fatty acid [1-^{14}C]lignoceric acid, much lower amounts appeared to be taken up into the cells, with 60–80 % of the substrate remaining unchanged in the media. In contrast, [1-^{14}C]palmitic acid was more actively taken up into the cells, with 45–58 % of the label recovered from the lipid extracts of both control and Zellweger cells. Unchanged substrate in the media lipid extract accounted for only 10–25 % of the added radioactivity. The low uptake of lignoceric acid into the cells is unlikely to be due to poor solubility since the fatty acid was bound to albumin as described in the Materials and methods section.

Very little of the ^{14}C-labelled fatty acids recovered in the cell lipid extract remained as free fatty acids. Most of the radioactivity was incorporated into a range of neutral and polar lipids. Labelled substrates added to control

Table 1. Individual fatty acid metabolites from labelled substrates: distribution in cells from normal and Zellweger skin fibroblast cultures

Fibroblast cell cultures were incubated with [1-^{14}C]arachidonic, [1-^{14}C]tetracosatetraenoic, [1-^{14}C]lignoceric and [1-^{14}]palmitic acids, and the products were identified as described in the Materials and methods section. Values are shown as a range from three cell lines incubated with substrate for 72 h and are expressed as a percentage of total recovered radioactivity. tr., trace.

		Distribution (% of total recovered radioactivity)							
	Substrate...	Arachidonic acid ($C_{20:4}$)		Tetracosatetraenoic acid ($C_{24:4}$)		Lignoceric acid ($C_{24:0}$)		Palmitic acid ($C_{16:0}$)	
Fatty acid	Fibroblasts...	Normal	Zellweger	Normal	Zellweger	Normal	Zellweger	Normal	Zellweger
$C_{14:0}$		tr.–3.2	0	tr.–2.5	0	2.2–2.6	0	0	0
$C_{16:0}$		2.2–4.6	0	7.0–26.6	0	15.3–19.7	0–2.5	64.2–75.5	84.5–91.1
$C_{18:0}$		1.0–2.7	0	3.4–16.1	0	8.4–9.2	0	17.5–19.0	5.4–11.0
$C_{20:0}$		tr.	0	tr.	0	0	0	0–tr.	0
$C_{22:0}$		tr.	0	tr.–1.7	0	0	0	0–tr.	0
$C_{24:0}$		tr.	0	tr.–3.2	0	59.1–62.3	75.5–79.2	0–1.5	0–tr.
$C_{26:0}$		0	0	0	0	2.9–4.0	16.3–20.9	0	0
$C_{28:0}$		0	0	0	0	0	2.0–3.6	0	0
$C_{16:1}$		tr.	0	tr.	0	0–3.9	0	1.0–2.8	0–1.7
$C_{18:1}$		tr.–2.9	0	1.0–4.9	0	0	0	1.0–5.7	tr.–1.2
$C_{20:1}$		tr.–1.4	0	tr.–1.9	0	0	0	0	0
$C_{22:1}$		tr.	0	tr.–1.1	0	0	0	0	0
$C_{24:1}$		tr.	0	tr.–2.9	0	0	0	0	0
$C_{20:4}$		68.7–81.0	59.6–72.5	0	0	0	0	0	0
$C_{22:4}$		12.4–15.8	20.0–27.4	0	0	0	0	0	0
$C_{24:4}$		1.5–2.5	5.8–9.6	23.5–64.1	59.1–76.6	0	0	0	0
$C_{26:4}$		0–0.5	1.7–3.4	7.8–9.0	10.8–19.5	0	0	0	0
$C_{28:4}$		0	tr.	0–1.6	1.3–3.0	0	0	0	0
Unknown polyunsaturate(s)		0	0	7.1–10.8	4.2–28.7	0	0	3.4–6.6	1.6–3.0

incubations without cells were recovered unchanged and no significant $^{14}CO_2$ or ^{14}C-labelled water-soluble-product production was observed.

Elongation and desaturation

Elongation of $C_{20:4}$, $C_{24:4}$ and $C_{24:0}$ was observed, to a maximum of C_{26} chain-length in normal fibroblast cultures, whereas in Zellweger cultures small amounts of C_{28} fatty acids were detected ($C_{28:4}$, $C_{28:4}$ and $C_{28:0}$ respectively). The principal elongation product in each case was formed by simple C_2 additions with formation of $C_{22:4}$, $C_{26:4}$ and $C_{26:0}$ respectively. Addition of further C_2 units to $C_{22:4}$ gave rise to small amounts of $C_{24:4}$ and $C_{26:4}$. A radiolabelled polyunsaturated product from $C_{24:4}$ which chromatographed faster than $C_{24:4}$ on reversed-phase t.l.c. and was particularly prominent in extracts from three of five different Zellweger fibroblast cultures was tentatively identified as $C_{24:5}$. At least two unidentified polyunsaturated fatty acids were observed in association with normal fibroblast metabolism of $C_{24:4}$, and up to three such metabolites were derived from $C_{16:0}$ in normal and Zellweger fibroblasts. No polyunsaturated fatty acids were observed as a result of $C_{24:0}$ metabolism in normal or Zellweger fibroblasts. The percentage contribution of each fatty acid metabolite to the total fatty acid profile after 3 days' incubation is shown in Table 1. Similar elongation profiles were observed for incubation time periods of 7 h, 1 day and 2 days. Although the proportion of metabolites was not measured quantitatively, autoradiography results suggest that the proportion of longer chain metabolites increased with time in most cases.

Media fatty-acid profiles were similar to cell profiles for the polyunsaturated substrates, although C_{28} polyunsaturated fatty acids and $C_{24:0}$ elongation products were not detectable in media from any of the cell lines.

Palmitic acid ($C_{16:0}$) was elongated to stearic acid ($C_{18:0}$), detectable in both cells and media, but only trace amounts of longer-chain products were detectable in control and some Zellweger cell lines.

Saturated and mono-unsaturated fatty acids synthesis

Small amounts of C_{14-24} saturated fatty acids were observed with normal fibroblast cell lines incubated with $C_{20:4}$ and $C_{24:4}$. Traces of mono-unsaturated fatty acids were also detected ($C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$ and $C_{24:1}$). With $C_{24:0}$, the maximum chain length of synthesized saturated fatty acid observed was stearic acid ($C_{18:0}$). Saturated and mono-unsaturated fatty acids synthesized from $C_{20:4}$ or $C_{24:4}$ were not detected in Zellweger fibroblast cultures. The distribution of saturated, mono-unsaturated and polyunsaturated fatty acids synthesized from the different substrates by normal and Zellweger fibroblast cultures is shown in Table 2. Cell lines used to obtain data for Table 2 were not necessarily the same as those used for Table 1.

$^{14}CO_2$ production

[^{14}C]Carbon dioxide was produced from all four fatty acids by control cell lines, the amount being dependent

Table 2. Fatty acid metabolites from labelled substrates: percentage distribution of saturates, mono-unsaturates and polyunsaturates in cells from normal and Zellweger skin fibroblasts

Fibroblast cell cultures were incubated as described in Table 1. Fatty acid metabolites from added 1-^{14}C-labelled substrates were characterized using argentation t.l.c. as described in the Materials and methods section. Values are shown as % of recovered metabolites and are a range obtained with at least three control and three Zellweger cell lines incubated with substrate for 72 h.

	Distribution (% of recovered radioactivity)							
	$C_{20:4}$		$C_{24:4}$		$C_{24:0}$		$C_{16:0}$	
	Control	Zellweger	Control	Zellweger	Control	Zellweger	Control	Zellweger
Saturates	3.8–12.3	0	12.7–64.5	0	96.1–100	97.5–100	92–92.8	92.9–98.2
Mono-unsaturates	0–2.6	0	4.5–11.1	0	0–3.9	0–2.5	3.5–4.5	0.7–2.9
Polyunsaturates	83.5–95.5	100.0	31.9–84.4	100.0	0	0	3.3–4.2	0–4.3

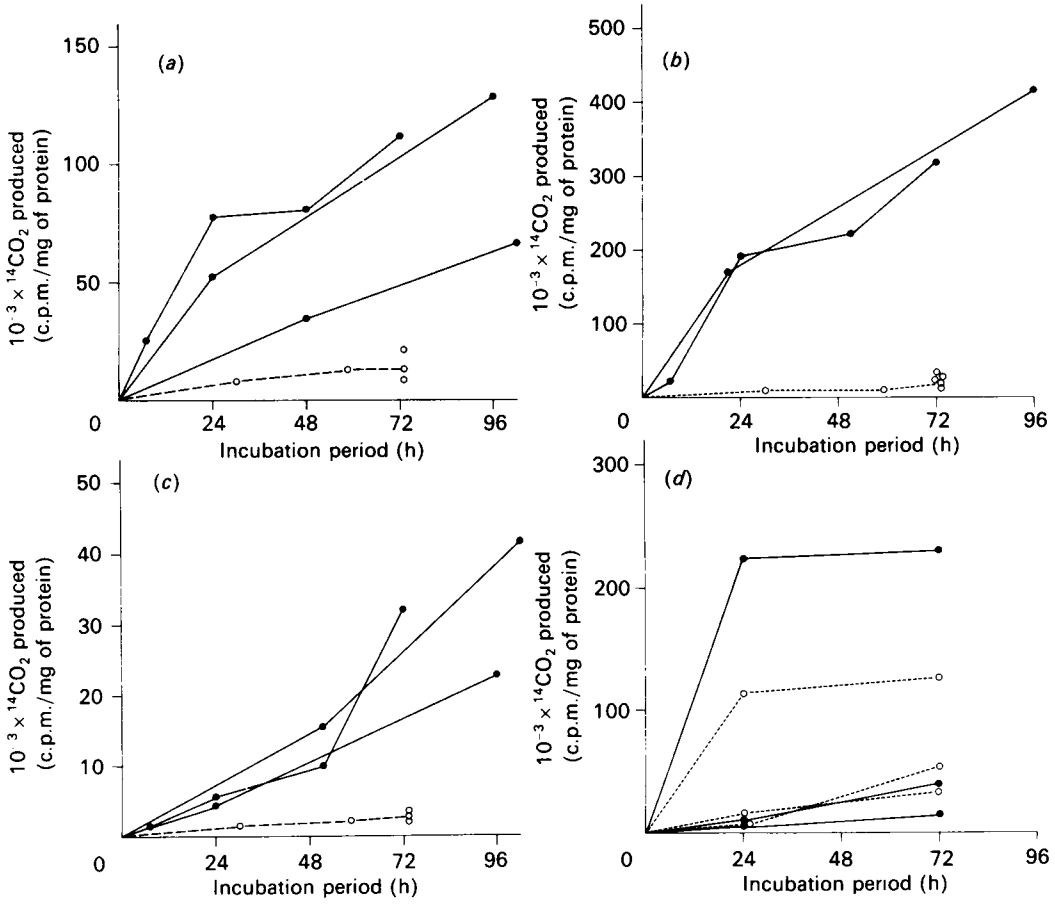

Fig. 1. Time course of $^{14}CO_2$ production from labelled fatty acids.

(*a*) [1-^{14}C]Arachidonic acid ($C_{20:4}$), (*b*) [1-^{14}C]tetracosatetraenoic acid ($C_{24:4}$), (*c*) [1-^{14}C]lignoceric acid ($C_{24:0}$) and (*d*) [1-^{14}C]palmitic acid ($C_{16:0}$). Flasks of confluent cells from 2–3 different control (●) and Zellweger (○) cell lines were incubated with the labelled substances as described in the Materials and methods section. One flask of cells was used to measure $^{14}CO_2$ production at each time point, and the same cell line was used for each time course shown.

on the type of fatty acid substrate, uptake efficiency and incubation period (Fig. 1). The polyunsaturated fatty acids, in particular $C_{24:4}$, produced much greater amounts of $^{14}CO_2$ than the saturated $C_{24:0}$ and $C_{16:0}$. $^{14}CO_2$ production from $C_{24:4}$ represented conversion of as much as 10% of the added labelled substrate. In contrast, only 1–2% of the added $C_{16:0}$ and $C_{24:0}$ label was detected as $^{14}CO_2$ after 3 days' incubation. In comparison with normal cell lines, $^{14}CO_2$ production was greatly reduced in Zellweger cell cultures with the longer-chain fatty-acid substrates $C_{20:4}$, $C_{24:0}$ and $C_{24:4}$ but was normal with $C_{16:0}$ (Fig. 1).

Formation of water-soluble product

Monitoring of labelled products, subsequent to partitioning of media and cell extracts from control cell cultures, showed that considerable radioactivity appeared in the upper aqueous phase, particularly in media extracts from polyunsaturated fatty acids. Water-soluble radiolabelled compounds comprised as much as 34% of the added radioactivity in normal fibroblast cultures incubated with [1-^{14}C]$C_{24:4}$, with 95% of the water-soluble product in the media extract and only 5% in the cell extract. Zellweger fibroblast cultures exhibited a markedly reduced capacity to produce the water-soluble product from the longer-chain radiolabelled fatty acid substrates $C_{20:4}$, $C_{24:0}$, and $C_{24:4}$. In contrast, synthesis of the water-soluble product from [1-^{14}C]-palmitic acid ($C_{16:0}$) was similar (5–8% of added labelled substrate) in both normal and peroxisome-deficient cell lines.

As with $^{14}CO_2$ synthesis, the amount of water-soluble product observed in normal cell cultures was dependent upon the fatty acid substrate, uptake efficiency and incubation period (Fig. 2). Synthesis of radiolabelled water-soluble product was greater than $^{14}CO_2$ production with all cell lines and all substrates.

Characterization of the water-soluble product

Preliminary experiments on volatility and solubility showed that acetate and acetyl-CoA were not major radiolabelled metabolic products from the long-chain fatty acids in normal fibroblast cultures. However, 45–64% of the water-soluble product(s) was retained by a strong cation-exchange column.

Amino acid analysis of this fraction suggests that these products are a series of amino acids including glutamine, asparagine and glutamic acid. The identity of the other one or two amino acid products has not as yet been established, although on a cation-exchange column radioactivity co-eluted with aspartic acid and glycine. The identity of the product(s) not retained on cation-exchange columns is not known.

DISCUSSION

The marked difference between normal and Zellweger skin fibroblasts in production of CO_2 and water-soluble compounds from $C_{20:4}$, $C_{24:0}$ and $C_{24:4}$ fatty acids suggests that in skin fibroblasts these substrates are predominantly oxidized in peroxisomes with mitochondrial oxidation contributing only a minor role. This

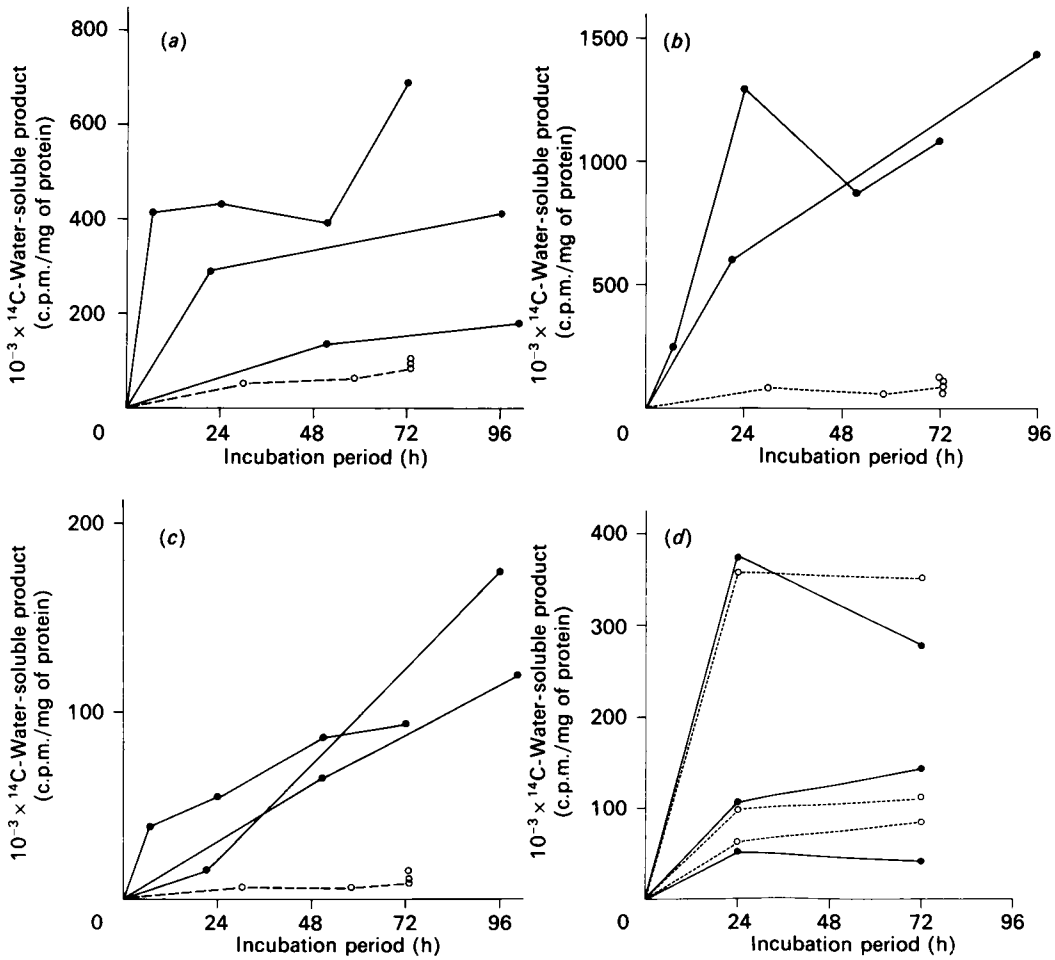

Fig. 2. Time course of synthesis of ^{14}C-labelled water-soluble product from labelled fatty acids

(*a*) [1-^{14}C]Arachidonic acid, (*b*) [1-^{14}C]tetracosatetraenoic acid, (*c*) [1-^{14}C]lignoceric acid, (*d*) [1-^{14}C]palmitic acid. ●, Control and ○, Zellweger cell lines. Conditions were as described in the legend to Fig. 1.

is in agreement with previous work using the saturated VLCFA $C_{24:0}$, in our laboratory (Singh & Poulos, 1986; Poulos *et al.*, 1986; Singh *et al.*, 1987*a*,*b*) and others (Osmundsen *et al.*, 1980; Singh *et al.*, 1984*a*,*b*). In contrast, palmitic acid, as shown in this study and others (Lazarow & de Duve, 1976; Singh *et al.*, 1984*a*; Kondrup & Lazarow, 1985) can be effectively degraded by β-oxidation in mitochondria. Our findings do not preclude some contribution of peroxisomal oxidation in the degradation of palmitic acid in the intact cell (Lazarow & de Duve, 1976; Kondrup & Lazarow, 1985). Although it has been demonstrated that hepatic peroxisomes can β-oxidize long-chain polyunsaturated fatty acids (Hiltunen *et al.*, 1986; Havik & Osmundsen, 1987), several studies have suggested that mitochondrial β-oxidation is the main route for $C_{20:4}$ degradation (Osmundsen & Bjornstad, 1985; Christensen *et al.*, 1986; Hiltunen *et al.*, 1986). In our present work, degradation of both the polyunsaturated fatty acids $C_{20:4}$ and $C_{24:4}$ to $^{14}CO_2$ and ^{14}C-labelled water-soluble products is markedly decreased in Zellweger fibroblasts compared with controls. These results suggest that peroxisomal function is necessary, at least in the initiation of oxidation of arachidonic acid, as well as for longer chain polyunsaturated fatty acids, in cultured skin fibroblasts. This discrepancy with previously described work may be due to tissue or species differences. However, the use by Christensen *et al.* (1986) of decanoylcarnitine to inhibit mitochondrial β-oxidation and hence show the relative roles of peroxisomal and mitochondrial β-oxidation pathways, is controversial, with the recent evidence that the carnitine acyltransferase found on the outer surface of mitochondria may be due to peroxisomal contamination of mitochondrial preparations (Vamecq, 1987; Healy *et al.*, 1988; Ramsay, 1988). The role of peroxisomal carnitine acyltransferase is not well defined, but it may be important in the transport of short-chain acyl-CoAs from the peroxisomes and hence determine their availability for further mitochondrial metabolism, or it may regulate fatty acid entry into the peroxisome (Vamecq, 1987). More recently, in accordance with our findings, β-oxidation of C_{22} unsaturated fatty acids was shown to be impaired in cultured Zellweger skin fibroblasts in comparison with controls (Christensen *et al.*, 1988). These results support our contention that chain shortening of fatty acids longer than C_{18} is principally a peroxisomal function.

With all the substrates used in this study, production of labelled water-soluble material from degradation of the fatty acids was greater than $^{14}CO_2$ production, in control ($\times 7.6 \pm 5.4$) and Zellweger ($\times 4.4 \pm 2.4$) fibroblasts. The distribution between CO_2 and water-soluble products is similar to that described previously for 1-^{14}C-labelled fatty acids (Kondrup & Lazarow, 1985; Christensen *et al.*, 1986). In our studies the ratio of ^{14}C-labelled water-soluble product/$^{14}CO_2$ decreased with time of incubation in all cases, suggesting that the water-soluble products were produced prior to CO_2 production. It is of interest that the water-soluble products were found almost exclusively in the media and constituted transformation of 17–34% of added [^{14}C]tetracosatetraenoic acid, 7–17% of added [^{14}C]arachidonic acid and 1–8% of added [^{14}C]lignoceric acid in normal fibroblast cultures. Much smaller amounts, decreasing with incubation time, were detected in the cells. Although water-soluble products from fatty acid oxidation have been described in rat liver homogenates (McGarry *et al.*, 1977; Kawamura & Kishimoto, 1981; Glatz *et al.*, 1983), rat skeletal muscle mitochondria (van Hinsberg *et al.*, 1978), rat brain homogenates (Uda *et al.*, 1981), isolated rat hepatocytes (Kondrup & Lazarow, 1985), fibroblast homogenates (Singh & Poulos, 1986), rat tissue homogenates (Veerkamp *et al.*, 1986) and human muscle preparations (Veerkamp *et al.*, 1986), few studies have attempted to identify them. Our preliminary experiments suggest that in fibroblast cultures, acetate and short-chain fatty acids or their CoA derivates, when produced, are converted to other products. However, from their behaviour on t.l.c. and ion-exchange column chromatography, there is evidence to suggest that a significant proportion of the product comprises a series of amino acids including glutamate, glutamine and asparagine. These amino acids are presumbaly produced by incorporation of labelled acetyl-CoA into the tricarboxylic acid cycle followed by transamination of labelled tricarboxylic acid cycle intermediates.

Although the amino acids formed in our studies from β-oxidation of fatty acids may represent a net production important in these and other functions within the fibroblast cell, their appearance may also represent simple dilution of label by exchange with the unlabelled amino acids in the culture medium. The high activity of the transaminase systems involved and the rapid exchange of glutamate across mitochondrial and plasma membranes would support this contention. Other studies using disrupted fibroblast cell homogenates (Singh & Poulos, 1986) and rat skeletal muscle mitochondria (van Hinsbergh *et al.*, 1978) identified the water-soluble products as acetyl-CoA and citric acid cycle intermediates respectively. The short incubation periods involved, disrupted systems and absence of excess unlabelled amino acids may well prevent significant transfer of label to amino acids in these systems. However, rat brain preparations with added amino acids in the assay medium and incubations of 6–10 min were shown to oxidize [1-^{14}C]-palmitic acid to amino acids (mainly glutamate and aspartate) and organic acids (mainly citrate) (Kawamura & Kishimoto, 1981). Similar experiments with rat liver preparations showed that in this tissue the principal water-soluble products were succinate, citrate and 3-hydroxybutyrate with minor amounts of amino acids (Kawamura & Kishimoto, 1981). Glutamate has been also identified as a major product of lignoceric fatty acid oxidation in rat brain preparations (Uda *et al.*, 1981). These preparations were incubated in the presence of considerable crude material from calf cerebellum and the amino acid composition of the incubation system is unknown.

The small amounts of labelled saturated and mono-unsaturated fatty acids observed in control fibroblast cultures presumably reflect β-oxidation and recycling of labelled acetate moieties in synthesis *de novo* or chain elongation of endogenous fatty acid. This contention is supported by the complete absence of saturated and mono-unsaturated fatty acids in media and cell extracts from Zellweger syndrome fibroblast cultures in which peroxisomal β-oxidation is absent or severely reduced. The longer-chain-length fatty acids ($> C_{18}$) and proportionately greater conversion to these products seen with polyunsaturated fatty acids is presumably due to increased amounts of available labelled acetate with these substrates. Synthesis of long-chain fatty acids

de novo has been described by other workers in fibroblast cell cultures (Tsuji *et al.*, 1984), rat brain preparations (Uda *et al.*, 1981) and endothelial cell cultures (Rosenthal & Hill, 1984). Recycling of labelled acetate in normal cell lines into shorter-chain fatty acids may also account for differences in mono-unsaturated and polyunsaturated fatty acid patterns observed between control and Zellweger cells lines incubated with $C_{24:4}$.

$C_{24:4}$, $C_{24:0}$ and also $C_{20:4}$ were all presumably elongated by addition of C_2 units by the action of microsomal or mitochondrial elongases. The longest chain lengths seen with normal fibroblast cultures were their corresponding C_{26} polyunsaturated or saturated fatty acids. The apparent termination of elongation at this point is possibly due to the low amounts of available substrate, because C_{28} elongation products were observed in Zellweger's syndrome, albeit in trace amounts; moreover, increased conversion to all elongation products occurred in Zellweger syndrome cell extracts. As the catabolism of VLCFA is severely impaired in Zellweger fibroblast cells, added labelled substrates are channelled principally into elongated and desaturated metabolites. Conversion of VLCFA substrates to more polar metabolites such as prostaglandins and leukotrienes (Samuelsson, 1983) was not assessed.

The use of Zellweger syndrome cell lines, in which peroxisomes are absent, permits assessment of the peroxisomal role in the metabolism of fatty acids. Our results show that peroxisomal function is essential for the effective catabolism of the VLCFA $C_{24:0}$ and $C_{24:4}$, as well as for $C_{20:4}$, and that prevention of their normal catabolism in peroxisomes channels these fatty acids into elongated metabolites. This provides a possible mechanism for regulation of availability of the VLCFA for elongated and oxygenated metabolites. The role of the VLCFA in normal tissues is not clear, but their accumulation in peroxisomal disorders (Poulos *et al.*, 1986; Sharp *et al.*, 1987) may contribute to the neurodegeneration and other clinical symptoms characteristic of some of these diseases. Analysis of the products of fatty acid catabolism in normal fibroblasts suggests that an important role of peroxisomes is provision of acetyl-CoA for synthetic functions, such as synthesis of saturated and monounsaturated fatty acids *de novo*. The role of conversion of label into other water-soluble products is less certain, but our work supports previous studies in showing that analysis of $^{14}CO_2$ alone is not a sufficient index of β-oxidation of fatty acids.

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REFERENCES

Arias, J. A., Moser, A. B. & Goldfischer, S. L. (1985) J. Cell Biol. **100**, 1789–1792

Banerjee, N. & Rosenthal, M. D. (1986) Biochim. Biophys. Acta **878**, 404–411

Bligh, B. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Brown, F. R., McAdams, A. J., Cummins, J. W., Konkol, R., Singh, I., Moser, A. B. & Moser, H. W. (1982) Johns Hopkins Med. J. **151**, 344–361

Christensen, E., Hagve, T.-A. & Christophersen, B. O. (1986) Biochim. Biophys. Acta **879**, 313–321

Christensen, E., Hagve, T.-A. & Christophersen, B. O. (1988) Biochim. Biophys. Acta **959**, 95–99

Folch, J., Lees, M. & Sloane-Stanley, G. (1957) J. Biol. Chem. **226**, 497–509

Gavino, V. C., Miller, J. S., Dillman, J. M., Milo, G. E. & Cornwell, D. G. (1981) J. Lipid Res. **22**, 57–62

Glatz, J. F. C., Wagenmakers, A. J. M., Veerkamp, J. H. & Van Moerkerk, H. T. B. (1983) Biochem. Pharmacol. **32**, 2489–2493

Goldfischer, S., Moore, C. L., Johnson, A. B., Spiro, A. J., Valsamis, M. P., Wisniewski, H. K., Ritch, R. H., Norton, W. T., Rapin, I. & Gartner, L. M. (1973) Science **182**, 62–64

Havik, R. & Osmundsen, H. (1987) Biochem. J. **247**, 531–535

Healy, M. J., Kerner, J. & Bieber, L. L. (1988) Biochem. J. **249**, 231–237

Hiltunen, J. K., Karki, T., Hassinen, I. E. & Osmundsen, H. (1986) J. Biol. Chem. **261**, 16484–16493

Igarashi, M., Schaumburg, A., Powers, J., Kishimoto, Y., Kolodny, E. & Suzuki, K. (1976) J. Neurochem. **26**, 851–860

Inomata, M., Tahahu, R., Naggie, Y. & Saita, M. (1982) Anal. Biochem. **125**, 197–202

Kawamura, N. & Kishimoto, Y. (1981) J. Neurochem. **36**, 1786–1791

Kondrup, J. & Lazarow, P. B. (1985) Biochim. Biophys. Acta **835**, 147–153

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

McGarry, J. D., Mannaerts, G. P. & Foster, D. W. (1977) J. Clin. Invest. **60**, 265–270

Moser, A. E., Singh, I., Brown, F. R., Solish, G. I., Kelley, R. I., Benke, P. J. & Moser, H. W. (1984) N. Eng. J. Med. **310**, 1141–1146

Osmundsen, H. & Bjornstad, K. (1985) Biochem. J. **230**, 329–337

Osmundsen, H., Neat, C. J. & Borresbeck, B. (1980) Int. J. Biochem. **12**, 625–630

Poulos, A. (1981) Clin. Gen. **20**, 247–253

Poulos, A., Singh, H., Paton, B., Sharp, P. & Derwas, N. (1986) Clin. Gen. **29**, 397–408

Poulos, A., Derwas, N., Fellenberg, A. J., Johnson, D. W., Paton, B., Sharp, P. & Singh, H. (1987) in Human Genetics: Proceedings of the 7th International Congress (Vogel, F. & Sperling, K. eds.), pp. 352–359, Springer-Verlag, Berlin

Ramsay, R. R. (1988) Biochem. J. **249**, 239–245

Rhodin, J. (1954) Ph.D. Thesis, Karolinska Institute Stockholm, Akitbolaget Godvil

Rosenthal, M. D. & Hill, J. R. (1984) Biochim. Biophys. Acta **795**, 171–178

Rosenthal, M. D. & Hill, J. R. (1986) Biochim. Biophys. Acta **875**, 382–391

Samuelsson, B. (1983) Science **220**, 568–575

Sharp, P., Poulos, A., Fellenberg, A. & Johnson, D. (1987) Biochem. J. **248**, 61–67

Singh, H. & Poulos, A. (1986) Arch. Biochem. Biophys. **250**, 171–179

Singh, H., Derwas, N. & Poulos, A. (1987*a*) Arch. Biochem. Biophys. **257**, 302–314

Singh, H., Derwas, N. & Poulos, A. (1987*b*) Arch. Biochem. Biophys. **254**, 526–533

Singh, I., Moser, A. E., Goldfischer, S. & Moser, H. W. (1984*a*) Proc. Natl. Acad. Sci. U.S.A. **81**, 4203–4207

Singh, I., Moser, A. E., Moser, H. W. & Kishimoto, Y. (1984*b*) Pediatr. Res. **18**, 276–290

Tsuji, S., Ohno, T., Miyatake, T., Suzuki, A. & Yamakawa, T. (1984) J. Biochem. (Tokyo) **96**, 1241–1247
Uda, M., Singh, I. & Kishimoto, Y. (1981) Biochemistry **20**, 1295–1300
Vamecq, J. (1987) Biochem. J. **241**, 783–791
van Hinsbergh, V. W. M., Veerkamp, J. H. & van Moerkerk, H.Th. B. (1978) Arch. Biochem. Biophys. **190**, 762–771
Veerkamp, J. H., van Moerkerk, H. T. B., Glatz, J. F. C., Zuurveld, J. G. E. M., Jacobs, A. E. M. & Wagenmakers, A. J. M. (1986) Biochem. Med. Met. Biol. **35**, 248–259

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