Preferential distribution of non-esterified fatty acids to phosphatidylcholine in the neonatal mammalian myocardium

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Non-esterified fatty acids are used to a limited extent as an energy source in the newborn-mammalian heart. Therefore additional roles for palmitic and oleic acids during this early period of growth and development were investigated in the cultured neonatal-rat heart cell model system. Our results indicate significant differences in non-esterified-fatty-acid metabolism exist in this system in comparison with the adult rat or embryonic chick heart. Initial rates of depletion of palmitate and oleate from serum-free growth medium by heart cells obtained from 2-day-old rats and maintained in culture for 10 or 11 days were 111 ± 2 and 115 ± 3 pmol/min per mg of protein respectively. In serum-containing medium, the initial depletion rates were 103 ± 3 and 122 ± 4 pmol/min per mg of protein respectively, when endogenous serum non-esterified-fatty-acid concentrations were included in rate calculations. Less than 1% of the intracellularly incorporated fatty acids were found in aqueous products at any time. After 25 h, 15.5% of the initial palmitate was deposited intracellularly in the phosphatidylcholine lipid fraction, 4.2% in the triacylglycerol + fatty-acid-ester fraction and 3.1% in the sphingomyelin fraction. These results contradict the classical view, based on findings with the lipid-dependent adult heart, that exogenous non-esterified fatty acids are directed intracellularly primarily to pathways of oxidation or to storage as triacylglycerol. More importantly, it underscores the significance of exogenous non-esterified fatty acids in membrane biosynthesis of the developing mammalian heart. Included here is a new method for one-dimensional t.l.c. separation of metabolically important polar lipids.

Long-chain non-esterified fatty acids are known to be a major source of energy in the mature or adult myocardium (Opie, 1969; Neely & Morgan, 1974) and in the chick-embryo heart (Rosenthal & Warshaw, 1973; Samuel *et al.*, 1976). Similar substrate preference in the newborn-rat heart has been reported (Rosenthal & Warshaw, 1977; Frelin *et al.*, 1979). However, these observations are inconsistent with evidence that indicates a decreased capacity for the oxidation of non-esterified fatty acids in the mammalian neonatal heart (Wittles & Bressler, 1965; Battaglia & Meschia, 1978; Maniscalco & Warshaw, 1978).

The newborn-mammalian myocardium depends primarily on glucose as an energy source (Battaglia & Meschia, 1978; Maniscalco & Warshaw, 1978).

During the suckling period, adaptive enzymic changes occur in the heart that allow non-esterified fatty acids, a more efficient source of energy, to be utilized when a change in diet is introduced at weaning (Wittles & Bressler, 1965; Greengard, 1971). The metabolic switch from a carbohydrate-dependent organ to one that is more competent in using lipids as an energy source leads to the necessity of a more careful delineation for the role of non-esterified fatty acids during this substrate-dependent transition period in the heart.

It should be emphasized that most of the understanding we possess concerning non-esterified-fatty-acid metabolism in the myocardium has been obtained with the adult-perfused-heart preparation or cultured chick-embryo heart cells. In order to investigate the multidimensional role of non-esterified fatty acids in the mammalian-newborn heart, we have chosen neonatal-rat heart

Abbreviation used: MEM, minimum essential medium.

cells in culture as a model system. Myocardial cells in culture contract spontaneously and rhythmically. They respond to cardioactive agents in the same manner as the intact heart (Schanne & Bkaily, 1981). This model system has been carefully characterized and is comparable in metabolism, protein/DNA content, and cellular dimensions with the newborn-rat heart *in vivo* (McCarl *et al.*, 1980; Schroedl *et al.*, 1982).

Although the use of non-esterified fatty acids as an energy source for the contractile process is only one of several important requirements for lipid during the development of neonatal cardiac tissue, previous reports have emphasized fatty acid oxidation (Rosenthal & Warshaw, 1977; Paris *et al.*, 1978; Frelin *et al.*, 1979). Thus it was necessary to define the additional purposes for uptake and utilization of non-esterified fatty acids in the newborn heart. We have compared the rates of growth-medium depletion of palmitic and oleic acids by cultured neonatal-rat heart cells. The intracellular distribution of these non-esterified fatty acids was also monitored throughout a 33 h period. Together with the determination of rates of $^{14}\text{CO}_2$ evolution from labelled palmitic and oleic acid, these results enable us to draw a clearly defined picture of non-esterified fatty acid usage in the neonatal heart.

Materials and methods

Cultured cells

Experiments were performed on neonatal-rat heart cells maintained for 10 or 11 days in culture. The procedures used to isolate, plate and maintain the cultured cells were described previously (Schroedl *et al.*, 1982). Cells were maintained in MEM growth medium or medium 199. To delineate both the age of the animals used as the source of cardiac tissue for culture and the age in days of the cells in tissue culture, experimental dishes of cells were labelled as follows: the age in days of the animal + the number of days the cells were maintained in culture (e.g., '2+10' means heart cells obtained from 2-day-old rats maintained in culture for 10 days).

Chemicals

Growth media and components of MEM were purchased from GIBCO, Grand Island, NY, U.S.A. Newborn-calf serum was from GIBCO or Flow Laboratories, McLean, VA, U.S.A. Enzymes and assay standards were from Sigma Chemical Co., St. Louis, MO, U.S.A. Radiochemicals and Aquasol-2 scintillation cocktail were supplied by New England Nuclear, Boston, MA, U.S.A. Scintiverse scintillation cocktail was obtained from Fisher Scientific Co., Pittsburgh, PA, U.S.A.

Lipid standards were purchased from Supelco, Bellefonte, PA, U.S.A., or Sigma.

Bovine serum albumin-palmitate or bovine serum albumin-oleate complex

The procedure for preparation of the bovine serum albumin-palmitate or bovine serum albumin-oleate complex has been outlined (Schroedl *et al.*, 1982). For experiments which examined growth-medium depletion of non-esterified fatty acid, a stock solution (10 $\times$) of the bovine serum albumin-fatty acid complex was prepared to contain 6% (w/v) bovine serum albumin and 1.0 mM-fatty acid, with an albumin/non-esterified-fatty-acid molar ratio of 1:1. The experimental growth medium was prepared in a more concentrated form (1.1 $\times$) to allow for dilution on addition of the bovine serum albumin-fatty acid complex. Depletion studies were performed in both serum-supplemented and serum-free media. Non-esterified-fatty-acid concentrations in excess of 0.1 mM above those in serum were found to cause cell lysis within 4 h of application of growth medium and were unsuitable to the long-term nature of our investigations. Owing to the direct contact of growth medium with the cell surface, and the detergent nature of non-esterified fatty acids, concentrations of palmitate and oleate were kept below 60 μM in serum-free media. In similar model systems, where serum-free media were used, fatty acid concentrations were typically less than 35 μM (Pinson & Padieu, 1974; Samuel *et al.*, 1976; Rogers, 1977; DeGrella & Light, 1980). Dry, crystalline bovine serum albumin was extracted with 20 vol. of chloroform/methanol (2:1, v/v) to remove any contaminating fatty acids. Before solubilization in 0.02 M-sodium phosphate buffer, pH 7.4, all traces of chloroform and methanol were removed. No denaturation of bovine serum albumin was apparent on solubilization. The 6%-bovine serum albumin solution was filter-sterilized, and the complex was formed by the addition of an appropriate amount of concentrated fatty acid in methanol and 1- ^{14}C -labelled fatty acid in chloroform (Schroedl *et al.*, 1982).

Medium-depletion studies and assays

Depletion of non-esterified fatty acid from the growth medium was measured by removal of growth-medium samples from dishes of cultured heart cells at various time intervals as described previously (Schroedl *et al.*, 1982). Levels of [1- ^{14}C]palmitate or [1- ^{14}C]oleate remaining in the medium were obtained by measurement of radioactivity in 100 μl portions of growth medium withdrawn from culture dishes at prescribed intervals between 0 and 48 h. To determine palmitic and oleic acid concentrations in the serum used during

some of the uptake studies, 10 ml portions of serum were extracted as described by Dole (1956). Non-esterified fatty acids were separated from other lipid fractions on preparative thin-layer plates as described below for neutral-lipid separations. The non-esterified-fatty-acid band was identified by standards, and was scraped from the thin-layer plate and extracted with chloroform. The extracted fatty acids were converted into their esters and were subjected to g.l.c. analysis by Lancaster Laboratories, Lancaster, PA, U.S.A. Values obtained were 0.34 mM for oleic acid and 0.16 mM for palmitic acid. Cultured cardiac cells contracted rhythmically throughout the time period of investigation.

Intracellular distribution of 1-¹⁴C-labelled fatty acid

Owing to decreases in glucose utilization and protein content in serum-free media (Schroedl *et al.*, 1982), serum-containing medium was used for intracellular-distribution experiments to maximize cell viability and beating. At prescribed intervals during the usage of radiolabelled fatty acid by cultured heart cells, three or four culture dishes were sacrificed for determination of intracellular 1-¹⁴C-labelled fatty acid. Each dish was washed three times with 3 ml portions of ice-cold saline (0.9% NaCl) and drained. Methanol (1.5 ml) was added to each dish, and the cells were transferred to clean test tubes. After a wash with 1.0 ml of methanol, freshly distilled chloroform (5 ml) was added to each sample. Each tube was mixed briefly with a vortex mixer and stored at -20°C until further extraction and chromatography. Complete extraction of the lipid from cultured heart cells was achieved by the procedure described by Slayback *et al.* (1977). Each cell pellet was triply extracted and the organic portions from each sample were combined and evaporated to dryness under N₂ gas. The residue was resolubilized immediately in 100 µl of chloroform. The aqueous phase obtained during extraction was retained and its volume accurately determined. Samples of the aqueous portion were used to determine the quantity of water-soluble ¹⁴C-labelled components. A portion (10 µl) of each lipid sample in chloroform was used to quantify the total amount of ¹⁴C label in the extracted cells. This value also served to indicate the quantity of labelled lipid in the sample applied to each t.l.c. plate, so recovery of ¹⁴C label from the plate could be determined. Because the individual lipid concentrations in each dish of cells were below detectable levels, each sample tube was 'spiked' with a 10 µl portion of lipid standard containing 5 mg/ml of each of the following lipids: cholesterol, palmitic acid, tripalmitin (tripalmitoylglycerol), methyl palmitate and cholesteryl palmitate for neutral-lipid separation and

palmitoyl-CoA (2.5 mg/ml), lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, palmitoylcarnitine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol for polar-lipid separation before chromatography.

T.l.c.

All chromatographic separations were performed at ambient temperature when the relative humidity was less than 40%. Separation of neutral lipids was achieved by unidirectional two-step chromatography on 20 cm × 20 cm thin-layer plates coated with silica-gel type D-O or precoated plates (Whatman K5DF, Pierce) by the procedure outlined by Skipski & Barclay (1969). Plates were prewashed in chloroform/methanol (2:1, v/v) and activated at 110°C for 1 h. The first solvent system, isopropyl ether/acetic acid (24:1, v/v) was allowed to ascend 13 cm on each plate. Chromatography in light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (80:20:1, v/v) was subsequently carried to 0.5 cm of the plate top. After solvent evaporation from the plate, the separated lipid fractions were located by spraying each plate with a 0.2% (w/v) solution of 1,1'-dichlorofluorescein in ethanol. Spots were revealed under u.v. light and their location indicated on the thin-layer plate.

Polar lipids were separated by unidirectional t.l.c. on Whatman K5DF pre-coated plates. The plates were acid-washed before chromatography in the same solvent system used for separation, and activated at 110°C for 1.5 h before use. Cross-contamination of several polar lipids by intermediates of non-esterified-fatty-acid metabolism (palmitoyl-CoA and palmitoylcarnitine) was observed in all of the commonly used polar-lipid-separation systems. Therefore a new single-step separation method was developed. Chloroform/acetone/methanol/acetic acid/water (18:4:8:4:1, by vol.) allowed the separation of palmitoyl-CoA, lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, palmitoylcarnitine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine, with *R_F* values of 0, 0.09, 0.14, 0.26, 0.30, 0.45, 0.54 and 0.67 respectively. Neutral lipids migrated at the front of the solvent system. Lipid fractions were located by I₂ vapour. Instability of phosphatidylserine led to a second iodine-reactive spot with an *R_F* value of 0.72, which was pooled with the lipid material with an *R_F* value of 0.54. The inability of Aquasol-2 to extract completely phosphatidylcholine from silica gel required extraction of this fraction with chloroform/methanol/ammonia (27:21:1, by vol.), as suggested by Kritchevsky & Malhotra (1970), before scintillation counting.

The quantity of ¹⁴C-labelled lipid that co-chromatographed with the lipid standards was

determined by scraping each lipid-containing area on to glassine weighing paper and transferring the powder to scintillation vials. The ^{14}C -labelled-lipid-impregnated silica gel was shaken thoroughly with the scintillation fluid (Scintiverse for neutral lipids and Aquasol-2 for polar lipids) and the samples counted for radioactivity in a Beckman or Intertechnique scintillation counter. No interference by the silica gel (either enhancement or quenching of radioactivity measurements) was detected. Extraction of all ^{14}C -labelled lipid, except phosphatidylcholine, from the silica gel was $97.6 \pm 4.5\%$ complete. Loss of radioactivity during the chromatography, scraping and transferring process was $6.9 \pm 3.7\%$ for neutral-lipid chromatography and $10.5 \pm 7.2\%$ for polar-lipid chromatography.

$^{14}\text{CO}_2$ evolution from cultured cells

A microradiospirometer designed to quantitatively monitor continuous evolution of labelled CO_2 from radiolabelled substrates by cultured cells *in situ* was used to compare oxidation of $[1-^{14}\text{C}]$ -palmitate and $[1-^{14}\text{C}]$ -oleate by cultured neonatal heart cells. Several modifications were incorporated into the original radiospirometer design of Ross *et al.* (1981) to improve accuracy and precision of measurements and to maintain the physical integrity of cultured cells (C. R. Hartzell & C. R. Bacon, unpublished work). These included (1) improved gas-flow regulation to precisely determine flow rates, (2) lid modifications to improve gas-tightness and reduce shear effects of bubbling on cells, (3) mixing of growth medium by rocking rather than oscillatory motion, (4) simultaneous changing of all samples and (5) enclosure of the unit in Plexiglass for better temperature regulation. The specific experimental methods have been previously described (Ross *et al.*, 1981).

Results

Growth-medium depletion of palmitic and oleic acid by cultured heart cells

Initial rates of depletion of 0.05 mM - $[1-^{14}\text{C}]$ -palmitate and $[1-^{14}\text{C}]$ -oleate from serum-free growth medium 199 were 111 ± 2 and $115 \pm 3\text{ pmol/min}$ per mg of initial protein (three experiments involving a total of 13 dishes) respectively in 2+10-day-old heart cells. Similar measurements were performed in serum-containing growth medium. The contribution of serum-non-esterified fatty acids added to the initial non-esterified-fatty-acid concentration was such that palmitic acid was present at 0.066 mM and oleic acid at 0.084 mM in these experiments. Initial rates of depletion were comparable with those obtained in the absence of serum: $103 \pm 3\text{ pmol/min}$ per mg of protein for

palmitate and $122 \pm 4\text{ pmol/min}$ per mg of protein for oleate.

Separate studies were undertaken to monitor intracellular distribution of radiolabelled free fatty acids using neonatal heart cell cultures obtained from slightly older rats (4–5 days old). Depletion of $[1-^{14}\text{C}]$ -palmitate from serum-containing medium in 5+11-day-old cells was comparable with the depletion curves for 2+10-day-old cells. For example, after 8 h, $52.4 \pm 2.6\%$ of the $1-^{14}\text{C}$ -labelled non-esterified fatty acid remained in the growth medium of 2+10-day-old cells, whereas $56.5 \pm 1.2\%$ remained for 5+11-day-old cells (Fig. 1).

DL-Carnitine at concentrations varying in the growth medium from 10 to $300\text{ }\mu\text{M}$ was tested for its ability to enhance the usage of $[1-^{14}\text{C}]$ -palmitate. Neither 24 h pretreatment with various concentrations of carnitine nor inclusion of 10– $300\text{ }\mu\text{M}$ -carnitine in the experimental growth medium changed the rate of usage of non-esterified fatty acid.

Intracellular localization of radiolabelled palmitate

Extraction of cultured heart cells with a chloroform/methanol mixture was performed on dishes sacrificed as prescribed after applying growth

Fig. 1. Distribution of $[1-^{14}\text{C}]$ -palmitate between growth medium and intracellular lipids as a function of time. Depletion of palmitate from the growth medium (Δ) and appearance of radiolabel in polar lipids (PL, $\circ$) and neutral lipids (NL, $\bullet$) of 5+11-day-old heart cells is illustrated. Percentage values were determined as a function of the total initial radiolabel in the growth medium for five experiments (Δ) or two experiments ($\circ$ and $\bullet$).

medium containing serum and 0.05 mM-[1- 14 C]palmitic acid. The intracellular distribution of 1- 14 C-labelled material in neutral- and polar-lipid classes was determined by t.l.c. Fatty acid esters and triacylglycerol exhibited very similar R_F values in the solvent system employed for neutral-lipid separation and cross-contamination of the two fractions was sometimes observed. When clean separations were obtained, $90.0 \pm 5.0\%$ (68 dishes) of the extractable radiolabel was in the triacylglycerol fraction. However, these two lipid classes were pooled for all calculations described below.

Fig. 2. Percentage of total initial radiolabel deposited in intracellular lipid storage locations as a function of time

(a) The scale on the left side of the Figure indicates the fraction of [1- 14 C]palmitate found in phosphatidylcholine (PC, ●) and triacylglycerol + fatty acid esters (TG + FAE, ○). (b) The scale on the left side shows the amount of fatty acid incorporated into the sphingomyelin (Sph, □), phosphatidylserine (PS, ■) and palmitoylcarnitine (PCarn, △) fractions. Seven dishes from two separate experiments with 5 + 11-day-old heart cells were used in the determinations.

During the first 8 h of exposure to [1- 14 C]palmitate, a linear decrease in growth-medium 14 C-labelled fatty acid and linear increases in intracellular polar and neutral lipids were observed (Fig. 1). Within the eleven lipid classes examined, [1- 14 C]palmitate was preferentially directed toward the phosphatidylcholine pool (Figs. 2a and 2b). After 25 h, 15.5% of the initial [1- 14 C]palmitate present in the growth medium was deposited in the phosphatidylcholine fraction, whereas 4.2% was found in the triacylglycerol + fatty acid ester fraction and 3.1% in the sphingomyelin fraction. The palmitoylcarnitine and phosphatidylserine fractions each contained less than 2% of the initial radioactivity (Fig. 2b), and palmitoyl-CoA, lysophosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, cholesterol and non-esterified-fatty-acid pools each represented less than 1% of the initial radiolabel after 25 h.

Quantification of radiolabel extracted into the aqueous phase accounted for an average of $0.52 \pm 0.28\%$ (55 dishes) of the initial radioactivity at all time points up to and including 33 h. 14 C label adsorbing to the cell surface at zero time accounted for $0.86 \pm 0.10\%$ (12 dishes) of the total 14 C label present at the start of the palmitate-utilization studies.

The incorporation of [1- 14 C]palmitate into intracellular lipid classes also was expressed as a fraction of the total 14 C extracted from the cells (Fig. 3). The proportion of [1- 14 C]palmitate that remained non-esterified decreased quickly within 4 h from 8.2 to 2.7% of the extractable radiolabel. The phosphatidylcholine fraction represented approx. 45% of the total extractable label throughout the 33 h experiment. Linear increases in 14 C label were observed in the phosphatidylserine and triacylglycerol + fatty acid ester fractions up to 25 h and in sphingomyelin up to 33 h, indicating the lipid pools that were preferentially labelled by palmitic acid.

Intracellular localization of [1- 14 C]oleic acid

Several significant differences between oleic and palmitic acids were observed in the pattern of deposition in neutral- and polar-lipid classes. Most of the intracellular fatty-acid-incorporation profiles were similar for palmitate and oleate, but Table 1 illustrates that a greater fraction of the initial [1- 14 C]oleic acid was directed to the fatty-acyl-CoA, lysophosphatidylcholine and sphingomyelin pools than that of palmitate, whereas the amount of [1- 14 C]oleate in the phosphatidylcholine fraction was one-half the amount of [1- 14 C]palmitate recovered in this pool. A similar ratio between palmitate and oleate in phosphatidylcholine has been observed in the intact newborn-rat heart (Gudmundsdottir & Gudbjarnason,

Table 1. *Comparison of intracellular localization of radioactivity from fatty acids expressed as percentage of initial radiolabel*
 Values are expressed as the average percentage of the total initial radiolabel present in the growth medium $\pm$ 1 s.d.
 Data were obtained from two different experiments containing seven dishes for palmitate at each time point and one experiment containing four dishes for oleate at 1 and 14 h. At 8 and 25 h, two dishes were used for oleate.

Time (h)	Localization (% of initial label)							
	1		8		14		25	
	Palmitate	Oleate	Palmitate	Oleate	Palmitate	Oleate	Palmitate	Oleate
Fatty acyl-CoA	0.11 $\pm$ 0.09	0.34 $\pm$ 0.04	0.18 $\pm$ 0.06	1.25 $\pm$ 0.29	0.23 $\pm$ 0.16	2.19 $\pm$ 0.16	0.25 $\pm$ 0.17	2.81 $\pm$ 0.40
Lysophosphatidylcholine	0.07 $\pm$ 0.03	0.17 $\pm$ 0.03	0.21 $\pm$ 0.07	0.64 $\pm$ 0.17	0.37 $\pm$ 0.07	0.92 $\pm$ 0.09	0.36 $\pm$ 0.13	1.01 $\pm$ 0.26
Sphingomyelin	0.21 $\pm$ 0.05	0.45 $\pm$ 0.06	1.57 $\pm$ 0.25	2.18	2.31 $\pm$ 0.33	2.82 $\pm$ 0.35	3.14 $\pm$ 0.16	3.53 $\pm$ 1.08
Phosphatidylcholine	1.94 $\pm$ 0.44	1.09 $\pm$ 0.22	10.09 $\pm$ 0.94	5.74 $\pm$ 0.55	13.61 $\pm$ 2.55	6.81 $\pm$ 0.83	13.07 $\pm$ 2.32	6.53 $\pm$ 0.21
Total polar lipids	3.46 $\pm$ 0.25	2.23 $\pm$ 0.95	17.44 $\pm$ 2.05	8.45 $\pm$ 2.05	25.45 $\pm$ 2.70	10.28 $\pm$ 2.70	28.59 $\pm$ 1.41	13.92 $\pm$ 3.70
Total neutral lipids	1.19 $\pm$ 0.25	0.95 $\pm$ 0.06	4.78 $\pm$ 1.50	3.85 $\pm$ 0.40	6.00 $\pm$ 1.35	5.40 $\pm$ 0.64	6.43 $\pm$ 1.72	10.91 $\pm$ 0.19

Table 2. *Comparison of intracellular localization of radioactivity from fatty acids expressed as percentage of extracted ^{14}C -labelled lipid*

Values are expressed as the average percentage of the total radiolabel extracted from each dish $\pm$ 1 s.d. Data were obtained from two different experiments containing seven dishes for palmitate at each time point and one experiment containing four dishes for oleate at 1 and 14 h. At 8 and 25 h, two dishes were used for oleate.

Time (h)	Localization (% of initial label)							
	1		8		14		25	
	Palmitate	Oleate	Palmitate	Oleate	Palmitate	Oleate	Palmitate	Oleate
Fatty acyl-CoA	2.9 $\pm$ 2.0	7.8 $\pm$ 1.2	0.8 $\pm$ 0.3	7.4 $\pm$ 1.5	0.8 $\pm$ 0.6	9.1 $\pm$ 1.1	0.8 $\pm$ 0.6	7.9 $\pm$ 1.0
Lysophosphatidylcholine	1.5 $\pm$ 0.6	3.9 $\pm$ 0.4	1.3 $\pm$ 0.9	3.8 $\pm$ 0.7	1.3 $\pm$ 0.2	3.8 $\pm$ 0.5	1.2 $\pm$ 0.4	2.8 $\pm$ 0.8
Sphingomyelin	3.8 $\pm$ 0.8	10.4 $\pm$ 1.2	7.4 $\pm$ 1.0	11.6	7.9 $\pm$ 0.9	11.6 $\pm$ 1.3	9.5 $\pm$ 0.6	9.9 $\pm$ 2.4
Phosphatidylcholine	42.8 $\pm$ 5.6	25.0 $\pm$ 3.6	47.6 $\pm$ 5.8	27.7	43.8 $\pm$ 5.6	27.9 $\pm$ 2.5	47.1 $\pm$ 4.3	18.5 $\pm$ 0.3
Triacylglycerol + fatty acid esters	6.0 $\pm$ 0.1	12.3 $\pm$ 4.7*	9.8 $\pm$ 0.6	17.1 $\pm$ 6.2	10.9 $\pm$ 1.6	18.5 $\pm$ 6.1	12.7 $\pm$ 2.6	20.9 $\pm$ 10.6
Total polar lipids	73.9 $\pm$ 7.7	75.9 $\pm$ 1.3*	81.2 $\pm$ 0.1	66.9 $\pm$ 0.3	84.4 $\pm$ 0.4	71.5 $\pm$ 4.4	85.0 $\pm$ 0.9	64.6 $\pm$ 9.9
Total neutral lipids	24.3 $\pm$ 4.2	21.7 $\pm$ 0.5	19.3 $\pm$ 2.4	23.3 $\pm$ 0.6	18.7 $\pm$ 2.6	22.1 $\pm$ 1.5	18.4 $\pm$ 2.4	30.9 $\pm$ 1.0

* The values for the quantity of radiolabelled oleate in the triacylglycerol + fatty acid ester fraction and total polar-lipid fraction were obtained from two different experiments containing seven dishes.

1983). The total intracellular deposit of labelled fatty acid in both polar- and neutral-lipid storage locations was greater with palmitate than with oleate. A calculation of the amount of radiolabel in the fatty-acyl-CoA, sphingomyelin and triacylglycerol + fatty acid ester fractions as a function of *extracted* radiolabel lead to the conclusion that a greater enrichment occurred in these pools by [^{14}C]oleate than by palmitate (Table 2). However, whereas the amount of incorporation into polar and neutral lipids was less for oleate than for palmitate as a function of initial radiolabel, the partitioning between polar- and neutral-lipid classes showed that more oleic acid was found in neutral lipids and more palmitic acid was located in the polar-lipid fraction as a function of the extractable radiolabel. This pattern is most evident

after 25 h exposure to radiolabelled non-esterified fatty acid.

Recovery of ^{14}C -labelled non-esterified fatty acid

A complete metabolic profile of non-esterified-fatty-acid utilization by beating heart cells is predicated on the ability to account for all of the [^{14}C]labelled substrate. Table 3 summarizes results obtained when values for intracellular label, growth-medium label and the non-esterified fatty acid metabolized or calculated to be released as CO_2 are summed at each time point sampled. By using the steady-state rates of $^{14}\text{CO}_2$ evolution determined by radiorespirometry, the amount of palmitic and oleic acids oxidized to CO_2 between 1.5 and 3 h were 29.8 and 43.7 pmol/min per mg of protein respectively.

Table 3. Recovery of radioactivity from fatty acids applied to culture dishes
Values are expressed as the average percentage of the total initial radiolabel present in the growth medium.

Time (h)	Recovery (%)							
	Intracellular ¹⁴ C*		Growth-medium ¹⁴ C†		(Estimated) ¹⁴ C as CO ₂		Total	
	Palmitate	Oleate	Palmitate	Oleate	Palmitate	Oleate	Palmitate	Oleate
0	0.9	N.D.	100	100	0.0‡	0.0‡	100.9	100
1	4.3	2.9	96.0	95.5	1.2	1.3	101.5	99.7
2	6.2	4.1	87.2	89.3	5.0	6.3	98.4	99.7
4	10.5	7.0	76.2	82.3	12.7	13.4	99.4	102.7
6	15.7	9.5	65.0	75.1	(19.3)§	(15.4)	(100)	(100)
8	20.2	10.9	56.5	68.5	(23.3)	(20.6)	(100)	(100)
11	25.5	12.0	45.0	57.3	(29.5)	(30.7)	(100)	(100)
14	29.5	13.1	35.7	52.8	(34.8)	(34.1)	(100)	(100)
25	33.4	18.6	20.4	34.9	(46.2)	(46.5)	(100)	(100)
33	27.2	12.1	15.8	29.6	(57.0)	(58.3)	(100)	(100)

* Percentage of initial radiolabel incorporated into the cells that was extractable into chloroform/methanol. The results were obtained from two experiments containing seven dishes for palmitate and three experiments containing ten dishes for oleate.

† Percentage of initial radiolabel remaining in the growth medium. The results were obtained from six experiments containing 34 dishes for palmitate and three experiments containing 23 dishes for oleate.

‡ Values were determined by quantification of the amount of ¹⁴CO₂ continuously released from cultured neonatal-rat heart cells. The results were obtained from two experiments containing seven dishes for palmitate and eight dishes for oleate.

§ Values in parentheses were calculated assuming that the radiolabelled fatty acids not found in intracellular lipid pools or remaining in the medium has been completely oxidized to CO₂.

Fig. 3. Percentage of total radiolabel extracted from 5+11-day-old heart cells deposited in intracellular storage locations as a function of time

Abbreviations shown on the Figure are the same as indicated in Fig. 2. The fraction of [1-¹⁴C]palmitate found in the phosphatidylcholine (●), sphingomyelin (□), triacylglycerol + fatty acid ester (○), phosphatidylserine (■), palmitoylcarnitine (△) and non-esterified-fatty-acid (NEFA) (▲) pools are shown. Seven dishes from two experiments were used in the determinations.

Discussion

Usage of palmitic and oleic acid

Cultured neonatal-rat heart cells were shown to deplete serum-containing or serum-free growth medium of palmitic and oleic acids at approximately equal rates. These values varied from 103 to 122 pmol/min per mg of protein. In perfused adult-rat hearts, usages of these two fatty acids have been reported to be equivalent (Vasdev & Kako, 1977), whereas in cultured chick-embryo heart cells, Samuel *et al.* (1976) showed that, in 15 min, 2 nmol of palmitate and 2.3 nmol of oleate had been utilized by 1 × 10⁶ cells. Maximum usage rates for palmitate of 200 pmol/min per 10⁶ cells in chick-embryo heart cells (Samuel *et al.*, 1976) and 780 pmol/min per mg of protein in perfused adult heart (DeGrella & Light, 1980) have been reported. Clearly, the developing mammalian heart takes up non-esterified fatty acids at a lower rate than does adult or embryonic avian heart.

It is interesting to note that uptake rates in the absence and presence of serum were nearly equal in cultured rat heart cells. The effect of bovine serum albumin on non-esterified-fatty-acid usage has been studied widely (Samuel *et al.*, 1976; Paris *et al.*, 1978; DeGrella & Light, 1980), and rates of usage were shown to be dependent on a number of variables. Although the serum used in the growth medium contained 0.1 mM-stearic acid and

0.13 mM-linoleic acid in addition to palmitate and oleate, little effect of these fatty acids on usage rates were observed. Since the intracellular distributions of palmitate and oleate differ, partitioning of the acids must occur once they enter the cell. Other serum-containing fatty acid esters would also be expected to compete for intracellular transport sites. Thus the ultimate cellular bioavailability of palmitic and oleic acids may not be equal in the neonatal rat heart.

Intracellular radiolabelled non-esterified fatty acids as aqueous products

Several groups have addressed the question of distribution of products of fatty acids taken up by the heart (Rogers, 1977; Paris *et al.*, 1978; Pinson *et al.*, 1979; DeGrella & Light, 1980). DeGrella & Light (1980) state categorically that, in adult-rat heart myocytes, only non-esterified fatty acids, CO₂, triacylglycerol and polar lipids are intracellular products. In cultured cardiac cells from chick embryo, Paris *et al.* (1978) reported that the rate of incorporation of [1-¹⁴C]palmitate into aqueous products was 13% of the rate of the total [¹⁴C]lipid incorporation. Pinson *et al.* (1979) reported an average of 17% of initial [16-¹⁴C]palmitate and 7% of [1-¹⁴C]palmitate applied to 5-day-old cultured rat heart cells were recovered in water-soluble products, primarily as L-(+)-3-hydroxybutyrate. Rogers (1977) found that 51% of [14-¹⁴C]erucic acid depleted from the growth medium by 4 + 7-day-old rat heart cells was in the non-lipid cell fraction after 24 h incubation. However, subsequent analysis of the radiolabelled components in this fraction was not performed.

Intracellular water-soluble products of non-esterified-fatty-acid degradation accounted for less than 1% of the total radioactivity used in our experiments. Extracellular (i.e., growth medium) ¹⁴C-labelled compounds could have included citric-acid-cycle intermediates and ketones, especially during later time points (Table 3). Orloff (1971) reported no detectable acetoacetate or β -hydroxybutyrate in growth medium of cultured neonatal-rat heart cells prepared and maintained much like our cells. Radioactivity in the growth medium in the experiments described herein was probably due to [1-¹⁴C]palmitate or [1-¹⁴C]oleate, although water-soluble ¹⁴C-labelled products arising from the ¹⁴C-labelled fatty acid were not determined.

Intracellular storage of radiolabelled non-esterified fatty acids

The present results clearly show that palmitic acid is as important for phospholipid synthesis as it is an energy source in the developing neonatal-rat heart. In this respect, cultured neonatal-rat heart

cells reflect the needs of growing, dividing cells for substrates necessary for membrane biosynthesis. More than 65% of the intracellular labelled oleate and over 74% of labelled palmitate were directed toward polar- rather than neutral-lipid stores (Table 2). We are unable to explain the results of Frelin *et al.* (1979), who reported that, during the first week in culture, newborn-rat heart cells oxidized 25 μ M-palmitic acid complexed with 25% (v/v) foetal-calf serum at the same rate at which the growth medium was depleted of non-esterified fatty acid, indicating that no membrane biosynthesis was detectable in spite of a large increase in cell population during this period. At later times in culture, intracellular accumulation of palmitate in triacylglycerol was observed by those authors.

In contrast, reports from researchers using perfused adult-rat heart (Apstein *et al.*, 1972; Crass & Shipp, 1972; Vasdev & Kako, 1977; Gloster *et al.*, 1978) or cultured cardiac cells from rat (Rogers, 1974) or chick-embryo (Samuel *et al.*, 1976) collectively have shown that nearly two-thirds of the radiolabelled palmitate or [14-¹⁴C]erucate in the extractable lipid fraction was deposited preferentially in triacylglycerol. These findings are logical in adult-heart and embryonic-chick heart, where non-esterified fatty acids are used as the primary energy source for the contractile process. However, it has been well documented (Wittles & Bressler, 1965; Maniscalco & Warshaw, 1978) that the newborn mammal has a reduced capability to oxidize non-esterified fatty acids. The oxidation rates we report here are comparable with those in newborn-rat heart (32 pmol/min per mg of protein; Wittles & Bressler, 1965) and 7-day-old cultured rat heart cells (36 pmol/min per mg; Frelin *et al.*, 1979), whereas adult-rat heart oxidizes palmitate at 204 pmol/min per mg (Wittles & Bressler, 1965).

During the suckling period, an increase in carnitine fatty-acyltransferase and fatty-acyl-CoA synthetase activities enables the newborn animal to adapt its carbohydrate-dependent metabolism to a lipid-enriched diet (Wittles & Bressler, 1965). There is much evidence to show that carnitine levels increase in developing rat heart (Wittles & Bressler, 1965; Borum, 1978), that carnitine palmitoyltransferase activity increases (Warshaw, 1972; Wood, 1975), and the addition of exogenous carnitine to neonatal cardiac preparations enhances oxidation of palmitic acid (Wittles & Bressler, 1965; Warshaw, 1972; Liu & Spitzer, 1978). Although the addition of carnitine did not increase the rate of palmitate usage in our preparation, it was evident that cultured neonatal-rat heart cells were able to oxidize [1-¹⁴C]palmitate to CO₂ (Rosenthal & Warshaw, 1973; Frelin *et al.*, 1979; McCarl *et al.*, 1980). Myocardial lipoprotein lipase activity also increases immediately during

the postnatal period (Cryer & Jones, 1978), but it reaches nearly adult levels within 2 days after birth, and should not be a limiting factor in fatty acid utilization by the neonatal heart.

We conclude that, of the fatty acids incorporated into cultured neonatal-rat heart cells, less than 1% can be found in aqueous products. Equivalent amounts of palmitate are oxidized to CO₂ and deposited into lipid-storage locations, primarily phosphatidylcholine, an important component of membrane structure. These results represent a departure from the classical observations made in adult heart, where non-esterified fatty acids predominate as the preferred substrates for energy production, and intracellular deposition of fatty acids occurs primarily in triacylglycerol.

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