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XENOBIOTIC TRIACYLGLYCEROL FORMATION IN ISOLATED HEPATOCYTES

KATHRYN G. MOORHOUSE,* PETER F. DODDS and DAVID H. HUTSON†‡

Department of Biochemistry and Biological Sciences, Wye College (University of London), Wye, Ashford, Kent TN25 5AH; and †Shell Research Limited, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, U.K.

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Abstract—The formation of neutral lipophilic metabolites from five xenobiotic carboxylic acids was studied in isolated rat hepatocytes. Oleic acid was used as a positive control. Rates of formation of lipids lay in the order: oleic acid > phytanic acid > ibuprofen > 3-phenoxybenzoic acid > indomethacin and 3-phenylbutanoic acid (rates were undetectable with the last two substrates). The process was saturable with the maximum rates at about 0.5 mM substrate concentration. Supplementation of the hepatocyte system with glycerol enhanced the yields of lipid products. The hepatocytes also effectively modelled the *in vivo* metabolism of ibuprofen, 3-phenoxybenzoic acid and 3-phenylbutanoic acid with oxidations and classical conjugation reactions predominating over xenobiotic lipid formation.

The participation of a range of xenobiotic carboxylic acids in lipid metabolism to form unnatural or "hybrid" triacylglycerols is now well established [1–3]. This is a relatively minor pathway in comparison with the other routes of biotransformation of carboxylic acids but it is one which requires assessment in terms of toxicological significance [1, 4, 5].

Biosynthetic [6] and structural [7] studies on 3-phenoxybenzoic acid (3PBA) have indicated that the acid may occupy any of the three positions in triacylglycerol. One route of biosynthesis is via the xenobiotic acyl-CoA ester from which acyl group transfer is catalysed by monoacylglycerol acyltransferase [8]. This hepatic microsomal enzyme utilizes as substrates palmitoyl-CoA, 3-phenoxybenzoyl-CoA (3PBA-CoA), benzoyl-CoA and 1-naphthylacetyl-CoA but not the CoA esters of clofibric acid or 2, 4-dichlorophenoxyacetic acid [8]. The activity of the enzyme is higher in microsomes from young (16–18-day-old) rats compared with those from adults. A diacylglycerol acyltransferase also catalyses triacylglycerol formation from 3PBA-CoA and 1,2-dipalmitoyl-*sn*-glycerol.

The present studies with isolated hepatocytes were undertaken with a view to the development of a relatively simple *in vitro* technique to enable comparisons to be made between compounds. The technique eliminates the requirement for the chemical synthesis of the CoA thioesters. This is time-consuming and, in addition, may afford misleading results when attempting to model biosynthesis where the action of the acyl-CoA ligase is rate-limiting.

The model compounds chosen, all ^{14}C-labelled, were 3PBA (an aromatic acid with which we have some experience *in vivo* and *in vitro*), 3-phenylbutanoic acid (a small but metabolically stable alkanoic acid), the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin and ibuprofen (the latter of which is known to be incorporated into triacylglycerols *in vivo* [9]) and phytanic acid (a plant-derived, branched chain C-20 alkanoic acid, 3,7,11,15-tetra-methylhexadecanoic acid). The latter is of interest in that failure of the body to metabolize it leads to its build-up in tissues and incorporation into neutral lipids and phospholipids [10]. This inherited condition, known as Refsum's disease, is progressive and is associated with peripheral polyneuropathy, cerebellar ataxia, nerve deafness and night blindness. The natural fatty acid, oleic acid, was used as a positive control substance to ensure the viability of the cells for lipid biosynthesis. The stuctures of the xenobiotic acids are shown in Fig. 1.

During the course of these studies Sallustio *et al.* [11] reported that the NSAID fenoprofen was incorporated into triacylglycerols by isolated rat hepatocytes and adipocytes.

MATERIALS AND METHODS

Chemicals. 3-Phenoxy-[^{14}C]benzoic acid (2.14 Ci/mol) and 3-[ring-^{14}C]phenylbutanoic acid (1.87 Ci/mol) were synthesized at Sittingbourne Research Centre. [propan-1-^{14}C]Ibuprofen (2.93 Ci/mol), [2-^{14}C-indole]-indomethacin (2.5 Ci/mol), [1-^{14}C]phytanic acid (1.73 Ci/mol) and [1-^{14}C] oleic acid (2.13 Ci/mol) were obtained from Amersham International (Amersham, U.K.). The compounds were diluted as necessary with authentic carrier material to achieve the specific radioactivities given above. Non-radioactive phytanic acid was obtained as a generous gift from Mr Charles Earl, The Rowett Research Institute, Aberdeen, U.K. 1,2-Dipalmitoyl-3-(3-phenoxybenzoyl)glycerol [7] and 3-phenylbutanoylglycine [12] were described previously. Ibuprofen metabolites [13] A, 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propanoic acid and

* Present address: Laboratory of Pesticide Chemistry and Toxicology, Department of Entomological Sciences, University of California, Berkeley, CA 94720, U.S.A.

‡ To whom requests for reprints should be addressed.

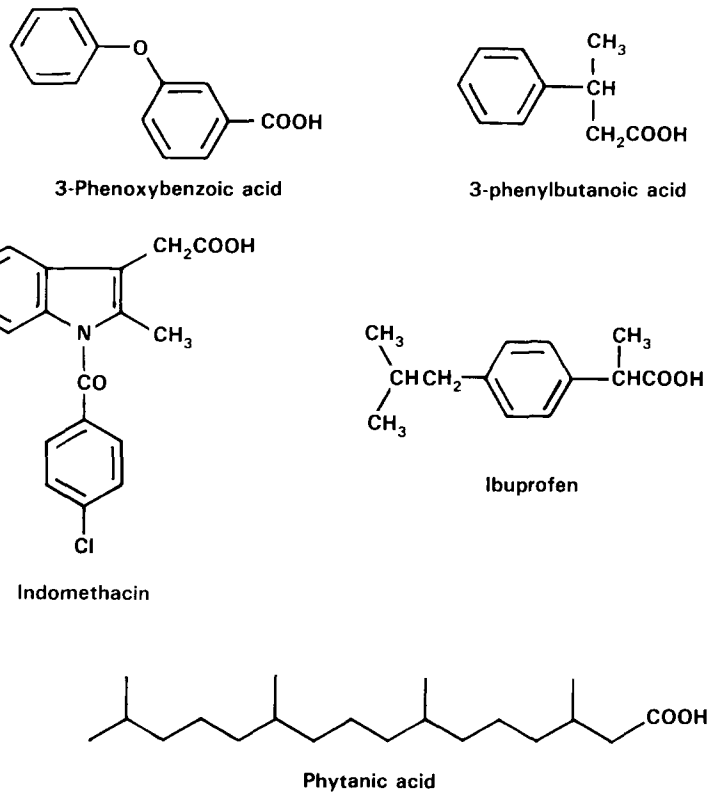

Fig. 1. Structures of the xenobiotic carboxylic acids used as substrates.

B, 2-[4-(2-carboxypropyl)phenyl]propanoic acid were isolated from the urine of rats dosed with [^{14}C]ibuprofen. They were characterized by proton N.M.R. spectroscopy. Enzymes and reagents were obtained from the Sigma Chemical Co. (Poole, U.K.).

Animals. Young female Fischer rats (28–35 days old, 60–100g), pregnant Wistar rats and male Wistar rats (49–56 days old, 150–200 g) were obtained from Charles River Ltd (Manston, Kent, U.K.). The Wistar rat pups were kept with their mother and used at 15–18 days of age.

Preparation of isolated hepatocytes. These were isolated from the livers of one or two of the older rats or from groups of 5 or 6 Wistar rat pups using the method described by Fry and co-workers [14, 15]. All cell preparations and incubations were carried out using siliconized glassware (prepared using dimethyldichlorosilane, 2% (w/v), in 1,1,1-trichloroethane). The liver slices were pre-incubated (10 min) with buffer containing 0.5 mM EGTA to chelate calcium ions and then for 60 min with Hank's buffer containing collagenase (0.5%, w/v) and hyaluronidase (1%, w/v) and 5 mM $CaCl_2$ at pH 7.5. The isolated, washed cells were finally suspended in Liebovitz L-15 solution (Gibco Ltd, Paisley, U.K.) containing 10% (v/v) new-born calf serum (Gibco Ltd). Cells were counted and viabilities were assessed using the trypan blue exclusion method [15]. Cell viabilities were 60–80%, reducing to 50–60% after 60 min incubation at 37°. A later modification for the comparative study which used the adult male Wistar rats was the inclusion of an extra centrifugation step in the preparation. The cell suspension was layered over a 30% (w/v) solution of Nycodenz (Nyegaard U.K. Ltd, Sheldon, Birmingham, U.K.) in L-15 medium. Intact hepatocytes collected at the interface.

Incubation conditions. Incubations were generally carried out in 25-mL glass Erlenmeyer flasks with 3×10^6 cells/mL in 4 mL of the Liebovitz solution described above. The reaction was started by the addition of the xenobiotic acid in 10 μL of acetonitrile to give a final concentration of 0.1 mM of the acid. Acetonitrile (50 μL) was shown to have minimal effect on the viability of the cells after 60 min at 37°. In the comparative study, using adult male Wistar rats, 5 mM glycerol was routinely included in the incubations. Samples (0.5 or 1 mL) were withdrawn at intervals between 0 and 120 min and added to ice-cold chloroform:methanol (2:1 by vol.). An acidic Hajra extraction [16] or a neutral Bligh–Dyer extraction [17] was then carried out on each sample. Total recoveries of radioactivity were greater than 95% in all cases (organic plus aqueous fractions). The phases were stored at −20° until further analysis, usually within 24 hr, was carried out.

Analytical methods. Thin-layer chromatography (TLC) was carried out using silica gel F_{254} plates (0.25 mm, Merck, Darmstadt, F.R.G.) using as solvents: A, hexane:diethyl ether:acetic acid (60:40:1, by vol.); B, chloroform:methanol:acetone:acetic acid:water (40:20:20:10:5, by vol.); C, toluene:acetic acid (90:10, by vol.); D,

chloroform : methanol : acetic acid (72 : 24 : 3. by vol.); E, ethyl acetate : formic acid : water (70 : 4 : 4, by vol.); F, *n*-butanol : formic acid : water (60 : 10 : 10, by vol.). Samples were applied using a Camag Linomat IV automatic applicator. Compounds were located using (i) iodine vapour, (ii) UV light (254 nm), (iii) radiochromatogram analyser (RITA-3200, Raytest, Sheffield), and (iv) autoradiography with Agfa-Gevaert Curix RPI X-ray film. Quantification of chromatograms in all cases was achieved by excision of silica gel and liquid scintillation counting (LSC). Organic and aqueous solutions were assayed by LSC using 10 mL of Optiphase Safe scintillation fluid (LKB). Silica gel was shaken with 0.5 mL of methanol before addition of the scintillation fluid. Rates of reaction derived from quantitative analyses of the TLC plates were normalized to that for 10^6 cells and expressed as pmol/min per 10^6 cells.

In the comparative study, 0.5 or 1 mL samples of the Bligh-Dyer bottom phase were subjected to column chromatography on basic alumina to remove the radioactive acid precursor. The eluant was subjected to LSC [6]. This method was validated for each acid by TLC before its routine adoption.

RESULTS

Biosynthesis of triacylglycerols containing 3-phenoxybenzoic acid (3PBA)

Initial experiments in which the organic phases of the neutral Bligh–Dyer extractions were analysed (solvent B for 10 cm then solvent A × 2 for the full length of the plate) [8] indicated the formation of two lipophilic products (R_f values 0.77 and 0.79 cf. 3PBA at 0.70) migrating slightly lower than a natural triacylglycerol (triolein) but in the expected postitions for the 3-phenoxybenzoyl-derived triacylglycerols [7]. The rate of formation of the products was 2.5 pmol/min per 10^6 cells. The Hajra acidic extraction also afforded these products but, in addition, the expected polar metabolites of 3PBA [18] were also detected in much greater yield. These were tentatively identified using solvents B, E and F as the glucuronic acid and glycine conjugates of 3PBA and 4-hydroxy-3PBA and its glucuronide and sulphate conjugates as described earlier [18]. The nature of the lipid product was not investigated further because this aspect has been covered *in vivo* [7] and *in vitro* [6] previously.

Biosynthesis of triacylglycerols containing ibuprofen

Ibuprofen was incubated with hepatocytes from young adults and neonates under a variety of conditions. Intial experiments utilized hepatocytes incubated in the absence of added glycerol and the organic phase of the acidic extraction was analyzed (solvents B then A and solvent C). The chromatogram shown in Fig. 2 (solvent C) indicates the formation of ibuprofen-derived lipid (IL, migrating at R_f 0.5 in the triacylglycerol region of the chromatogram ahead of unchanged ibuprofen, R_f 0.4). Three other metabolites were formed [R_f values 0.38 (B); 0.16 (A) and 0 (C)], which migrated as one zone in solvent B/A. These three metabolites co-chromatographed with three metabolites present in the urine of ibuprofen-dosed rats: A, 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propanoic acid; B, 2-[4-(2-carboxypropyl)phenyl]propanoic acid; C, probably the glucuronic acid conjugate of ibuprofen. The relative proportions of the compounds in the organic phase in this experiment (>90% of the radioactivity) were: ibuprofen 36%, metabolite A 15%, metabolite B 11%, metabolite C 36% and ibuprofen-derived lipid 1.5%. In a series of experiments the rates of formation of the lipid metabolite in cells from adult female Fischers, adult male Wistars and suckling Wistars were, respectively: 2.0 ± 0.9 (SD, N = 3), 2.1 (N = 1) and 3.7 (range 0.5, N = 2) pmol/min per 10^6 cells. Glucose (5 mM) did not stimulate lipid formation.

A preparation of the ibuprofen-derived triacylglycerol was purified from an incubation using female Fischer rat hepatocytes (supplemented with 2 mM glycerol) by extraction and TLC. One sample of this was subjected to methanolysis by refluxing with toluene : methanol : sulphuric acid (10 : 20 : 1, by vol.); a second sample was exposed to diazomethane under gentle conditions which will methylate unesterified fatty acids but not glycerol esters.

Whereas all the radioactivity co-chromatographed in solvent A with the methyl ester of ibuprofen after methanolysis, none did so after diazomethane treatment. This indicated that the radioactive content of the 'ibuprofen-derived triacylglycerol' was not due to contamination with unreacted ibuprofen. The ibuprofen-derived triacylglycerol was relatively resistant to lipolysis by pancreatic lipase (compared to lard), but using high concentrations of the enzyme and longer incubation times it was possible to achieve a partial degradation. TLC in solvent A of the products showed that a portion of the radioactivity was to be found in the diacylglycerol region of the TLC plate and that a new non-radioactive spot appeared which co-chromatographed with standard oleic acid or, after diazomethane treatment, standard methyl oleate. The degradation evidence is consistent with the proposed triacylglycerol structure for the ibuprofen derived lipid.

Studies with indomethacin and 3-phenylbutanoic acid

Indomethacin exhibited very little metabolism. Only traces of metabolite were detected in the organic and aqueous phases. None migrated as mono-, di- or tri-acylglycerols. 3-Phenylbutanoic acid was approximately 50% converted to two more polar metabolites, one migrating as 3-phenylbutanoylglycine and the other a more polar unknown (not 3-phenyl-butanoyltaurine). However, no metabolites were detected in the acylglycerol region.

Comparative studies with oleic acid, phytanic acid, ibuprofen and 3-phenoxybenzoic acid

The variability between hepatocyte preparations necessitates the acquisition of many replicates if absolute results are to be quoted. In order to avoid this requirement, a series of experiments were conducted in which four substrates were studied simultaneously under a variety of conditions thus enabling direct comparison of the results between substrates. Where replicates were obtained (oleic

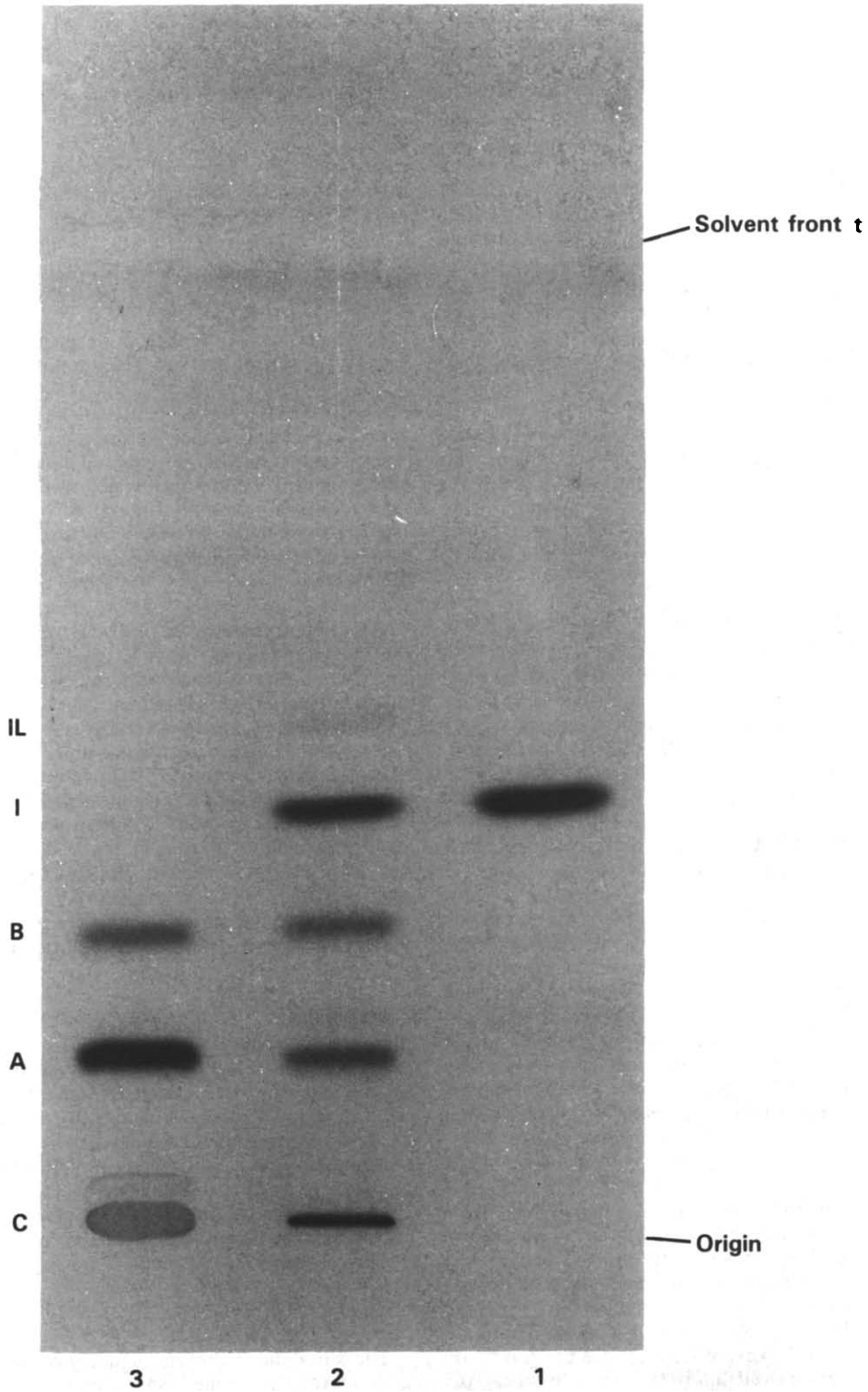

Fig. 2. Autoradiogram of TLC separation of the metabolites of ibuprofen. The samples were prepared, developed in solvent C, and subjected to autoradiography as described in the text. Track 1 shows the extract of hepatocytes incubated for 0 min with [^{14}C]ibuprofen; track 2 shows the extract of hepatocytes incubated for 60 min with [^{14}C]ibuprofen; track 3 shows urine collected between 0 and 24 hr after dosing rats with [^{14}C]ibuprofen. The following abbreviations are used: IL, ibuprofen-derived lipid, I, ibuprofen; A, B and C are the metabolites described in the Results section.

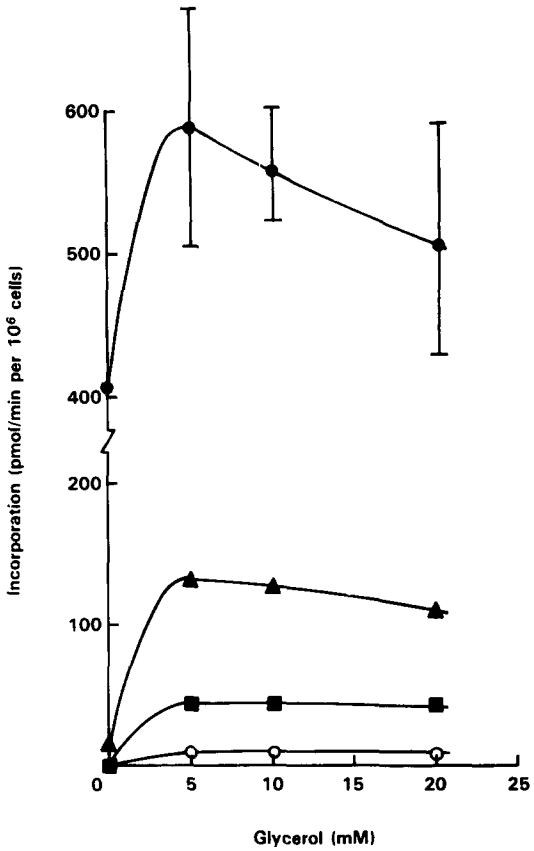

Fig. 3. The effect of glycerol concentration on the incorporation of carboxylic acids into lipids by isolated rat hepatocytes. The standard incubation conditions (see Materials and Methods section) were used except that the indicated concentration of glycerol was used. The ^{14}C-substrates were: (●) oleic acid; (▲) phytanic acid; (■) ibuprofen; (○) 3-phenoxybenzoic acid. The error bars represent the range from two experiments.

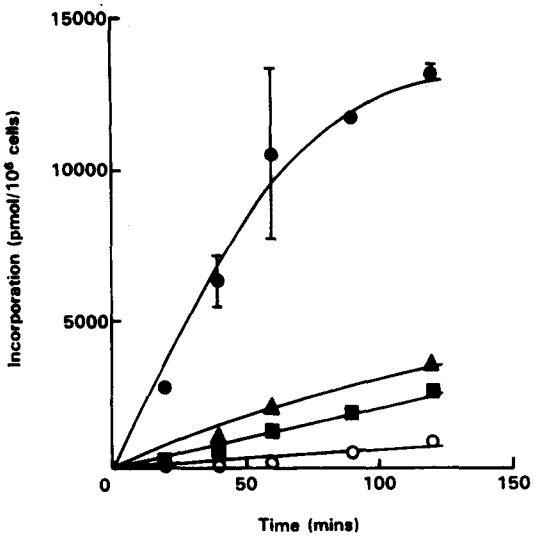

Fig. 4. The effect of incubation time on the incorporation of carboxylic acids into lipids by isolated rat hepatocytes. The standard conditions were used except that incubations were conducted for the indicated times. The ^{14}C-substrates were as shown under Fig. 3. The error bars represent the range from two experiments.

acid), error bars are shown; the single points shown were derived from single experiments (Figs 3–6). However, in each case, four substrates were studied simultaneously using one of the preparations. The effect of glycerol concentration at 0.1 mM acid substrate concentration is shown in Fig. 3. The relative effectiveness of the four acids at 0.1 mM as substrates for incorporation into triacylglycerols is shown in Fig. 4. This experiment also demonstrates the robust nature of the preparation under the conditions of incubation. Incorporation was first order with respect to time for at least the first 60 min. The effect of cell concentration is shown in Fig. 5. The incorporation was first order up to 2×10^6 cells/mL. The effect of substrate concentration is shown in Fig. 6. The biosynthetic processes are clearly saturable. Maximum rates were obtained at concentrations of acids which were greater than the 0.1 mM used to obtain Figs 3–5. The optimum concentration was 0.5 mM but the relative activities changed little between 0.1 and 0.5 mM. At a concentration of 1 mM oleic or 1 mM phytanic acid, incorporation was much lower than at 0.5 mM. The

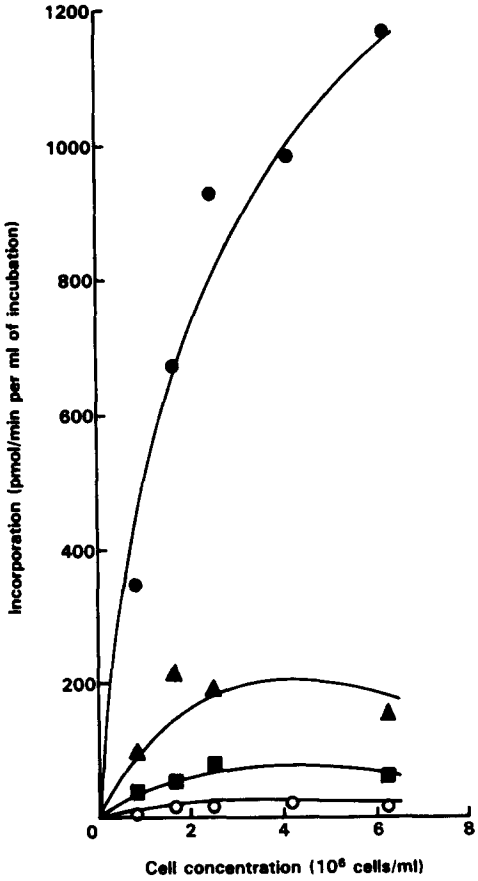

Fig. 5. The effect of cell concentration on the incorporation of carboxylic acids into lipids by isolated rat hepatocytes. The standard conditions were used except that the indicated concentrations of hepatocytes were used. The ^{14}C-substrates were as shown under Fig. 3.

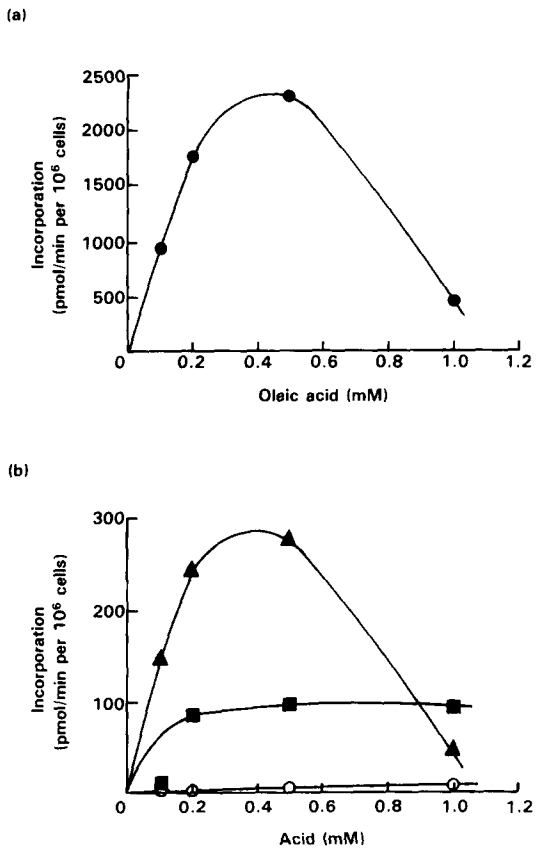

Fig. 6. The effect of substrate concentration on the incorporation of carboxylic acids into lipids by isolated rat hepatocytes. Fig. 6a, oleic acid; Fig. 6b, xenobiotic acids as shown under Fig. 3. The standard incubation conditions were used except that the indicated concentrations of the ^{14}C-acids were used.

Table 1. Relative rates of incorporation of carboxylic acid substrates into the neutral lipids of isolated rat hepatocytes

Substrate	Rate of incorporation (pmol/min per 10^6 cells)
Oleic acid	636 ± 247(6)
Phytanic acid	111 ± 52(4)
Ibuprofen	28 ± 15(4)
3-Phenoxybenzoic acid	6.3 ± 4(4)

Values are mean ± SD (N determinations).

medium in which the cells were incubated contained 1.8–2.5 mM unesterified fatty acid (data from Gibco Ltd) in addition to the added acid. The effective concentration of unesterified fatty acid depends on the concentration in the medium of serum albumin (which binds fatty acids). Direct comparisons, therefore, between experiments are difficult. Nevertheless, 2 mM fatty acid caused a similar decline in triacylglycerol synthesis in chicken hepatocytes [19]. The activities of the four substrates derived from the various time points at 0.1 mM acid and 0.5 mM glycerol are summarized in Table 1.

DISCUSSION

These experiments have demonstrated that the incorporation of certain xenobiotic carboxylic acids into lipophilic metabolites, previously shown to occur *in vivo*, can be modelled in the isolated rat hepatocyte. The rates of incorporation lay in the order: oleic acid > phytanic acid > ibuprofen > 3-phenoxybenzoic acid > 3-phenylbutanoic acid and indomethacin (both below the limit of detection). This order is in agreement with the degree of similarity of the substrates to a natural fatty acid (Fig. 1). The use of a positive control substrate (oleic acid) and one other xenobiotic substrate (e.g. ibuprofen) in addition to the test carboxylic acid substrates would be advisable in a routine assay of incorporation potential. This would avoid the requirement for time-consuming replication of the assays.

The precise chemical structures of the triacylglycerols formed have not been elucidated. Although the products appeared to be single compounds (apart from those from 3-phenoxybenzoic acid which formed two products [7]) they are probably mixtures deriving from a variety of natural fatty acids esterifying the glycerol molecule. This is certainly the case *in vivo*. The 3-phenoxybenzoyldipalmitin isolated and identified from the tissues of rats dosed with 3-phenoxybenzoic acid [7] represented only a small fraction of the total (radiolabelled) triacylglycerol fraction. It is likely that other analogues such as those involving stearic, oleic and myristic acid were also present. These deductions are borne out by the results of the degradation by lipase of [^{14}C]ibuprofen-containing triacylglycerol which produced a [^{14}C]diacylglycerol analogue and a non-radioactive fatty acid. Such multiplicity has been found in the few cases where detailed chromatographic analysis of the xenobiotic triacylglycerol fraction has been carried out [20].

Supplementing of the hepatocytes with glycerol improved the yields of xenobiotic lipids, presumably by providing more "backbone" for the triacylglycerol synthesis. Glucose had no effect. The other substrate for the mixed-acid triacylglycerols are natural fatty acids. No experiment was conducted to determine the effects of supplementing the medium (which itself contained about 2 mM unesterified fatty acids) with natural fatty acids because of the complications likely to be caused by competition for the acyl-CoA ligase and acyltransferase enzymes. It is known that added fatty acid stimulates triacylglycerol synthesis from glycerol and glucose in chicken [19] and rat [21] hepatocytes. It is possible that variation in availability of exogenous fatty acids (between batches of serum) and endogenous fatty acids may have been a cause of the variation noted above between hepatocyte preparations. It is debatable how much modulation of the system should be attempted if the objective is to model the *in vivo* situation. Furthermore, it is unlikely that yields can be substantially improved because, as *in vivo*, there exist competing reactions which utilize major proportions of the carboxylic acid substrates.

The finding that hepatocytes from neonatal rats synthesize xenobiotic lipids more efficiently from

ibuprofen than do those from adult rats is a further interesting aspects of the model. The increased activity is in accord with our findings at the enzyme level (microsomal monoacylglycerol acyltransferase) reported earlier [8]. It is also in accord with the results of Coleman and Haynes [22] who reported high rates of natural triacylglycerol synthesis in rat pups. The toxicological significance of this phenomenon has yet to be assessed.

Interestingly, the hepatocytes proved to mimic *in vivo* metabolism in several other aspects: (i) the glycine conjugation of 3-phenylbutanoic acid, (ii) the aryl hydroxylation and sulphate conjugation of 3-phenoxybenzoic acid [18], and (iii) the hydroxylation and further oxidation to afford the two major *in vivo* metabolites of ibuprofen. A recent paper [23] on the metabolism and chiral inversion of ibuprofen in isolated rat hepatocytes confirms this last observation.

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