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Drugs Affecting the Synthesis of Glycerides and Phospholipids in Rat Liver

THE EFFECTS OF CLOFIBRATE, HALOFENATE, FENFLURAMINE, AMPHETAMINE, CINCHOCAINE, CHLORPROMAZINE, DEMETHYLIMIPRAMINE, MEPYRAMINE AND SOME OF THEIR DERIVATIVES

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1. The effects on glycerolipid synthesis of a series of compounds including many drugs were investigated in cell-free preparations and slices of rat liver. 2. *p*-Chlorobenzoate, *p*-chlorophenoxyisobutyrate, halofenate, D-amphetamine, adrenaline, procaine and *N*-[2-(4-chloro-3-sulphamoylbenzoyloxy)ethyl]norfenfluramine had little inhibitory effect on any of the systems investigated. 3. Two amphiphilic anions, clofenapate and 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetate, both inhibited glycerol phosphate acyltransferase and diacylglycerol acyltransferase at approx. 1.6 and 0.7mM respectively. Clofenapate (1mM) also inhibited the incorporation of glycerol into lipids by rat liver slices without altering the relative proportions of the different lipids synthesized. 4. The amphilic amines, mepyramine, fenfluramine, norfenfluramine, hydroxyethylnorfenfluramine, *N*-(2-benzoyloxyethyl)norfenfluramine, cinchocaine, chlorpromazine and demethylimipramine inhibited phosphatidate phosphohydrolase by 50% at concentrations between 0.2 and 0.9mM. The last four compounds inhibited glycerol phosphate acyltransferase by 50% at concentrations between 1 and 2.6mM. None of the amines examined appeared to be an effective inhibitor of diacylglycerol acyltransferase. 5. Norfenfluramine, hydroxyethylnorfenfluramine and *N*-(2-benzoyloxyethyl)norfenfluramine produced less inhibition of glycerol incorporation into total lipids than was observed with equimolar clofenapate. The major effect of these amines in liver slices was to inhibit triacylglycerol and phosphatidylcholine synthesis and to produce a marked accumulation of phosphatidate. 6. The results are discussed in terms of the control of glycerolipid synthesis. They partly explain the observed effects of the various drugs on lipid metabolism. The possible use of these compounds as biochemical tools with which to investigate the reactions of glycerolipid synthesis is considered.

It has recently been shown that two classes of drugs, namely the derivatives of clofibrate [ethy l2-(4-chlorophenoxy)-2-methylpropionate] and fenfluramine [2-ethylamino-1-(*m*-trifluoromethylphenyl)-propane hydrochloride], inhibit glycerolipid synthesis *in vitro*. First, fenfluramine and its derivatives inhibit the synthesis of glycerides in preparations of rat and human liver (Marsh & Bizzi, 1972; Bowley *et al.*, 1973), rat intestine (Dannenburg *et al.*, 1973) and human adipose tissue (Wilson & Galton, 1971). There is also evidence to suggest that fenfluramine and *N*-(2-benzoyloxyethyl)norfenfluramine (S780, Servier Laboratories Ltd.) are hypolipidaemic agents (Duhault & Boulanger, 1965; Duhault & Malen, 1970; Rivilene, 1974).

Secondly, *p*-chlorophenoxyisobutyrate inhibits the esterification of *sn*-glycerol 3-phosphate in cell-free preparations from rat liver (Scott & Hurley, 1969; Adams *et al.*, 1971; Lamb & Fallon, 1972; Fallon *et al.*, 1972). Clofenapate [sodium 4-(4'-chlorophenyl)phenoxyisobutyrate] was more effective than *p*-chlorophenoxyisobutyrate in decreasing the concentrations of serum triacylglycerol (Craig, 1972) and in inhibiting glycerol phosphate esterification in homogenates of human liver and in mitochondrial and microsomal fractions of rat liver (Brindley *et al.*, 1973). In addition clofenapate was more potent in inhibiting the esterification of dihydroxyacetone phosphate than that of glycerol phosphate in rat liver mitochondrial fractions (Bowley *et al.*, 1973).

The present series of experiments was performed to investigate the relative abilities of compounds related to fenfluramine, halofenate and *p*-chlorophenoxyisobutyrate to inhibit glyceride synthesis in preparations from rat liver. Studies have shown that fenfluramine and some of its derivatives are effective inhibitors of phosphatidate phosphohydrolase (EC 3.1.3.4) (Brindley & Bowley, 1975).

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This observation was thought to be particularly significant since phosphatidate phosphohydrolase is at a branch point in glycerolipid synthesis and appears to have a regulatory function. In liver its activity is enhanced in conditions where there is an increased flux of fatty acids into glycerides (Vavrečka *et al.*, 1969; Mangiapane *et al.*, 1973; Lamb & Fallon, 1974). In synaptosomal preparations the activity of phosphatidate phosphohydrolase is thought to be stimulated by acetylcholine (Yagihara *et al.*, 1973; Schacht & Agranoff, 1973, 1974) leading to an enhanced incorporation of ^{32}P into phosphatidate and phosphatidylinositol.

It also became evident from work with pig lymphocytes (Allan & Michell, 1975) that the effects of a wide variety of amphiphilic amines on glycerolipid synthesis could also be explained in terms of a decrease in the rate of conversion of phosphatidate into diacylglycerol. It was therefore decided to test whether compounds such as the local anaesthetics chlorpromazine, demethylimipramine and mepyramine might, in fact, directly inhibit phosphatidate phosphohydrolase.

Materials and Methods

Male Wistar rats (180–270g) were obtained from the Nottingham University Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K. They were allowed free access to food and water before the experiments.

Materials

Unless stated to the contrary, cofactors, substrates and reagents were prepared or purchased as described previously (Sánchez *et al.*, 1973; Mangiapane *et al.*, 1973). Adrenaline was purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; chlorpromazine and mepyramine maleate were from May and Baker Ltd., Dagenham, Essex, U.K.; cinchocaine was from Sandoz Products Ltd., 98 The Centre, Feltham, Middx., U.K.; procaine was from Macarthies Ltd., Lyn Estate, Great Barr, Birmingham B41 IDU, U.K.; demethylimipramine was from Geigy (U.K.) Ltd., Hurdsfield Industrial Estate, Macclesfield, Cheshire, U.K.; t.l.c. plates (Kieselgel 60/Kieselgur F254) were from E. Merck, Darmstadt, Germany; microgranular chromatographically tested cellulose was from Reeve Angel Scientific Ltd., 14 New Bridge Street, London E.C.4, U.K.

Fenfluramine, norfenfluramine [2-amino-1-(*m*-trifluoromethylphenyl)propane hydrochloride], hydroxyethylnorfenfluramine, *N*-(2-benzoyloxyethyl) norfenfluramine (S780), its methanesulphonate (S1513), *N*-[2-(4-chloro-3-sulphamoyl)benzoyloxyethyl]norfenfluramine (S1204) and D-amphetamine sulphate were gifts from Servier Laboratories Ltd., Servier House, Horsenden Lane South, Greenford, Middx. UB6 7PW, U.K. *p*-Chlorophenoxyisobutyrate and clofenapate were gifts from Imperial Chemical Industries Ltd., Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K. Halofenate was donated by Merck, Sharp and Dohme Ltd., Hertford Rd., Hoddesdon, Herts. HN11 9BU, U.K.

Preparation of 2-(p-chlorophenyl)-2-(m-trifluoromethylphenoxy)acetic acid. To 500mg of halofenate was added 25ml of 1M-NaOH and 1ml of methanol. The mixture was warmed to 40°C and shaken until most of the halofenate had been hydrolysed and the powder had disappeared. The mixture was cooled to 20°C and 40ml of light petroleum (b.p. range 40–60°C)–diethyl ether (1:1, v/v) was added. The mixture was shaken and the top phase removed. The bottom aqueous phase was washed four times with diethyl ether and then taken to pH1 with 6M-HCl. 2-(*p*-Chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetic acid was then extracted with diethyl ether and the extract was washed four times with water. The diethyl ether was dried over $CaCl_2$ and the solvent was removed by evaporation. The residue was finally dried in a vacuum desiccator over H_2SO_4 and weighed 314mg. Samples of the residue were chromatographed on plates of silica gel G developed in acetic acid–ethyl acetate (1:19, v/v) or NH_3 (sp.gr. 0.88)–propan-2-ol (1:19, v/v) (Hucker *et al.*, 1971). The residue was free from halofenate and had chromatographic properties consistent with its being 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)-acetic acid (Hucker *et al.*, 1971). Standard solutions of this compound and halofenate were prepared in ethanol and their E_{274} measured. Assuming that the extinction coefficients of the two compounds are identical, the 2-(*p*-chlorophenyl-2-(*m*-trifluoromethylphenoxy)acetic acid was pure.

Preparation of drug solutions. Unless stated to the contrary stock solutions of the drugs were adjusted to pH7 where necessary with NaOH and were stored at −20°C. Solutions of compounds S780, S1513 and adrenaline (2–33mM) were prepared immediately before use.

Fatty acid-poor bovine serum albumin (1mg; Pentex Inc., Kankakee, Ill., U.S.A.) was added to each 4mg of compounds S780, S1204, adrenaline or halofenate to aid dispersion. The mixture was then sonicated at 21KHz until a uniform emulsion was obtained.

Preparation of cell fractions. Cell fractions were prepared from rat liver essentially as described by Mangiapane *et al.* (1973). However, the livers were perfused *in situ* with ice-cold 0.9% NaCl when the particle-free supernatant was to be used as a source of enzyme. Protein concentrations were determined by a biuret method (Brindley & Hübscher, 1965).

Preparation of membrane-bound [^3H]phosphatidate. The method was based on procedures described

previously (Smith *et al.*, 1967; Mitchell *et al.*, 1971). [^{3}H]Phosphatidate was synthesized on endoplasmic reticulum membranes which were obtained from rat liver. The incubation system used for this preparation contained, in a volume of 400ml, 16.6mM-potassium phosphate buffer (pH7.4), 1mM-dithiothreitol, 50mM-NaF, 13.4mM-$MgCl_2$, 8mM-ATP, 20mM-*rac*-glycerol 3-phosphate, 25μM-CoA, 0.8mM-potassium [^{3}H]-palmitate (0.5μCi/μmol; prepared by warming palmitic acid with a 20% molar excess of KOH), 3mg of fatty acid-poor bovine serum albumin/ml and 3mg of microsomal protein/ml. The incubation was for 30min at 37°C.

The reaction was stopped by adding sufficient ice-cold EDTA solution (adjusted to pH7.0 with KOH) to give a final concentration of 20mM. The mixture was frozen and stored overnight at −20°C. After thawing it was sonicated at 0°C for 2min at 21KHz and then layered over 0.3M-sucrose containing 1mM-EDTA. The tubes were centrifuged for 80min at 4°C and 76000*g* ($r_{av.}$ = 7.62cm) and the pellets resuspended in about 35ml of 0.3M-sucrose.

Analysis of the ^{3}H-labelled lipids from this preparation showed that approx. 97% of the radioactivity was in phosphatidate with the remainder in lysophosphatidate, diacylglycerol, unesterified fatty acid and triacylglycerol.

Methods

Determination of the rate of glycerolipid synthesis from sn-glycerol 3-phosphate. The biosynthesis of glycerolipids was determined by measuring the rate of incorporation of *sn*-[1,3-^{3}H]glycerol 3-phosphate into lipids. The assay medium contained, in a volume of 0.25ml, 25mM-Tris buffer (adjusted to pH7.4 with HCl), 5mM-dithiothreitol, 2.4mM-$MgCl_2$, 3mM-ATP, 60μM-CoA, 0.8mM-potassium palmitate, 15.9mM-*sn*-[1,3-^{3}H]glycerol 3-phosphate (0.5μCi/μmol) and 1.5mg of fatty acid-poor bovine serum albumin. Where indicated solutions of drugs were added to this medium. The reaction was started by the addition of rat liver homogenate (400μg of protein). After 10min the reaction was stopped by the addition of 1.88ml of chloroform–methanol (1:2, v/v) and the lipids were extracted and analysed as described elsewhere (Brindley, 1973).

Measurement of phosphatidate phosphohydrolase activity. The enzyme was measured by following the synthesis of diacylglycerol from the membrane-bound [^{3}H]phosphatidate (Smith *et al.*, 1967; Mitchell *et al.*, 1971). The assay system contained, in a volume of 0.25ml, 25mM-potassium phosphate buffer, pH6.8, 6mg of fatty acid-poor bovine serum albumin/ml, 1mM-$MgCl_2$ and 25μl of membrane-bound phosphatidate. Drugs were added as indicated and the reaction was started with particle-free supernatant (300μg of protein) which contained phosphatidate phosphohydrolase. The reaction was stopped after 20min at 37°C by adding 1.88ml of chloroform–methanol (1:2, v/v). The phases were separated by adding 0.625ml of chloroform [containing 0.4% (w/v) of olive oil as carrier] and 0.875ml of water. After centrifugation and removal of the top phase, samples of the bottom phase were analysed for glycerides on columns of alumina (Mitchell *et al.*, 1971).

Measurement of diacylglycerol acyltransferase activity (*EC* 2.3.1.20). Diacylglycerol acyltransferase activity was measured by following the incorporation of radioactivity from [^{3}H]palmitoyl-CoA into glycerides with 1,2-diacyl-*sn*-glycerol as an acyl acceptor. The assay system contained, in a final volume of 0.35ml, 25mM-Tris buffer (adjusted to pH7.4 with HCl), 5mM-dithiothreitol, 50μM-[^{3}H]-palmitoyl-CoA (2.6μCi/μmol), 4mM-1,2-diacyl-*sn*-glycerol, 18mM-$MgCl_2$ and 1.5mg of fatty acid-poor bovine serum albumin/ml. The diacylglycerol emulsion was prepared by pipetting a hexane solution of diacylglycerol into a tube, evaporating off the hexane under N_2, adding the reagents described above and sonicating at 21KHz until a uniform emulsion was obtained. The drug solutions were then added and the reaction was started by the addition of 70μg of microsomal protein. After shaking for 10min at 37°C the reaction was stopped with 1.88ml of chloroform–methanol (1:2, v/v). The phases were separated by the addition of 0.625ml of chloroform and 0.775ml of water. After centrifuging, the bottom phase was washed once with 2ml of 50mM-Tris buffer (adjusted to pH8.0 with HCl), to remove unchanged palmitoyl-CoA. The glycerides were then purified by alumina chromatography (Mitchell *et al.*, 1971).

Synthesis of lipids from [1,3-^{3}*H*]*glycerol by rat liver slices.* The methods used have been described by Manning & Brindley (1972). The reactions were stopped by rapid filtration under suction. The tissue was washed with approx. 15ml of ice-cold 0.9% NaCl and placed in a weighed tube containing methanol–water (5:4, v/v). The weight of the tissue recovered was 100±10mg (S.D.). The slices were sonicated and the lipids extracted by the method of Hajra *et al.* (1968). Water-soluble contaminants were removed by washing the extract two times with synthetic top phase. This was obtained by mixing 1litre of chloroform, 1litre of methanol, 400ml of water, 500ml of 2M-KCl–0.2M-H_3PO_4 and 40ml of conc. HCl and adding 4g of glycerol and 1g of *rac*-glycerol 3-phosphate to the resulting top phase.

The radioactivity in the total lipids was determined directly by scintillation counting and lipid classes were analysed on t.l.c. plates of Kieselgel 60. The plates were developed for about 60% of their length with chloroform–methanol–acetic acid–acetone–water (10:2:2:4:1, by vol.). After being

dried the plates were then developed for their full length with hexane–diethyl ether–acetic acid (60:4:1, by vol.) in order to achieve a separation of the various neutral lipid classes from the phospholipids. The lipids were detected with iodine vapour, scraped into counting vials, and their radioactivity was determined (Sánchez *et al.*, 1973).

Characterization of lipids. Lipids were identified by co-chromatography with authentic samples (Sánchez *et al.*, 1973). In addition, the phosphatidate synthesized in selected incubations with rat liver slices was characterized further. The lipids were analysed by two-dimensional chromatography on Kieselgel 60/Kieselgur F254 t.l.c. plates in the following solvent systems. The plates were developed in the first direction with chloroform–methanol–water–NH_3 (sp.gr. 0.88) (24:14:1:2, by vol.) and in the second direction with the system described above for the one-dimensional chromatography. The distribution of radioactivity in lipids was the same in the one- and two-dimensional systems.

The area corresponding to phosphatidate was eluted from the gel with 4×5ml extractions of chloroform–methanol (1:1, v/v) and the solvent removed. To the residue were added 5μmol of *rac*-glycerol 3-phosphate, 0.5ml of methanol–toluene (1:1, v/v) and 0.5ml of methanolic 0.2M-KOH. The mixture was subjected to mild alkaline hydrolysis by leaving for 1h at 0°C in order to obtain glycerol phosphate from phosphatidate. After the addition of 0.5ml of water the pH was adjusted to between 1 and 2 with Dowex 50 (H^+ form). The aqueous phase was then washed with 2×2.5ml of diethyl ether. Samples of the aqueous extract were chromatographed in two dimensions on 0.6mm cellulose t.l.c. plates. The solvents were phenol-saturated water–acetic acid–ethanol (100:10:12, by vol.) in the first direction and methanol–98% (v/v) formic acid–water (80:13:7, by vol.) in the second as recommended by Dawson (1963). The percentage of the water-soluble radioactivity which migrated with glycerol phosphate was 88% compared with authentic radioactive glycerol phosphate. This confirms that the original lipid was phosphatidic acid.

Samples of the total lipid fraction of control incubations and those to which drugs had been added were degraded by a more rigorous alkaline hydrolysis (Manning & Brindley, 1972). More than 94% of the ^3H was recovered in water-soluble compounds with the remainder almost equally distributed between non-saponifiable lipids and unesterified fatty acids. This indicated that the [1,3-^{3}H]glycerol was incorporated almost exclusively into the glycerol backbone of the lipids.

Measurement of radioactivity. Radioactivity was determined by liquid-scintillation counting (Sánchez *et al.*, 1973).

Results

The first series of experiments involved measuring the effects of a variety of drugs on the various stages of triacylglycerol synthesis in subcellular preparations from rat liver. In the first system rat liver homogenates were used to esterify *sn*-[1,3-^{3}H]glycerol 3-phosphate with palmitate. The mean rate of glycerol phosphate incorporation into the total lipid fraction was 4.3±1.0 (S.D.) nmol/min per mg of protein [13 independent preparations (Brindley & Bowley, 1975)]. Of the total lipid synthesized 14±5% (S.D.) was isolated as diacylglycerol and triacylglycerol. Most (about 83%) of the remainder was phosphatidate, which indicates that phosphatidate phosphohydrolase was rate-limiting in this system. The determination of the rate of glycerol phosphate incorporation into the total lipid fraction effectively measures the activity of glycerol phosphate acyltransferase, whereas the incorporation into glycerides indicates the relative activity of phosphatidate phosphohydrolase.

The activity of the soluble phosphatidate phosphohydrolase of rat liver was also assayed directly with membrane-bound [^{3}H]phosphatidate as substrate. This method is thought to determine the activity of the enzyme which participates in triacylglycerol synthesis (Hübscher, 1970; Brindley, 1974). The mean activity of this enzyme (nine preparations) was 0.9±0.5 (S.D.) nmol of glyceride formed/min per mg of supernatant protein.

Finally, diacylglycerol acyltransferase was assayed with the microsomal fraction of rat liver and an emulsion of 1,2-diacyl-*sn*-glycerol. Its mean activity from six preparations was 2.5±2.4 (S.D.) nmol of triacylglycerol synthesized/min per mg of microsomal protein.

The compounds which were tested in these systems will be divided into two groups, amphiphilic anions and amphiphilic cations. The results are summarized in Table 1.

Effects of amphiphilic anions

p-Chlorophenoxyisobutyrate was not a good inhibitor of any of the systems tested but it was more potent than the related compound *p*-chlorobenzoate, which is not a hypolipidaemic agent. Clofenapate, a more hydrophobic derivative of *p*-chlorophenoxyisobutyrate, produced 50% inhibitions of glycerol phosphate incorporation into total lipids and into glycerides at concentrations of 1.6 and 0.7mM respectively (Table 1). This decreased rate of glyceride synthesis from glycerol phosphate does not appear to result from a direct inhibition of phosphatidate phosphohydrolase. When this enzyme was assayed directly a concentration of 6.8mM-clofenapate was required to produce a 50% inhibition and stimulations of up to 2-fold were obtained at 1–4mM-clofenapate. In addition, *p*-chlorobenzoate and *p*-chlorophenoxy-

Table 1. *Effects of various drugs and other compounds on the enzymes of glycerolipid synthesis*

The concentrations of the compounds which were required to produce a 50% inhibition of the activities indicated are listed. The numbers in parentheses are the numbers of rats used and at least five different concentrations of each active compound were tested with each rat. The combined results were plotted as log concentration against the percentage inhibition, and the concentration required to produce a 50% inhibition was calculated from these graphs. Unless indicated to the contrary in the Table, the results are accurate to within ±10% (S.D.). Some compounds produced no significant inhibitions up to the concentration shown and this is indicated by the symbol, *. n.m. means not measured. For comparative purposes, some of the results obtained by Brindley & Bowley (1975) have been added to the Table and are indicated by †.

	Concentration (mM) required to produce a 50% inhibition in the activity of:			
	Glycerol phosphate incorporation into:			
Compound	Total lipids	Glycerides	Phosphatidate phosphohydrolase	Diacylglycerol acyltransferase
p-Chlorobenzoate	~80 (6)	~50 (6)	20 (8)*	n.m.
p-Chlorophenoxyisobutyrate	24 (5)	14 (5)	20 (8)*	13 (5)*
Clofenapate	1.6 (6)	0.7 (6)	6.8 (6)	~0.6 (6)
2-(*p*-Chloromethyl)-2-(*m*-trifluoromethylphenoxy)acetate	1.5 (5)	0.5 (5)	7.8 (6)	0.7 (3)
Halofenate	20 (3)*	20 (3)*	n.m.	n.m.
Adrenaline	1.5 (4)*	1.5 (4)*	1.5 (4)*	n.m.
D-Amphetamine	20 (6)*	10 (6)	3.2 (6)	n.m.
Fenfluramine	12 (6)†	1.3 (6)†	0.8 (6)†	12 (5)*
Norfenfluramine	11 (5)†	1.5 (5)†	0.9 (6)†	8 (5)*
Hydroxyethylnorfenfluramine	15 (5)†	~1.3 (5)†	~0.4 (6)†	8 (5)*
Compound S780	2.4 (5)†	0.8 (5)†	0.5 (6)†	n.m.
Compound S1513	1.9 (10)†	~0.7 (10)†	0.6 (6)†	3 (6)*
Compound S1204	18 (5)*†	18 (5)*†	~10 (6)†	n.m.
Cinchocaine	2.6 (4)	0.5 (4)	0.4 (4)	n.m.
Procaine	7 (4)*	7 (4)*	7 (4)*	n.m.
Chlorpromazine	1 (4)	0.2 (4)	0.2 (4)	~5 (6)
Demethylimipramine	1 (4)	0.3 (4)	0.3 (4)	n.m.
Mepyramine	3 (4)*	~1.9 (4)	0.7 (4)	n.m.

isobutyrate (3–20mM) stimulated the activity of the phosphohydrolase. Lower concentrations of clofenapate (0.1–0.4mM) also stimulated the activity of diacylglycerol acyltransferase by up to 2-fold. However, at a concentration of approx. 0.6mM clofenapate inhibited diacylglycerol acyltransferase activity by 50% (Table 1).

Halofenate is also used as a hypolipidaemic agent but this compound had no inhibitory effect in any of the systems tested (Table 1). However, *in vivo* it is rapidly metabolized to 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetic acid (Hucker *et al.*, 1971), and this compound closely resembled clofenapate in its inhibitory effects.

Effects of amphiphilic amines

Of the amines examined adrenaline, procaine and compound S1204 had no inhibitory effects at the concentrations that were used (Table 1). All of the other amines, except D-amphetamine, were effective inhibitors of phosphatidate phosphohydrolase in the range 0.2–0.9mM (Table 1). They also inhibited the incorporation of glycerol phosphate into glycerides at similar concentrations.

None of the amines tested in the present study appeared to be an effective inhibitor of diacylglycerol acyltransferase activity. Neither did fenfluramine, norfenfluramine, hydroxyethylnorfenfluramine and mepyramine inhibit glycerol phosphate acyltransferase activity except at relatively high concentrations (Table 1).

In contrast, compounds S1513, S780, cinchocaine, chlorpromazine and demethylimipramine did inhibit glycerol phosphate acyltransferase activity by 50% at concentrations between 1 and 2.6mM.

Effects of clofenapate and some derivatives of fenfluramine on the incorporation of glycerol into lipids by rat liver slices

The synthesis of glycerolipids was determined by incubating rat liver slices with [1,3-^{3}H]glycerol at a physiological concentration (0.1mM) as described by Manning & Brindley (1972). The time-course for this reaction is shown in Figs. 1(*a*) and 1(*b*). The accumulation of [1,3-^{3}H]glycerol in phosphatidate and in diacylglycerol reached a steady state in approx. 8 and 15min respectively. In contrast, there was an initial lag of 8–12min in the synthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine. Thereafter, these lipids were syn-

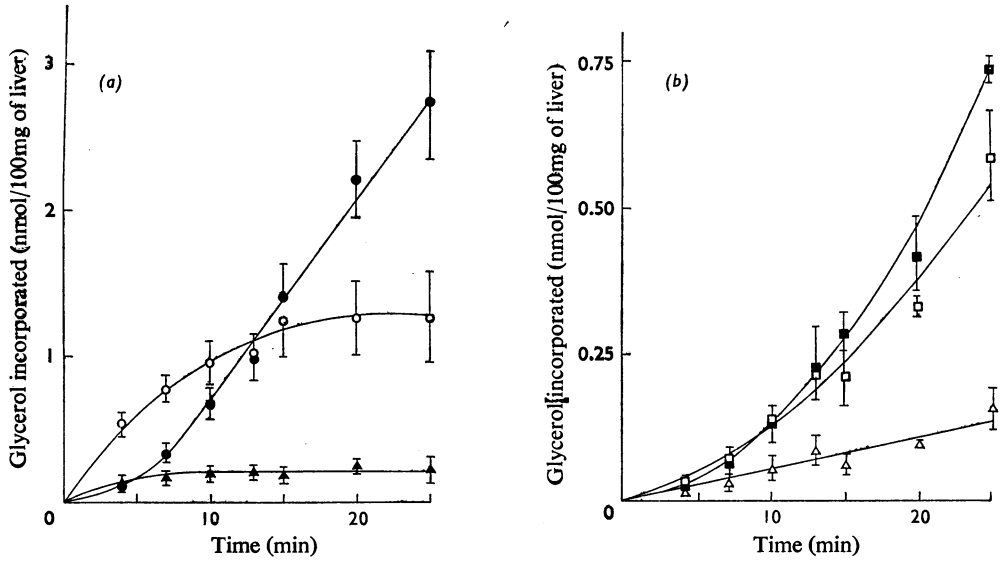

Fig. 1. *Time-course of glycerol incorporation into lipids by rat liver slices*

Liver slices were incubated with 0.1mM-[1,3-^{3}H]glycerol as indicated in the Materials and Methods section. The incorporation of ^{3}H into (*a*) triacylglycerol (●), diacylglycerol (○), phosphatidate (▲), and (*b*) phosphatidylethanolamine (□), phosphatidylcholine (■) and phosphatidylserine plus phosphatidylinositol (△) is shown at different times of incubation. The rates are indicated as means±1S.D. (a total of 14 rats). The time-points for 4, 7 and 13min were obtained with slices from ten different preparations, those at 15, 20 and 25min from four different preparations and that at 10min from 14 different preparations.

thesized at approximately constant rates (Figs. 1*a* and 1*b*). The mean rate of glycerol incorporation into the total lipid fraction was 0.22±0.02 (S.D. from 14 independent preparations) nmol/min per 100mg of liver slice, and this rate was constant throughout the 25min of incubation.

The rate of lipid synthesis obtained by Sundler *et al.* (1974) was 3–8.5nmol of glycerol incorporated/min per 100mg of isolated rat hepatocytes, but they used 2mM-glycerol instead of the 0.1mM-glycerol used in the present studies. Maximum rates of glycerol incorporation into lipids in rat liver slices are obtained at approx. 10mM and are approximately ten times higher than at 0.1mM-glycerol (Liberti & Jezyk, 1970; Manning & Brindley, 1972).

The potential of the slices to synthesize glycerolipids is therefore only slightly less than the isolated cells. The rates obtained with the slices are also in the same range as those obtained by Kohout *et al.* (1971) who studied triacylglycerol accumulation with perfused liver. Although liver slices may not provide a good model system for studying some aspects of metabolism, they do provide a fairly active and reproducible system for studying the sites of action of various drugs which alter the rate of hepatic glycerolipid synthesis. In order to do this an incubation time of 20min was chosen, by which time phosphatidate and diacylglycerol had reached steady-state concentrations and the other lipids were being synthesized at constant rates (Figs. 1*a* and 1*b*).

Treatment of the slices with up to 1mM-clofenapate inhibited the incorporation of glycerol into the total lipid fraction. During this inhibition there was no significant change in the relative composition of the individual lipid classes (Fig. 2*a*).

When the derivatives of fenfluramine were added to liver slices there was much less inhibition of total lipid synthesis than was observed with the same concentrations of clofenapate (Fig. 2*b*). The major effect of the fenfluramine derivatives was to alter the distribution of [1,3-^{3}H]glycerol among the various lipid fractions. The compounds tested were *N*-(2-benzoyloxyethyl)norfenfluramine (S780 and S1513), and norfenfluramine and hydroxyethylnorfenfluramine, which are rapidly formed as metabolites of compounds S780 and S1513 (Beckett *et al.*, 1972). Since the effects of these fenfluramine derivatives appeared to be identical, the results obtained with them have been averaged to produce Fig. 2(*b*). This finding is different from that of Wilson & Galton (1971), who showed that, compared with fenfluramine,

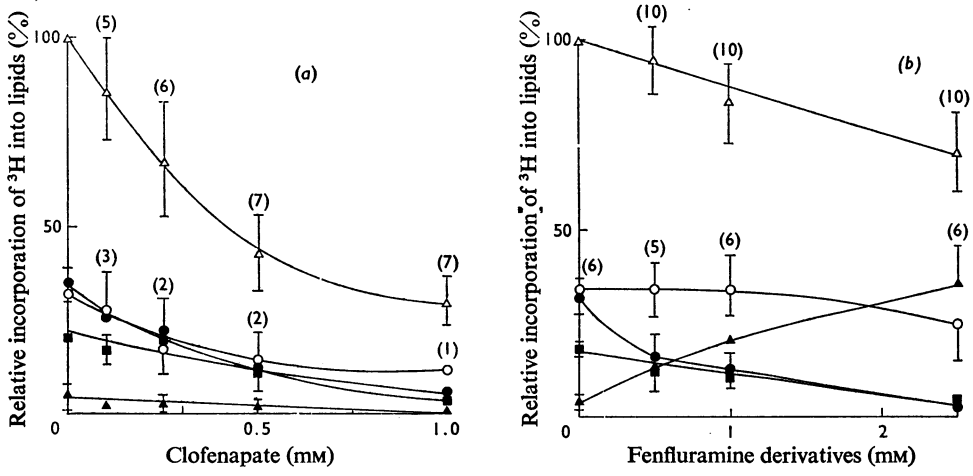

Fig. 2. *Effects of clofenapate and compounds S780, S1513, norfenfluramine and hydroxyethylnorfenfluramine on the synthesis of lipids by rat liver slices*

Rat liver slices were incubated for 20min with 0.1mM-[1,3-^{3}H]glycerol as indicated in the Materials and Methods section. Analysis of the total lipid fraction was performed on selected incubations. The mean incorporation±1S.E.M., based on the number of different preparations shown in parentheses, is expressed relative to the synthesis of total lipid when no drugs were added to the assay system. The activities are indicated as follows: total lipids (△), triacylglycerol (●), diacylglycerol (○), phosphatidate (▲) and phosphatidylcholine (■). The rates of glycerol incorporation into the other lipids were less than 10% of the total incorporation and have been omitted from the Figure. The effects on total lipid synthesis of compounds S780, S1513, norfenfluramine and hydroxyethylnorfenfluramine (*b*) were obtained with seven, eight, three and three different preparations respectively, whereas the analyses of the different lipid classes were performed on three, four, three and three different preparations. Since these compounds appeared to produce the same effect, the results from them have been averaged.

compound S1513 had little effect on the synthesis of glycerides by intact adipocytes.

From Fig. 2(*b*) it can be seen that the major effect of the fenfluramine derivatives was to decrease the synthesis of triacylglycerol and phosphatidylcholine, and to increase the accumulation of phosphatidate. There was no significant change in the steady-state concentration of labelled diacylglycerol.

Discussion

Of the anionic amphiphilic drugs, *p*-chlorophenoxyisobutyrate did inhibit glycerol phosphate acyltransferase as previously reported (Adams *et al.*, 1971; Lamb & Fallon, 1972), but at relatively high concentrations compared with clofenapate or 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetate. Diacylglycerol acyltransferase was also inhibited by the last two compounds but phosphatidate phosphohydrolase was not very sensitive to these reagents. The results from the liver slice experiments (Fig. 2*a*) are compatible with clofenapate inhibiting an enzyme early in glycerolipid synthesis, such as glycerol phosphate acyltransferase or dihydroxyacetone phosphate acyltransferase. Since the latter enzyme seems to be more susceptible to inhibition by clofenapate (Bowley *et al.*, 1973), this compound might serve as a fairly selective inhibitor with which to investigate the relative activities of the glycerol phosphate and dihydroxyacetone phosphate pathways.

Recent experiments with isolated hepatocytes have shown that *p*-chlorophenoxyisobutyrate stimulates rather than inhibits the incorporation of glycerol and palmitate into glycerides (Capuzzi *et al.*, 1974). These results are consistent with the observations that the acyltransferases of glyceride synthesis are not inhibited until very high concentrations of *p*-chlorophenoxyisobutyrate (Table 1), and that 3–20mM-*p*-chlorophenoxyisobutyrate stimulates phosphatidate phosphohydrolase activity. Capuzzi *et al.* (1974) conclude that *p*-chlorophenoxybutyrate could not lower plasma triacylglycerols by inhibiting hepatic lipid synthesis but this is contrary to the conclusions of Fallon *et al.* (1972). Halofenate decreases the conversion of acetate into fatty acids and lipid by isolated rat hepatocytes and produces an equal decrease in all lipid classes (Horney & Margolis, 1974). In the experiments performed with clofenapate no selective inhibition of lipid synthesis was observed (Fig. 2*a*).

Serum concentrations of 0.5–1.5mM for *p*-chlorophenoxyisobutyrate (Fallon *et al.*, 1972) and approx. 0.4mM for clofenapate (Thorp, 1970) and 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetate (Hucker *et al.*, 1971) have been reported. The concentrations found in rat liver are approx. 0.3mM for *p*-chlorophenoxyisobutyrate (Daae & Aas, 1973) and approx. 40% of the serum concentration for 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetate (Hucker *et al.*, 1971). The intracellular concentration may be considerably lower than this owing to the presence of blood in the liver sample. It therefore appears unlikely that *p*-chlorophenoxyisobutyrate could inhibit glyceride synthesis *in vivo* by inhibiting acyltransferase activity. Clofenapate and 2-(*p*-chlorophenyl)-2-(*m*-trifluoromethylphenoxy)acetate, which are 20–50 times more potent than *p*-chlorophenoxyisobutyrate (Table 1: Horney & Margolis, 1974), might have this effect. However, this action could be complicated by strong binding of these amphiphilic anions to albumin (Thorp, 1970) and their ability to displace fatty acids from it, thus tending to increase fatty acid esterification and oxidation (Horney & Margolis, 1974).

In contrast with the anionic compounds, the active amphiphilic cations inhibited phosphatidate phosphohydrolase at relatively low concentrations. Compounds S780, S1513, cinchocaine, chlorpromazine and demethylimipramine were also able to inhibit glycerol phosphate acyltransferase (Table 1). The action of fenfluramine derivatives on the incorporation of glycerol into lipids by liver slices was compatible with an inhibition of glyceride and phospholipid synthesis at the level of phosphatidate phosphohydrolase: phosphatidate accumulated and the synthesis of triacylglycerol and phosphatidylcholine was depressed. However, the steady-state concentration of diacylglycerol was not significantly changed (Fig. 2*b*). This might have resulted from a simultaneous decrease in the utilization of diacylglycerol. Dannenburg *et al.* (1973) demonstrated that fenfluramine inhibits acylglycerol palmitoyltransferase (EC 2.3.1.22) and we thought that diacylglycerol acyltransferase activity might also be inhibited. However, no evidence for a direct inhibition was obtained under the experimental conditions used. It is not known whether CDP-choline–1,2-diacyl-*sn*-glycerol cholinephosphotransferase (EC 2.7.8.2) might be inhibited by fenfluramine derivatives, although D-amphetamine has been postulated to have this effect (Hitzemann & Loh, 1973; Leonard & Neuhoff, 1974). Another possibility is that the concentration of diacylglycerol has to reach a threshold before it can be metabolized further.

In the past there have been a number of reports about the effects of amphiphilic cations on phospholipid synthesis, the common feature of which is an increased labelling with ^{32}P of phosphatidate or phosphatidylinositol. These effects, which have now been investigated further and which are discussed in greater detail elsewhere (Allan & Michell, 1975), can also be explained in terms of an inhibition of phosphatidate phosphohydrolase. This results in an accumulation of phosphatidate which in some tissues leads to a redirection of glycerolipid synthesis into CDP-diacylglycerol, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol.

The mode of action of the amphiphilic cations on phosphatidate phosphohydrolase has not been fully investigated. However, it is likely that the inhibition is caused by a physical interaction between the cations and the phosphatidate resulting in the neutralization of the negatively charged phosphate group, which may be accompanied by the displacement of bivalent cations (Dawson & Hauser, 1970). This view is supported by the following observations. (1) The structure of the hydrophobic region of the amphiphilic amines seems to be relatively unimportant provided that it is bulky (Table 1). (2) The amphiphilic anions are not good inhibitors of phosphatidate phosphohydrolase activity; in fact, at lower concentrations they all stimulate this activity. (3) The introduction of a polar group into compound S780 to yield compound S1204 removes the inhibitory effect (Table 1). However, a direct comparison of the relative effectiveness of these compounds is made difficult because of their low solubilities at the concentrations used in the assay systems. (4) The concentration of chlorpromazine required to produce half-maximum synthesis of phosphatidylinositol in pig lymphocytes is sufficient to cause the neutralization of half of the acidic phospholipids in the assay system used (Allan & Michell, 1975). It is thought that this stimulation of phosphatidylinositol synthesis results directly from the inhibition of phosphatidate phosphohydrolase activity.

The absolute concentration of the amphiphilic amines that is required to produce a 50% inhibition of phosphatidate phosphohydrolase activity would therefore depend on the concentration of acidic phospholipids in a particular assay medium. Thus the values quoted in Table 1 should only be considered in terms of the relative inhibitory effects of the different amines. The amphiphilic amines are known to be concentrated in membranes. Local anaesthesia is obtained in frog sciatic-nerve membranes at concentrations of 6–16mmol of chlorpromazine or local anaesthetics per kg of dry membranes. Approx. 5–10% of this concentration is required for general anaesthesia (Seeman, 1972). In rats treated with 5mg of fenfluramine/kg of body weight, concentrations of 40–80μM-fenfluramine plus norfenfluramine can be detected in the whole liver (D. B. Campbell, personal communication); the concentration of these amines in the hepatocyte

membranes would be even higher. The concentrations of the amphiphilic amines which inhibit phosphatidate phosphohydrolase (Table 1) are therefore reasonable in comparison with those expected *in vivo*.

The ability of the amphiphilic amines to inhibit phosphatidate phosphohydrolase may be of importance in controlling glycerolipid synthesis, since this enzyme is thought to have a regulatory function in this process (Hübscher, 1970; Mangiapane *et al.*, 1973; Brindley, 1974; Lamb & Fallon, 1974). The arguments presented in the present paper indicate that this inhibition can be obtained with a wide variety of cationic drugs in a number of tissues *in vitro*. However, the extent to which such an action might be responsible for their therapeutic actions or their side effects is unknown.

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