Effect of ethanol on lipid metabolism in cultured hepatocytes

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1. Isolated rat hepatocytes were cultured in a modified HI-WO/BA medium for 16 h. In the following 24 h oleate or oleate plus ethanol was added to the medium. After this period the medium was changed again and the cultures were further incubated with [$1\text{-}^{14}\text{C}$]oleate alone or with [$1\text{-}^{14}\text{C}$]oleate plus ethanol for 6 h. This allowed a comparison of effects of short-term (6 h) and long-term (24+6 h) exposure to ethanol on fatty acid metabolism. 2. The increased intracellular accumulation of triacylglycerol in the presence of ethanol was quantitatively accounted for by increased fatty acid uptake, by decreased fatty acid oxidation in the tricarboxylic acid cycle and by decreased VLDL (very-low-density lipoprotein)-triacylglycerol secretion. Ketone-body production was not affected. 3. After short-term exposure the rate of accumulation of triacylglycerol was increased by 50%. This increase was accounted for by increased fatty acid uptake (44%), decreased tricarboxylic acid-cycle activity (49%) and decreased VLDL-triacylglycerol secretion (7%). 4. After long-term exposure, the rate of accumulation of triacylglycerol was increased by 74%. This increase was accounted for by increased fatty acid uptake (34%), decreased tricarboxylic acid-cycle activity (34%) and decreased VLDL-triacylglycerol secretion (32%). 5. The larger increase in accumulation of triacylglycerol after long-term exposure to ethanol was entirely accounted for by increased inhibition of secretion of VLDL-triacylglycerol. 6. The biochemical mechanisms underlying the observations are discussed.

It is well established that both acute and prolonged ethanol administration can cause accumulation of triacylglycerol in the liver. This has been ascribed to effects of ethanol on the oxidation of fatty acids, on the secretion of VLDL-triacylglycerol, on the esterification of fatty acids and on the synthesis and uptake of non-esterified fatty acids by the liver. However, conflicting results have been obtained by different laboratories (see Baraona & Lieber, 1979; Kondrup, 1984). The effects of ethanol on all these reactions have never been investigated simultaneously in order to account quantitatively for a chemically measured increase in the cellular triacylglycerol. This has been due to the lack of a model *in vitro* of the ethanol-induced fatty liver. Primary cultures of hepatocytes have provided such a model, allowing long-term exposure to ethanol with the formation of fatty liver (Dich *et al.*, 1983).

Abbreviation used: VLDL, very-low-density lipoprotein(s).

Previous work with hepatocyte cultures (Grunnet *et al.*, 1984) has demonstrated a time lag of 6 h before the effect of ethanol on triacylglycerol accumulation was discernible. In the present study the effect of ethanol on the metabolism of 1 mM- [$1\text{-}^{14}\text{C}$]oleate was studied over a period of 6 h in cell cultures that had been preincubated for 24 h either with 1 mM-oleate or with 1 mM-oleate plus 50 mM-ethanol.

Experimental

Materials

[$1\text{-}^{14}\text{C}$]Oleate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was of sufficient purity for direct use as judged by t.l.c. analysis. $^{60}\text{Co}(\text{NO}_3)_2$ was from Risø, Roskilde, Denmark. Serum albumin, from Armour, Eastbourne, Sussex, U.K., was delipidated as described by Chen (1967). Silica glass-fibre t.l.c. plates were from Gelman Instrument Co., Ann Arbor, MI, U.S.A. Enzymes and coenzymes were

from C. F. Boehringer, Mannheim, Germany, except collagenase (type II), which was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Insulin was purchased from Novo, Copenhagen, Denmark, and dexamethasone from Merck, Sharp and Dohme, Haarlem, The Netherlands. Horse serum was obtained from Gibco, Glasgow, Scotland, U.K., and collagen was prepared from rat tails (Michalopoulos & Pitot, 1975).

Hepatocyte cultures

Hepatocytes were prepared from 200g starved female rats and plated on collagen-coated Petri dishes in a modified HI-WO/BA medium containing horse serum (10%, v/v), insulin (0.1 μ M) and dexamethasone (1 μ M) as previously described (Dich *et al.*, 1983). At 20h after plating (on the morning of day 2), the dishes were divided into three groups. In groups 1 and 2, the medium was changed to a serum- and hormone-free HI-WO/BA medium with 1.0mM-oleate and 0.29mM-albumin. Group 3 was incubated with the same medium plus 50mM-ethanol. On the morning of day 3, the medium in all three groups was replaced with the same medium as mentioned above except for the addition of [1- 14 C]oleate to a specific radioactivity of about 200c.p.m./nmol. [1- 14 C]Oleate was mixed with unlabelled oleate before complexing with albumin. To groups 2 and 3 were further added ethanol (50mM). At 1.5, 3, 4.5 and 6h after the last change of medium, the medium was removed. Cells were washed with a buffer containing 0.15M-KCl, 0.01M- Na_2HPO_4 and 5mM-tolbutamide in order to inhibit lipolysis (Debeer *et al.*, 1977) and afterwards disrupted by sonication (Sonifier B-12; Branson, Danbury, CT, U.S.A.; 40W for 10s).

A sample of the medium was used for isolation of VLDL, for determination of radioactivity and concentration of fatty acids and radioactivity of water-soluble compounds. To another sample of the medium (3.0ml) was added 0.375ml of 62.5% (w/v) citric acid, and the radioactivity in C-1 of acetoacetate and the amount of ketone bodies were determined. The cells were used for determination of the amount and radioactivity of phospholipids, diacylglycerol, triacylglycerol and cholesterol esters.

For the determination of the radioactivity in CO_2 , cells were plated in culture flasks under conditions identical with those mentioned above. After addition of [1- 14 C]oleate the flasks were sealed by serum caps. At the end of the incubation 100 μ l of 5M-NaOH was injected into the flasks. On the next day a sample of the alkaline medium was transferred to Warburg flasks with filter paper containing 100 μ l of 2M-NaOH in the centre cup and 350 μ l of 62.5% citric acid in the side arm. After closing the flasks, the citric acid was tilted

into the main vessel. The flasks were incubated for 1h at 37°C and radioactivity absorbed to the filter-paper was counted.

Analysis of lipids

Lipids were extracted from the cell or the VLDL fraction as described previously (Grunnet & Kondrup, 1983) after addition of 0.2ml of 1M-citric acid per 0.9ml fraction and separated by t.l.c. (Breckenridge & Kuksis, 1968). Bands corresponding to phospholipids, diacylglycerol and triacylglycerol were cut out and eluted quantitatively with 5ml of Folch's lower phase (Folch *et al.*, 1957; Kondrup, 1979). Of this 0.5ml was used for determination of radioactivity and 4.5ml was evaporated and used for glycerol determination after alkaline hydrolysis (Eggstein & Kuhlmann, 1974) or phosphate determination after acid hydrolysis (Kates, 1972) for acylglycerol and phospholipid compounds respectively. T.l.c. fractions corresponding to non-esterified fatty acids and cholesterol esters were used for determination of radioactivity only and were counted directly.

VLDL were isolated by ultracentrifugation at 12–16°C in a SW-40 rotor at 187000g for 21h in an L 65-5 ultracentrifuge (Beckman Spinco); 12.7ml of medium of density 1.006g/ml was used. After centrifugation the upper 2ml was removed from the centrifuge tubes and used for lipid analysis as described above.

Radioactivity of non-esterified fatty acids in the medium was determined as the sum of radioactivity in non-esterified fatty acid in the top fraction (measured after t.l.c. as described above) and lipid-soluble radioactivity in the bottom fraction from the ultracentrifugation. More than 97% of the radioactivity in the latter fraction migrated as non-esterified fatty acids in the t.l.c. system described above.

The concentration of non-esterified fatty acids in the medium was measured as the ^{60}Co -complex (Ho & Meng, 1969).

Analysis of water-soluble compounds

Water-soluble radioactivity was measured in the water phase from the lipid extraction of the top fraction and the bottom fraction after ultracentrifugation for isolation of VLDL.

The citric acid-treated medium was used for measurement of radioactivity of C-1 of acetoacetate by aniline-catalysed decarboxylation (Ontko, 1972) and for determination of 3-hydroxybutyrate (Williamson & Mellanby, 1974) and acetoacetate (Mellanby & Williamson, 1974). DNA was determined in the cell fraction by the method of Kissane & Robins (1958).

Radioactivity

^{14}C radioactivity was determined in a scintillation mixture of Instafluor/Triton X-100 or X-114 (2:3, v/v) with a total water volume of 23%.

Calculation and expression of results

All determinations were performed in duplicate. The recovery of radioactivity for each sample through the separation procedure was used to correct the radioactivity and the amount of the various lipid fractions to 100% recovery. The lowest recovery was observed for the samples that went through the whole separation procedure, including t.l.c., and was $91 \pm 9\%$ (mean $\pm$ S.D., $n = 120$).

The results of the chemical measurements are presented as fatty acid units/mg of DNA and are means $\pm$ S.E.M. for five experiments, except cellular triacylglycerol and fatty acid uptake, which are from four experiments, owing to obvious technical errors. In the calculations of total fatty acid oxidation (see the Results section), a fatty acid chain length of 18 carbon atoms was assumed. The incorporation of $[1-^{14}\text{C}]$ oleate into metabolites is expressed as $[1-^{14}\text{C}]$ oleate/mg of DNA, designated C_{18} /mg of DNA, and values are means $\pm$ S.E.M. for five experiments. Statistically, the results were treated as paired data and levels of significance were estimated by Student's t test. Rates were calculated by linear regression analysis by the least-squares method, except for ketone-body production and incorporation of ^{14}C into VLDL-triacylglycerol, as specified in the Results section. Correlation coefficients (r) were larger than 0.89, except for the triacylglycerol accumulation in one experiment with control cells ($r = 0.63$) and for the incorporation of ^{14}C into diacylglycerol in one experiment with control cells ($r = 0.81$).

For the comparison of results with those obtained by other laboratories it was assumed that 1 g liver wet wt. corresponds to 2.5 mg of DNA (Fischer-Szafarz *et al.*, 1981) and that 1 g of liver protein corresponds to 16 mg of DNA (Durrington *et al.*, 1982).

Results

Fatty acid uptake

The concentration of fatty acids in the medium declined to about 0.6 mM at 6 h (results not shown). The fatty acid uptake in the 0–6 h period, as determined by chemical analysis, was 6290 ± 288 nmol/h per mg of DNA (Fig. 1a), which is similar to the uptake measured in perfused rat liver at this fatty acid concentration (Heimberg *et al.*, 1969; Kondrup, 1979). Ethanol addition to cell cultures which had been preincubated with oleate or oleate plus ethanol caused a (statistically insigni-

ficant) 9% and 12% increase in the uptake of fatty acids respectively.

The corresponding uptake of $[1-^{14}\text{C}]$ oleate was 6588 ± 299 nmol C_{18} /h per mg of DNA (Fig. 1b) in control cells. Ethanol caused a statistically significant increase in the uptake by 12% in cells preincubated with oleate ($P < 0.01$) and by 16% in cells preincubated with oleate plus ethanol ($P < 0.01$). Ethanol has previously been shown to increase the fatty acid uptake in the perfused rat liver by 15% (Kondrup *et al.*, 1979a).

Secretion of VLDL-triacylglycerol

The secretion of triacylglycerol as VLDL occurred at a rate of 1455 ± 247 nmol of fatty acid/h per mg of DNA (Fig. 1c). In liver perfusions with 1 mM-oleate, Topping & Mayes (1972) and Goh & Heimberg (1979) measured a rate of secretion of 1.3 and 1.6 μmol of fatty acids/h per mg of DNA respectively. In hepatocyte cultures incubated with 1.0–1.5 mM-oleate, the VLDL-triacylglycerol secretion was 0.4–0.7 μmol of fatty acids/h per mg of DNA (Dashti *et al.*, 1980; Davis & Boogaerts, 1982).

Ethanol had no significant effect on the rate of VLDL-triacylglycerol secretion when added to cells that had been preincubated with oleate only (rate 1356 ± 261 nmol of fatty acids/h per mg of DNA; Fig. 1c), although the amount of VLDL-triacylglycerol in the incubation medium after 6 h was 14% lower ($P < 0.01$) than in control cells (Fig. 1c). In cell cultures that had been preincubated with oleate plus ethanol, ethanol caused a 46% inhibition of the secretion of VLDL-triacylglycerol ($P < 0.01$; Fig. 1c).

Because of a time lag in the appearance of radioactivity in VLDL (Fig. 1d), the rate of incorporation of $[1-^{14}\text{C}]$ oleate into this product was calculated by linear regression analysis of the data from 1.5 to 6.0 h. The lower secretion rate of radioactivity during the first 1.5 h of incubation was probably due to the existence of preformed VLDL particles in the hepatocytes at zero time. Incorporation of $[1-^{14}\text{C}]$ oleate into VLDL-triacylglycerol was 927 ± 164 nmol of C_{18} /h per mg of DNA in control cells. The inhibition by ethanol of the secretion of ^{14}C -labelled VLDL-triacylglycerol was 48% in the cells preincubated with oleate plus ethanol ($P < 0.01$; Fig. 1d).

Production of ketone bodies and labelled water-soluble products

As shown in Fig. 1(e), the rate of ketone-body production was not linear with time, and therefore the rate of ketone-body production is given as a time-averaged value for the 6 h period. The rate was 995 ± 125 nmol of fatty acids/h per mg of DNA. With 1 mM fatty acids as substrate, ketone-

Fig. 1. Time course of fatty acid uptake, secretion of VLDL-triacylglycerol, ketone-body production and formation of water-soluble products in cultured hepatocytes

Isolated hepatocytes were cultured in a modified HI-WO/BA medium as described in the text (○) in the absence of ethanol in both the preincubation and the incubation periods, (●) in the presence of ethanol (50 mM) only in the incubation period (6 h) or (■) in the presence of ethanol in both the preincubation (24 h) and the incubation periods. Oleate (1 mM) was added to all plates in both the preincubation and in the incubation periods. Upper panels (a, c, e): chemical determinations. Lower panels (b, d, f): incorporation of [^{14}C]oleate. Bars indicate S.E.M.; $n = 5$, except in (a), where $n = 4$. * indicates that the value is significantly different from the control value ($P < 0.05$ or less).

body production in the perfused liver from fed rats has been reported to be 1.3 and 0.9 μmol of fatty acids/h per mg of DNA respectively by Heimberg *et al.* (1969) and Laker & Mayes (1982).

Ethanol caused no change in the production of ketone bodies in cells preincubated with oleate or with oleate plus ethanol (Fig. 1e).

The incorporation of [^{14}C]oleate into ketone bodies and other water-soluble products amounted to $648 \pm 60 \text{ nmol}$ of C_{18} /h per mg of DNA in control cells, and was inhibited to 76% and 77% of this in cells preincubated with oleate and with oleate plus ethanol respectively ($P < 0.02$, Fig. 1f).

CO_2 production

The oxidation of [^{14}C]oleate to CO_2 was

$296 \pm 33 \text{ nmol}$ of C_{18} /h per mg of DNA (Fig. 2). In liver from fed rats perfused with 1 mM-[^{14}C]-palmitate, a $^{14}\text{CO}_2$ production of 0.5 μmol of fatty acids/h per mg of DNA was reported (Kondrup *et al.*, 1979a).

Ethanol caused a 49% inhibition of the $^{14}\text{CO}_2$ production in cells preincubated with oleate only ($P < 0.01$). Preincubation with oleate plus ethanol caused a further depression ($P < 0.02$) of the formation of $^{14}\text{CO}_2$ to 37% of that in control cells ($P < 0.01$; Fig. 2).

Total fatty acid oxidation

Total fatty acid oxidation is taken to be the sum of ketone bodies, 'other water-soluble products' and CO_2 . The rate of total oxidation of exogenous

Fig. 2. Time course of $^{14}\text{CO}_2$ production in cultured hepatocytes

Results are shown from the same experiments as depicted in Fig. 1 ($n = 4$). * and ** respectively indicate that the value is significantly different ($P < 0.05$ or less) from the control value and the value in experiments where ethanol was present only in the incubation period.

and endogenous fatty acids can be calculated from the data given above plus the specific radioactivity of C-1 of acetoacetate. It was assumed that acetyl-CoA is formed exclusively from fatty acids and that a single pool of acetyl-CoA is the precursor of these oxidation products. Total production of water-soluble products was calculated by dividing the measured rate of appearance of radioactivity in water-soluble products by the specific radioactivity of C-1 of acetoacetate. When the chemically measured rate of ketone-body formation is subtracted from this value, the total production of 'other water-soluble products' is obtained. Total production of CO_2 was calculated by dividing the rate of appearance of radioactivity in CO_2 by the specific radioactivity of C-1 of acetoacetate. The sum of total production of 'other water-soluble products' and CO_2 was taken to represent the flux of acetyl-CoA through the tricarboxylic acid cycle. In this calculation, the specific radioactivity of C-3 of acetoacetate is assumed to equal that of C-1. A radioactivity ratio of C-3 to C-1 of acetoacetate of 0.65, as observed in isolated hepatocytes from fed rats (Grunnet & Kondrup, 1983), results in a 15%, 40% and 50% higher estimate of the tricarboxylic acid-cycle flux in the absence of ethanol, in the presence of ethanol after preincubation with oleate and in the presence of ethanol after preincubation with ethanol plus oleate respectively. However, the absolute change in the tricarboxylic acid-cycle flux caused by ethanol is unaffected by the

specific radioactivity of C-3 of acetoacetate. In the present experiments, the specific radioactivity of C-1 of acetoacetate relative to that of the added oleate was 0.45 ± 0.04 ($n = 4$) at 6 h. No effect of ethanol addition was observed.

In control cells, total oxidation of acetyl-CoA through the tricarboxylic acid cycle was 1102 nmol of C_{18}/h per mg of DNA, and the total fatty acid oxidation was 2097 nmol/h per mg of DNA. In cells preincubated with oleate only, ethanol inhibited the oxidation of acetyl-CoA through the tricarboxylic acid cycle by 61%, to 435 nmol of C_{18}/h per mg of DNA, and the total fatty acid oxidation by 32%, to 1431 nmol/h per mg of DNA. In cells preincubated with oleate plus ethanol, ethanol caused a 68% inhibition of the oxidation of acetyl-CoA through the tricarboxylic acid cycle and a 36% inhibition of the total oxidation of fatty acids. The effect of ethanol on fatty acid oxidation is thus accounted for by the effect on the tricarboxylic acid cycle activity (cf. Table 1).

Accumulation of triacylglycerol in the cells

At the start of the experimental period the cells contained considerable amounts of triacylglycerol, owing to the 24 h preincubation with oleate (Fig. 3a). In the control cells the accumulation rate was 2620 ± 554 nmol of fatty acids/h per mg of DNA. The accumulation rate in the present study is higher than that in the previous study (Dich *et al.*, 1983), probably because of the higher mean concentration of oleate in the present shorter experimental period.

Addition of ethanol to cells that had been preincubated with oleate or with oleate plus ethanol caused a 50% and 74% increase in the rate of triacylglycerol accumulation respectively ($P < 0.02$; Fig. 3a). The magnitude of the effect of ethanol is comparable with that observed previously in 24 h incubations of hepatocyte cultures (Dich *et al.*, 1983).

The incorporation of [$1\text{-}^{14}\text{C}$]oleate into cellular triacylglycerol was 3857 ± 336 nmol of C_{18}/h per mg of DNA (Fig. 3b), i.e. about 1.5 times the rate of accumulation of triacylglycerol, indicating a turnover of the intracellular triacylglycerol pool. Ethanol caused a 26% and 42% increase in the [$1\text{-}^{14}\text{C}$]oleate incorporation when added to cells that had been preincubated with oleate or with oleate plus ethanol respectively ($P < 0.001$; Fig. 3b). Ethanol caused a 15% higher rate of triacylglycerol accumulation (not significant) and a 13% higher rate of ^{14}C incorporation into triacylglycerol ($P < 0.001$) in cells that had been preincubated with oleate plus ethanol when compared with cells that had been preincubated with oleate only. In perfused liver from fed rats, Kondrup *et al.* (1979a) measured an incorporation of [^{14}C]fatty

Table 1. *Rates of fatty acid metabolism and the changes caused by ethanol during the 6 h experimental period*
 The rates (nmol/h per mg of DNA) of fatty acid metabolism for control cells are shown in column (1) and the changes caused by ethanol are shown for cells preincubated with oleate only in column (2) and for cells preincubated with oleate plus ethanol in column (3). Rates were calculated as described in the Experimental section.

24h preincubation ... 6h experimental period ...	Rate	Change caused by ethanol	
		— Ethanol	Ethanol Ethanol
1 Fatty acid uptake	(1) 6290	(2) 597	(3) 734
2 VLDL-triacylglycerol secretion	1455	—99	—675
3 Ketone-body formation	995	1	1
4 Acetyl-CoA oxidation through the tricarboxylic acid cycle	1102	—667	—747
5 Total fatty acid oxidation	2097	—666	—746
6 Cellular triacylglycerol	2620	1331	1934
7 Cellular diacylglycerol	0	0	0
8 Cellular phospholipids	0	0	0
9 Total cellular acylglycerol	2620	1331	1934
Line 2+5+9/1	0.98	—	—
Line 1—2—5/9	—	1.02	1.12

Fig. 3. *Time course of content of triacylglycerol, diacylglycerol and phospholipids in cultured hepatocytes*
 Results are shown for the same experiments as depicted in Fig. 1; $n = 5$, except in (a), where $n = 4$. Upper panels (a, c, e): chemical determinations. Lower panels (b, d, f): incorporation of $[1-^{14}\text{C}]$ oleate.

acids into triacylglycerol of $6.2\mu\text{mol}$ of fatty acids/h per mg of DNA and an 18% stimulation by ethanol.

Diacylglycerol in the cells

At the start of the experimental period, control cells contained $3.7\mu\text{mol}$ of fatty acids/mg of DNA as diacylglycerol and this amount did not change during the experiments (Fig. 3c). The content in rat liver has been reported to be about $1\mu\text{mol}$ of fatty acids/mg of DNA (Kondrup *et al.*, 1979a). Preincubation of the cell cultures with oleate plus ethanol caused a 22% increase in the diacylglycerol content ($P < 0.01$, Fig. 3c). Ethanol addition during the 6 h experimental period had no effect on the diacylglycerol content of the cells (Fig. 3c).

Incorporation of $[1-^{14}\text{C}]$ oleate into diacylglycerol was $240 \pm 18\text{nmol}$ of C_{18}/h per mg of DNA in control cells (Fig. 3d). Ethanol caused a 28% and 42% increase when added to cells that had been preincubated with oleate or with oleate plus ethanol, respectively ($P < 0.05$ and < 0.001 respectively).

Phospholipids in the cells

The initial content of phospholipids was $25.4\mu\text{mol}$ of fatty acids/mg of DNA (Fig. 3e),

which is about the same as found in rat liver (Kondrup, 1979). The content did not change during the experimental period (Fig. 3e).

Incorporation of $[1-^{14}\text{C}]$ oleate into phospholipids was $557 \pm 76\text{nmol}$ of C_{18}/h per mg of DNA in control cells, and was increased by 17% in cells that had been preincubated with oleate only ($P < 0.02$; Fig. 3f). On the contrary, ethanol had no effect on the incorporation of radioactivity into phospholipids in cells that had been preincubated with oleate plus ethanol (rate $104 \pm 7\%$ of control rate; Fig. 3f).

Labelling of cholesterol esters

The incorporation of $[1-^{14}\text{C}]$ oleate into cholesterol esters is shown in Fig. 4. In the control cells $75 \pm 9\text{nmol}$ of C_{18}/h per mg of DNA was incorporated. Addition of ethanol in the experimental period only did not change this rate, but addition of ethanol also in the preincubation period decreased the incorporation to 45% ($P < 0.02$; Fig. 4). These results are in accordance with the suggestion that the turnover of cholesterol esters directly reflects the rate of VLDL secretion (Mathe *et al.*, 1984). A similar picture was seen for the ^{14}C labelling of cholesterol esters in the VLDL (results not shown).

Discussion

Stoichiometry of fatty acid metabolism

The total contents of non-esterified and esterified fatty acids in the hepatocyte cultures at the start of the 6 h experimental period were 166161 and $177753\text{nmol}/\text{mg}$ of DNA for cells preincubated with oleate and with oleate plus ethanol respectively. The recovery of fatty acids after 1.5, 4.5 and 6 h of incubation was $99 \pm 5\%$ (mean $\pm$ s.d., $n = 48$). Further, in the control cells, $98 \pm 7\%$ of the fatty acid uptake was accounted for by oxidation products, and fatty acids accumulated in cellular triacylglycerol and VLDL-triacylglycerol (Table 1). In the presence of ethanol $98 \pm 8\%$ and $98 \pm 5\%$ of the fatty acid uptake was accounted for in these metabolites in cells preincubated with oleate and with oleate plus ethanol respectively (results not shown).

The ethanol-induced accumulation of triacylglycerol was accounted for in increased fatty acid uptake, decreased fatty acid oxidation and decreased VLDL-triacylglycerol secretion by 102% and 112% in cells preincubated with oleate and with oleate plus ethanol respectively (Table 1). These values are considered equal to 100%, as they represent a difference between a small difference between two large numbers. Fatty acid synthesis in the present system corresponds to 407nmol of fatty acids/h per mg of DNA (Grunnet *et al.*, 1984) and was not changed by ethanol.

Fig. 4. Time course of the incorporation of $[1-^{14}\text{C}]$ oleate into cholesterol esters in cultured hepatocytes

Results are shown from the same experiments as depicted in Fig. 1 ($n = 5$).

It is apparent from the above data that some endogenous fatty acids in acylglycerol and phospholipids were exchanged with exogenous fatty acids, since incorporation of [$1-^{14}\text{C}$]oleate exceeded the chemically measured net accumulation of esterified products. In control cells the excess incorporation of [$1-^{14}\text{C}$]oleate amounted to 2034 nmol/h per mg of DNA (1237 into triacylglycerol, 240 into diacylglycerol and 557 into phospholipids). Conversely the total oxidation of fatty acids, and the secretion of fatty acids as VLDL-triacylglycerol, exceeded the conversion of [$1-^{14}\text{C}$]oleate into these products: in control cells by a total of 1933 nmol of fatty acids/h per mg of DNA (1149 in total oxidation and 784 in VLDL-triacylglycerol). It appears that the exchanged endogenous fatty acids were quantitatively accounted for by conversion into oxidation products and triacylglycerol in VLDL; it appears that the assumption that fatty acids are the only precursor for acetyl-CoA (see above) is essentially correct. With ethanol present the exchange of endogenous fatty acids amounted to 1961 nmol/h per mg of DNA; the conversion of endogenous fatty acids into oxidation products and triacylglycerol in VLDL amounted to 1493 nmol/h per mg of DNA. With ethanol present, after preincubation with ethanol, the exchange of endogenous fatty acids amounted to 1825 nmol/h per mg of DNA; endogenous fatty acids in total oxidation and triacylglycerol in VLDL amounted to 1209 nmol/h per mg of DNA. The exchanged endogenous fatty acids not accounted for in the presence of ethanol could have been liberated into the medium, according to the mean values of uptake of fatty acids (uptake of [$1-^{14}\text{C}$]oleate versus chemically determined fatty acid uptake); however, the confidence limits of these measurements do not allow any firm conclusions.

Effect of ethanol

In the present experiments 44% of the ethanol effect on triacylglycerol accumulation was accounted for by increased fatty acid uptake, 49% by decreased fatty acid oxidation and 7% by decreased VLDL-triacylglycerol secretion during 6 h incubation with ethanol (Table 1). After 24+6 h incubation with ethanol, 34% of the ethanol effect on triacylglycerol accumulation was accounted for by increased fatty acid uptake, 35% by decreased fatty acid oxidation and 31% by decreased VLDL-triacylglycerol secretion (Table 1).

All the effects of ethanol observed in the present system have been described previously; however, this is the first study to present a quantitative account for a chemically measured increase in the accumulation of triacylglycerol. Further, the relative importance of the effect of ethanol on the indi-

vidual metabolic pathways has not previously been determined.

An increase in fatty acid uptake has previously been reported *in vivo*, and it was ascribed to an ethanol-induced increase in hepatic blood flow (Abrams & Cooper, 1976a,b). Ethanol also increases the fatty acid uptake in perfused liver (Kondrup *et al.*, 1979a) and in freshly isolated hepatocytes (Wiebe & Belfrage, 1980). There is no explanation to this effect of ethanol; it is probably not a simple biophysical action of ethanol, since the effect is independent of the concentration of ethanol and is not always present (Kondrup *et al.*, 1979a,b).

Previous investigators have reported variable effects of ethanol on total fatty acid oxidation *in vitro* (see Table 4 in Grunnet & Kondrup, 1983). In all previous reports ethanol decreased CO_2 production from fatty acids. The decreased tricarboxylic acid-cycle activity is probably due to the increased NADH/NAD⁺ ratio in the mitochondria during ethanol oxidation (Ontko, 1973); cultured hepatocytes also exhibit an increased mitochondrial NADH/NAD⁺ ratio in the presence of ethanol (Grunnet *et al.*, 1984).

The effect of ethanol on ketone body production is variable, however; Topping *et al.* (1979) found an increase, McGarry & Foster (1971) found a decrease and Fellenius & Kiessling (1973) found no effect. One explanation for this discrepancy may be that ethanol itself may be converted into ketone bodies under some conditions. In freshly isolated hepatocytes, ethanol supplied about 20% of the carbon atoms of ketone bodies and increased ketone-body formation by 10% (Grunnet & Kondrup, 1983). In the present study ethanol did not decrease the specific radioactivity of C-1 of acetoacetate and therefore did not contribute significantly to ketone-body formation. Since β -oxidation can be taken to equal the sum of conversion of fatty acids into ketone bodies and tricarboxylic acid-cycle products, it is concluded that ethanol inhibits β -oxidation. It is, however, not understood why a decreased tricarboxylic acid-cycle activity is not simply associated with an increased ketone-body formation; usually, when the capacity of the tricarboxylic acid cycle is exceeded, the excess acetyl-CoA is converted into ketone bodies.

Inhibition of the secretion of triacylglycerol has been reported previously *in vitro* (Schapiro *et al.*, 1964; Kondrup *et al.*, 1979a; Topping *et al.*, 1979). The decreased secretion of VLDL-triacylglycerol in isolated hepatocytes was suggested to be due to the concomitant inhibition of protein synthesis, including the apoproteins of VLDL (Lakshmanan *et al.*, 1980). In preliminary studies ethanol has been found to inhibit protein synthesis by about 40% in cultured hepatocytes (J. Dich & N. Grunnet,

unpublished work). *In vivo*, the effect of a single dose of ethanol on VLDL-triacylglycerol production is variable; a decrease was reported by Annable & Cooper (1974), an increase by Hernell & Johnson (1973) and no effect by Baraona *et al.* (1973). Ethanol treatment *in vivo* increases the concentration of non-esterified fatty acids in plasma under some conditions (Brodie & Maickel, 1963; Kondrup & Grunnet, 1973); an increased fatty acid concentration will increase the secretion of triacylglycerol by the liver (Heimberg *et al.*, 1969). This indirect effect of ethanol *in vivo* may under some conditions supervene the direct inhibitory effect of ethanol on the liver.

In conclusion, the short term effects of ethanol were to increase the uptake and to decrease the oxidation of fatty acids. These effects of ethanol will increase the hepatic accumulation of triacylglycerol (Heimberg *et al.*, 1969; Ide & Ontko, 1981). Long-term exposure to ethanol caused a similar increase in the total production of triacylglycerol (i.e. intra- + extra-cellular), but in this situation the secretion of triacylglycerol was inhibited, causing an additional increase in the intracellular accumulation of triacylglycerol.

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