Effects of dexamethasone and insulin on the synthesis of triacylglycerols and phosphatidylcholine and the secretion of very-low-density lipoproteins and lysophosphatidylcholine by monolayer cultures of rat hepatocytes

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1. Rat hepatocytes in monolayer culture were preincubated for 19 h with 1 μ M-dexamethasone, and the incubation was continued for a further 23 h with [14 C]oleate, [3 H]glycerol and 1 μ M-dexamethasone. Dexamethasone increased the secretion of triacylglycerol into the medium in particles that had the properties of very-low-density lipoproteins. The increased secretion was matched by a decrease in the triacylglycerol and phosphatidylcholine that remained in the hepatocytes. 2. Preincubating the hepatocytes for the total 42 h period with 36 nM-insulin decreased the amount of triacylglycerol in the medium and in the cells after the final incubation for 23 h with radioactive substrates. However, insulin had no significant effect on the triacylglycerol content of the cell and medium when it was present only in the final 23 h incubation. 3. Insulin antagonized the effects of dexamethasone in stimulating the secretion of triacylglycerol from the hepatocytes, especially when it was present throughout the total 42 h period. 4. The labelling of lysophosphatidylcholine in the medium when hepatocytes were incubated with [14 C]oleate and [3 H]glycerol was greater than that of phosphatidylcholine. The appearance of this lipid in the medium, unlike that of triacylglycerol and phosphatidylcholine, was not stimulated by dexamethasone, or inhibited by colchicine. However, the presence of lysophosphatidylcholine in the medium was decreased when the hepatocytes were incubated with both dexamethasone and insulin. 5. These findings are discussed in relation to the control of the synthesis of glycerolipids and the secretion of very-low-density lipoproteins and lysophosphatidylcholine by the liver, particularly in relation to the interactions of glucocorticoids and insulin.

INTRODUCTION

The injection of glucocorticoids into rats stimulates the synthesis of triacylglycerols in the liver (Glenny & Brindley, 1978) and can lead to the production of a fatty liver (Hill & Droke, 1963; Ožegović *et al.*, 1975). When the corticosteroid analogues methylprednisolone (Reaven *et al.*, 1974), triamcinolone (Krausz *et al.*, 1981), or dexamethasone (Cole *et al.*, 1982) were injected for several days, there was also an increase in the secretion of very-low-density lipoproteins. However, the direct addition of dexamethasone to a liver perfusion did not alter this secretion, except at very high hormone concentrations (Cole *et al.*, 1982).

The present experiments were performed to investigate whether some direct effects of glucocorticoids on the synthesis, accumulation and secretion of glycerolipids could be observed in isolated hepatocytes. The long-term objective of this work is to determine the mechanisms by which these changes are brought about. The hepatocytes were maintained in monolayer cultures, and they had previously been shown to respond to glucocorticoids and insulin and to be stable over several days (Pittner *et al.*, 1985a,b). This also enabled us to determine the effects of insulin on the synthesis, accumulation and secretion of glycerolipids. The literature concerning the effects of insulin on the secretion of very-low-density lipoproteins is contradictory: it has been shown to increase (Topping & Mayes, 1972; Laker & Mayes, 1984), not to change (Woodside & Heimberg, 1976; Beynen *et al.*, 1981; Beynen & Geelen, 1982; Haagsman & van Golde, 1984),

or to decrease (Nikkilä, 1974; Durrington *et al.*, 1982; Patsch *et al.*, 1983a; Pullinger & Gibbons, 1985) this process.

EXPERIMENTAL

Animals and materials

The sources of the rats and most of the materials have been described (Jennings *et al.*, 1981; Pollard & Brindley, 1984; Cascales *et al.*, 1984; Pittner *et al.*, 1985a). Colchicine was purchased from Sigma (London) Chemical Co. Primaria tissue-culture dishes (60 mm diameter) and flasks (50 ml capacity) were purchased from Becton Dickinson U.K. Ltd., Cowley, Oxford, U.K. T.l.c. was performed with plastic sheets of silica gel 60 obtained from Merck through British Drug Houses, Poole, Dorset, U.K., or on glass plates of K6 silica gel obtained from Whatman. The [14 C]oleate-albumin complex was prepared by dissolving 233 μ mol of [14 C]oleate (0.11 Ci/mol) in diethyl ether and neutralizing it with ethanolic 0.1 M-KOH, by using phenolphthalein as an indicator. The solvents were removed by rotary evaporation, and the potassium oleate was dispersed in about 1 ml of water. Fatty-acid-poor bovine serum albumin (2.79 g) which had been neutralized with KHCO_3 in about 8 ml of water was added and the total volume was adjusted to 10 ml.

Preparation and incubation of hepatocytes

Hepatocytes were prepared from male Wistar rats as described by Cascales *et al.* (1984), and approx. 2×10^6

cells were applied to 60 mm-diam. Primaria (Falcon) tissue-culture dishes (Pittner *et al.*, 1985a). These dishes were used without further treatment, since coating with collagen was not necessary because of the positive charge on the plastic. Our initial work with this culture system established that coating normal tissue-culture dishes with collagen (Cascales *et al.*, 1984) or the use of Primaria dishes gave much better attachment of the cells during long-term cultures compared with coating with fibronectin as described by Durrington *et al.* (1982). The medium was the modified Leibovitz L-15 containing 10% (v/v) newborn-calf serum as described by Cascales *et al.* (1984). The cells were incubated for 1–2 h at 37 °C in water-saturated air, and the unattached and non-viable cells were removed with the medium. The monolayer of cells was then incubated for 17 h with fresh medium that contained 1 μ M-dexamethasone or 36 nM-insulin as indicated. After incubation for a further 2 h in fresh medium containing the appropriate hormones, the medium was removed and the cells were washed with 2 $\times$ 2 ml of modified Leibovitz medium which was serum-free. The cells were then incubated in 3 ml of serum-free modified Leibovitz medium which contained 0.94 mM-[1,3-³H]glycerol (0.784 Ci/mol) and 0.72 mM-[1-¹⁴C]oleate (0.11 Ci/mol) which was complexed to albumin. Furthermore, the medium was supplemented with a final concentration of 315 μ M-choline chloride to ensure that the synthesis of phosphatidylcholine was not limited by choline availability. Phosphatidate phosphohydrolase activity was measured (Pollard & Brindley, 1984) in parallel incubations with the same concentrations of non-radioactive substrates.

The medium was collected after various times of incubation and was centrifuged at 1200 *g* for 10 min at room temperature to remove detached cells and cell debris. Samples (2 ml) of the supernatant were taken for lipid analysis. The monolayer of cells was washed with 2 $\times$ 2 ml of modified Leibovitz medium (serum-free) and then scraped from the dishes in 1 ml of ice-cold 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes adjusted to pH 7.4 with KOH. The suspension was sonicated for 5 s at 22 kHz with an amplitude of 8 μ m peak-to-peak. Samples (0.8 ml) of the homogenate were used for lipid analysis and the remainder was stored at –20 °C before the determination of lactate dehydrogenase (Saggerson & Greenbaum, 1969) and of protein by the method of Bradford (1976), except that PAGE Blue G-90 was used. The results in the paper are expressed relative to lactate dehydrogenase, which was used as an indicator of viable cells in preference to measuring DNA or protein (Pittner *et al.*, 1985a,b). Non-viable cells attached to the dishes could contribute to these latter values. The ratio of lactate dehydrogenase to protein remained relatively constant during the incubation of hepatocytes with radioactive precursor in this type of system (R. A. Pittner & D. N. Brindley, unpublished work).

Extraction and analysis of lipids

Lipids were extracted by the method of Bligh & Dyer (1959) from samples of the cell homogenates or incubation medium. The bottom phase of the extracts was washed twice with the appropriate volume of top phase prepared from chloroform/methanol/water (10:10:9, by vol.) and containing 1% (w/v) glycerol. Samples of the

bottom phase were dried with a stream of N₂ and dissolved in 100 μ l of chloroform. Samples (25 μ l) were taken for scintillation counting after evaporation, and 50 μ l samples were applied to 20 cm t.l.c. plates. These were developed for 6 cm with chloroform/methanol/acetic acid/acetone/water (10:2:2:4:1, by vol.), dried, and then developed for their full lengths with hexane/diethyl ether/acetic acid (60:40:1, by vol.). Lipids were detected with I₂ vapour and eluted from the silica gel by adding 1 ml of water and 10 ml of FisoFluor scintillant. Radioactivity was determined after 48 h and by correction for counting efficiency by the external-standard method; recoveries of ³H and ¹⁴C were greater than 95%. The identity of lipids was confirmed by co-chromatography with standards. The radioactivity in lysophosphatidylcholine was further characterized both by chromatography in two dimensions in the solvent systems described by Arthur & Sheltawy (1980) and by completely separating it from sphingomyelin by a double development in one dimension with chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.). The lysophosphatidylcholine was identified by spraying the plates with Dragendorff's reagent (Dawson *et al.*, 1969).

The concentration of triacylglycerol was determined after separating neutral lipids by t.l.c. in hexane/diethyl ether/acetic (70:30:1, by vol.). The areas containing triacylglycerols were scraped from the plates and eluted by washing with 2 $\times$ 2 ml of chloroform/methanol (1:4, v/v). The solvents were evaporated under a stream of N₂, and triacylglycerol was determined by the charring method of Kabara & Chen (1976). The concentration of triacylglycerols was calculated by comparison with standards of trioleoylglycerol that were chromatographed at the same time.

Precipitation of very-low-density lipoproteins

The method was adapted from that of Burstein *et al.* (1970). To 2.75 ml of cell-free medium was added 1.1 ml of fresh human serum, 113 μ l of 2 M-MgCl₂, 63 μ l of 100 mM-sodium phosphotungstate, pH 7.0, and 475 μ l of water. After centrifuging at 1200 *g* for 60 min at room temperature, lipid was analysed in the supernatant and precipitate after the latter had been resuspended in 0.16 M-NaCl.

Ultracentrifugation of lipoproteins

This was performed on 4 ml of the cell-free medium from incubations essentially as described by Vance *et al.* (1983). The samples were centrifuged for 90 h at 10 °C in the AH627 rotor (*r*_{av} = 11.7 cm) at 94 500 *g* in a Sorvall OTD-50 ultracentrifuge. Fractions (1 ml) were collected from the tops of the gradients by using a peristaltic pump, and these were used for lipid analysis.

Determination of fatty acid oxidation

The formation of ¹⁴CO₂ and acid-soluble ¹⁴C from the [1-¹⁴C]oleate-albumin complex was measured as described by Pollard & Brindley (1984), except that the monolayers of hepatocytes were maintained in 50 ml Primaria tissue-culture flasks. After addition of [1-¹⁴C]oleate, the flasks were closed with a Subaseal, through which Hyamine hydroxide and HClO₄ were added after a 30 min incubation.

Fig. 1. Effects of dexamethasone on the synthesis and secretion of triacylglycerol by rat hepatocytes

Hepatocytes were preincubated for 19 h in the absence (□, △, ○) or presence (■, ▲, ●) of $1 \mu\text{M}$ -dexamethasone. The media were then replaced and 0.94 mM - $[1,3\text{-}^3\text{H}]$ glycerol and 0.72 mM - $[1\text{-}^{14}\text{C}]$ oleate were added, and the cells and the medium were analysed for lipids at the times indicated (see the Experimental section). The Figure shows: (a) the incorporation of [^{14}C]oleate; (b) the incorporation of [^3H]glycerol; and (c) and (d) the concentration of triacylglycerol as determined chemically (continuous lines) and the concentration calculated from the incorporation of [^{14}C]oleate and assuming that 3 mol of oleate were incorporated per mol of triacylglycerol (broken lines). The symbols indicate cell triacylglycerol (□, ■), medium triacylglycerol (△, ▲) and the sum of these two (○, ●). Each value is the mean $\pm$ range from two independent experiments. The 23 h time point was reproduced in two further independent experiments (Table 1).

RESULTS

Effect of dexamethasone on the synthesis and secretion of glycerolipids by hepatocyte monolayers

Hepatocytes were preincubated for 19 h at 37°C with $1 \mu\text{M}$ -dexamethasone so that it could induce long-term

changes in metabolism before the beginning of studies on lipid synthesis and secretion. When the hepatocytes were incubated with [^3H]glycerol and [^{14}C]oleate, there was a constant rate of incorporation of these precursors into the sum of the cell and secreted triacylglycerols for about 4 h (Figs. 1a and 1b). After this time the rate of synthesis

Table 1. Effects of insulin and dexamethasone on the synthesis and secretion of glycerolipids in isolated hepatocytes

Hepatocytes were preincubated for 19 h in the absence or presence of 1 μ M-dexamethasone or 36 nm-insulin as indicated in rows I–IV and VI. The medium was then replaced by the equivalent fresh medium to which 0.72 mM- 14 C-oleate and 0.94 mM- 3 H-glycerol had been added and, for rows V and VI, 36 nm-insulin was added only at this stage (see the Experimental section). The cells were then incubated for a further 23 h, and the cells and the medium were then analysed for 14 C- and 3 H-labelled lipids and for their total triacylglycerol contents. Results are expressed as means $\pm$ s.d. relative to the controls for three independent experiments, except for the radioactive incorporations for rows I and II, where there were four experiments. The absolute values for the controls are given in parentheses for row I, as nmol of triacylglycerol/unit of lactate dehydrogenase for the concentration, or as nmol of precursor incorporated/unit of lactate dehydrogenase for the incorporations. Lysophosphatidylcholine was not found in significant quantities in cell lipids. The significances of differences between the various treatments in sections (a)–(c) were calculated by using a paired *t* test, and they are indicated by: **P* < 0.05; ***P* < 0.02; †*P* < 0.01; ††*P* < 0.005.

	Additions	Relative triacylglycerol concn. (%)	Relative incorporation (%) into:					
			Triacylglycerol		Phosphatidylcholine		Lysophosphatidylcholine	
			[14 C]Oleate	[3 H]Glycerol	[14 C]Oleate	[3 H]Glycerol	[14 C]Oleate	[3 H]Glycerol
(a) Cell lipids	(I) None (control)	100 (148 $\pm$ 28)	100 (379 $\pm$ 45)	100 (16 $\pm$ 10)	100 (54 $\pm$ 11)	100 (5.5 $\pm$ 2.1)		
	(II) + dexamethasone (42 h)	58 $\pm$ 6.0 I versus II†	64 $\pm$ 14 I versus II†	51 $\pm$ 13 I versus II††	81 $\pm$ 5.3 I versus II†	71 $\pm$ 9.8 I versus II†		
	(III) + insulin (42 h)	56 $\pm$ 7.9 I versus III**	61 $\pm$ 5.4 I versus III†	44 $\pm$ 8.4 I versus III†	78 $\pm$ 9.7 I versus III*	71 $\pm$ 8.6 I versus III*		
	(IV) + dexamethasone (42 h)	51 $\pm$ 7.4 I versus IV†	56 $\pm$ 12 I versus IV*	33 $\pm$ 12 I versus IV†	62 $\pm$ 8.6 I versus IV*	53 $\pm$ 3.2 I versus IV††		
	+ insulin (42 h)							
	(V) + insulin (23 h)	84 $\pm$ 21	103 $\pm$ 0.59 I versus V** III versus V†	98 $\pm$ 24 II versus IV** III versus V*	94 $\pm$ 4.6	105 $\pm$ 28		
(b) Medium lipids	(VI) + dexamethasone (42 h)	61 $\pm$ 11 I versus VI**	75 $\pm$ 13	66 $\pm$ 23	74 $\pm$ 4.0 I versus VI†	66 $\pm$ 4.3 I versus VI†		
	+ insulin (23 h)							
	(I) None (control)	100 (25 $\pm$ 8.2)	100 (48 $\pm$ 19)	100 (6.1 $\pm$ 3.5)	100 (2.3 $\pm$ 0.7)	100 (0.63 $\pm$ 0.47)	100 (12 $\pm$ 3.7)	100 (4.4 $\pm$ 3.9)
	(II) + dexamethasone (42 h)	220 $\pm$ 125	280 $\pm$ 112 I versus II*	190 $\pm$ 78 I versus III*	243 $\pm$ 136	192 $\pm$ 101	104 $\pm$ 26	97 $\pm$ 23
	+ insulin (42 h)							
	(III) + insulin (42 h)	76 $\pm$ 35	66 $\pm$ 3 I versus III††	63 $\pm$ 9.5 I versus III*	74 $\pm$ 31	98 $\pm$ 47	85 $\pm$ 6.3	87 $\pm$ 14
(c) Cell + medium lipids	(IV) + dexamethasone (42 h)	113 $\pm$ 67	115 $\pm$ 19	85 $\pm$ 21	131 $\pm$ 76	105 $\pm$ 31	75 $\pm$ 13 II versus IV†	65 $\pm$ 11 I versus IV*
	+ insulin (42 h)							
	(V) + insulin (23 h)	93 $\pm$ 13	84 $\pm$ 12	95 $\pm$ 7.7	93 $\pm$ 58	86 $\pm$ 21	98 $\pm$ 12	100 $\pm$ 9.0
	(VI) + dexamethasone (42 h)	115 $\pm$ 48	147 $\pm$ 27	122 $\pm$ 31	135 $\pm$ 80	109 $\pm$ 48	90 $\pm$ 1.5	88 $\pm$ 7.7
	+ insulin (23 h)							
	(I) None (control)	100 (173 $\pm$ 30)	100 (428 $\pm$ 61)	100 (22 $\pm$ 13)	100 (57 $\pm$ 11)	100 (6.2 $\pm$ 2.6)		
(c) Cell + medium lipids	(II) + dexamethasone (42 h)	79 $\pm$ 10	90 $\pm$ 13	93 $\pm$ 34	88 $\pm$ 4.7 I versus II*	81 $\pm$ 16		
	+ insulin (42 h)							
	(III) + insulin (42 h)	57 $\pm$ 6.3 I versus III†	62 $\pm$ 4.6 I versus III††	49 $\pm$ 4.9 I versus III††	78 $\pm$ 7.9 I versus III*	73 $\pm$ 10 I versus III*		
	(IV) + dexamethasone (42 h)	58 $\pm$ 15 I versus IV*	62 $\pm$ 12 I versus IV*	45 $\pm$ 13 I versus IV**	65 $\pm$ 6.5 I versus IV**	57 $\pm$ 4.6 I versus IV††		
	+ insulin (42 h)							
	(V) + insulin (23 h)	84 $\pm$ 17	101 $\pm$ 1.3 III versus V†	97 $\pm$ 17 III versus V*	93 $\pm$ 4.8	103 $\pm$ 26		
(c) Cell + medium lipids	(VI) + dexamethasone (42 h)	67 $\pm$ 3.5 I versus VI††	83 $\pm$ 9.1	81 $\pm$ 24	77 $\pm$ 1.9 I versus VI††	70 $\pm$ 7.4 I versus VI**		
	+ insulin (23 h)

Fig. 2. Effects of dexamethasone on the synthesis and secretion of phosphatidylcholine by rat hepatocytes

The results are taken from the same experiments as shown in Fig. 1. The symbols indicate the phosphatidylcholine present in the cells ($\square$, $\blacksquare$) and in the medium ($\triangle$, $\blacktriangle$). The closed symbols show results when $1\ \mu\text{M}$ -dexamethasone was present throughout the preincubation and incubation periods, whereas the absence of dexamethasone is indicated by open symbols. Results are means $\pm$ ranges from two independent experiments, and the 23 h time point was reproduced in two further independent experiments (Table 1).

began to decline. Dexamethasone did not significantly alter the total rate of triacylglycerol synthesis, as determined by measuring ^{14}C incorporation (Fig. 1a) or the mass of triacylglycerol (Figs. 1c and 1d). However, it did alter the balance between the triacylglycerol present in the cell and in the medium (Fig. 1 and Table 1). After 23 h the amount of ^{14}C triacylglycerol was about 3.7-fold higher in the medium when the cells had been preincubated and incubated with $1\ \mu\text{M}$ -dexamethasone (Figs. 1a and 1b). The increases obtained in two further experiments were 1.9- and 1.8-fold (Table 1). In the latter two experiments, where the relative increase was lower, $2.5\ \mu\text{g}$ of amphotericin B/ml was added to prevent the contamination by yeasts. There are insufficient experiments to be sure that amphotericin B is consistently decreasing the effects of dexamethasone on triacylglycerol secretion, but this compound is known to alter cell permeability (Kotler-Brajtberg *et al.*, 1979). The effect of dexamethasone in stimulating the secretion of triacylglycerol can also be seen as early as 4 h after adding radioactive precursors. This was at a time when the total rate of glycerolipid synthesis was constant with time (Fig. 1).

The increased secretion of triacylglycerol into the medium was approximately balanced by an equivalent decrease in the cell triacylglycerols (Fig. 1; Table 1). Similar effects were seen when the incorporation of ^3H glycerol was measured, and there was a 2.7-fold increase in the ^3H triacylglycerol secreted after 23 h when

Fig. 3. Time course of the appearance of lysophosphatidylcholine in the incubation medium from cultures of hepatocytes

The results for cells incubated in the absence of added hormones are taken from the same experiments as shown in Fig. 1. The appearance of lysophosphatidylcholine was detected by labelling from ^{14}C oleate ($\circ$) and ^3H glycerol ($\bullet$). Results are means $\pm$ ranges from two independent experiments, and the 23 h time point was reproduced in two further independent experiments.

dexamethasone was present (Fig. 1b and also Table 1). However, the total amount of ^3H triacylglycerol in the cell and medium declined after about 4 h, whereas ^{14}C triacylglycerol increased (Figs 1a and 1b). This probably indicates a continuous turnover of triacylglycerol in the hepatocytes, and a low rate of re-incorporation of ^3H glycerol compared with ^{14}C oleate.

The patterns of incorporation of ^{14}C oleate (Fig. 2 and Table 1) and ^3H glycerol (Table 1) into phosphatidylcholine were similar to those for triacylglycerol. Dexamethasone again increased the proportion of the phosphatidylcholine that was secreted in all four experiments (Table 1).

There was also a significant incorporation of ^3H and ^{14}C into a lipid that was identified as lysophosphatidylcholine (Fig. 3). This was found in the medium rather than in the cells. The amount of radioactivity isolated in lysophosphatidylcholine was not significantly altered when the cells were preincubated with dexamethasone (Table 1).

Characterization of the radioactive lipids present in the medium after incubation

The release of triacylglycerol into the medium was inhibited on average by 54 and 62% by $20\ \mu\text{M}$ -colchicine

Table 2. Effects of colchicine on the incorporation by isolated hepatocytes of [14 C]oleate into medium and cell lipids

Hepatocytes were preincubated in the absence or presence of 1 μ M-dexamethasone for 19 h. The medium was then replaced by the equivalent fresh medium that contained 0.72 mM-[14 C]oleate plus 0.94 mM-[3 H]glycerol and 20 μ M-colchicine as indicated, and the cells were incubated for a further 23 h (see the Experimental section). The incorporations of radioactivity in the presence of colchicine are expressed relative to the equivalent values obtained in the absence of colchicine. The results are the means $\pm$ S.D. from three independent experiments. The significances of differences compared with the corresponding value in the absence of colchicine are indicated by: * P < 0.05; ** P < 0.02.

Lipid	Relative incorporation (%) in the absence of dexamethasone into:			Relative incorporation (%) in the presence of dexamethasone into:		
	Medium	Cells	Total	Medium	Cells	Total
Triacylglycerol	46 $\pm$ 16*	110 $\pm$ 11	104 $\pm$ 7	38 $\pm$ 13**	136 $\pm$ 42	104 $\pm$ 17
Phosphatidylcholine	101 $\pm$ 40	105 $\pm$ 8	105 $\pm$ 9	51 $\pm$ 11**	114 $\pm$ 14	108 $\pm$ 7
Lysophosphatidylcholine	110 $\pm$ 29	Not detected	110 $\pm$ 29	99 $\pm$ 17	Not detected	99 $\pm$ 17

in the absence or presence of dexamethasone respectively (Table 2). The appearance of phosphatidylcholine in the medium from dexamethasone-treated cells was decreased by about 49% in the presence of colchicine. However, the effect of colchicine was not significant for the control cells. Colchicine had no significant effects on the total incorporation of [14 C]oleate into triacylglycerols and phosphatidylcholine (Table 2). These results indicate that the appearance of triacylglycerols and at least some of the phosphatidylcholine in the medium depended on a secretory process, since colchicine is known to inhibit lipoprotein secretion (Le Marchand *et al.*, 1973).

Treatment of the medium with phosphotungstate resulted in the precipitation of all of the radioactive triacylglycerol. This reagent precipitates low-density and very-low-density lipoproteins that contain apolipoprotein-B (Burstein *et al.*, 1970), which indicates that the secreted triacylglycerol was associated with this apoprotein. After ultracentrifugation, more than 99% of the radioactive triacylglycerol was recovered at the top of the gradient where very-low-density lipoprotein was expected, and it was absent from the regions where low- and high-density lipoprotein should have been found. The distribution of phosphatidylcholine paralleled that of triacylglycerol.

By contrast, lysophosphatidylcholine was not precipitated by phosphotungstate, and it was recovered at the bottom of the tube after density-gradient centrifugation. Its appearance in the medium was also not significantly altered by the presence of 20 μ M-colchicine (Table 2).

Fatty acid oxidation

There was a substantial uptake of oleate by the hepatocytes, and its concentration in the medium fell dramatically in the first 8 h (Fig. 4). By 24 h less than 10% of the original [14 C]oleate remained in the medium. In four experiments more than 82% of the 14 C recovered could be accounted for in glycerolipids in the hepatocytes, and in the medium (Fig. 1). The rate of β -oxidation measured over the first 30 min of the incubation was less than 10% of the rate of esterification at this time. The oxidation was accounted for entirely by the formation of ketones (measured as water-soluble products), and no significant incorporation into 14 CO₂ was detected (results not shown). The oxidation of [14 C]oleate is therefore relatively less important than esterification in this experimental model, and further work concentrated on

Fig. 4. Effects of the time of incubation on the [14 C]oleate remaining in the incubation medium

The results are taken from the same experiments as shown in Fig. 1. The symbols indicate the [14 C]oleate present in the medium of cells incubated in the absence (Δ) or presence (□) of 1 μ M-dexamethasone. The concentration of [14 C]oleate present at zero time was 0.72 mM. Results are means $\pm$ ranges from two independent experiments, and the 23 h time point was reproduced in two further independent experiments.

the effects of dexamethasone and insulin on the latter process.

Effect of insulin on the synthesis and secretion of glycerolipids in the presence or absence of dexamethasone

The effect of insulin on lipid synthesis and secretion was investigated in three ways. First, insulin was added to the preincubation mixture at the same time as the dexamethasone, and it was then maintained throughout the incubation procedure to determine how these compounds interacted. Secondly, insulin was added with the radioactive precursor after the preincubation to detect how it might modify changes that had already been produced by dexamethasone. Thirdly, it was added in the absence of dexamethasone for either the whole 42 h or only during the final 23 h incubation with radioactive precursors.

Insulin when added throughout the 42 h incubation period decreased the total amount of radioactive triacylglycerol and phosphatidylcholine isolated in both the cells and in the medium. There was also a significant decrease in the total amount of triacylglycerol that was measured chemically (Table 1c). These effects of insulin were observed in the presence and absence of dexamethasone (Table 1c), and the decreases are reflected in the cell-associated lipids (Table 1a). However, insulin did not significantly decrease the accumulation of radioactive precursors in the combined or cell-associated lipids when it was present only for the last 23 h of the incubation (rows V in Tables 1a and 1c).

Insulin when added alone significantly decreased the secretion of triacylglycerols into the medium (Table 1). In all three experiments it decreased the appearance of triacylglycerol and phosphatidylcholine in the medium of the cells that were preincubated with dexamethasone. The appearance of lysophosphatidylcholine in the medium was decreased in those cells that had been incubated with both dexamethasone and insulin. These effects of insulin were observed when it was added in the preincubation period, or only during the time when lipid synthesis was determined (Table 1).

Effects of dexamethasone and insulin on the activity of phosphatidate phosphohydrolase in hepatocytes

The total activity of phosphatidate phosphohydrolase in the hepatocytes was also measured to demonstrate further the effectiveness of insulin and dexamethasone and to examine whether phosphohydrolase activity could be related directly to the rate of glycerolipid metabolism. The basal phosphohydrolase activity measured after 19 h in two independent experiments in the absence of dexamethasone was maintained during the subsequent 23 h incubation with the oleate-albumin complex (Table 3). The basal activity was not significantly altered by the presence of insulin. Incubation of the cells for 19 h with dexamethasone increased the total phosphohydrolase activity by an average of 11-fold in four independent experiments, and this high activity was partly maintained in both experiments when it was measured after the subsequent 23 h incubation with the oleate-albumin complex. The presence of insulin in the medium for 23 or 42 h decreased the effect of dexamethasone in increasing the phosphohydrolase activity. Further experimental evidence which justifies these interactions of insulin and dexamethasone in controlling the phosphohydrolase activity is given in Lawson *et al.* (1982a,b) and Pittner *et al.* (1985a,b).

DISCUSSION

The present results demonstrate that a glucocorticoid, dexamethasone, increases the secretion of triacylglycerol and phosphatidylcholine in particles that have the properties expected of very-low-density lipoproteins (Figs. 1 and 2; Tables 1 and 2). Essentially similar results were obtained when the synthesis and secretion of glycerolipids was measured by using [14 C]oleate or [3 H]glycerol, or when the absolute amount of secreted triacylglycerol was determined (Fig. 1, Table 1). The latter measurement was made to compensate for differences that might have existed in the effective specific

Table 3. Effects of dexamethasone, insulin and the incubation conditions on the activity of phosphatidate phosphohydrolase activity in hepatocytes

Hepatocytes were preincubated for 19 h in the absence or presence of 1 μ M-dexamethasone, and homogenates were prepared (rows I+II). Alternatively, the medium was replaced with fresh medium that contained 1 μ M-dexamethasone or 36 nM-insulin as indicated plus 0.72 mM-oleate and 0.94 mM-glycerol, and the incubation was continued for a further 23 h (rows III-VIII). Phosphatidate phosphohydrolase activities are expressed relative to the rate in row I, in which the activity was 217 ± 56 pmol of diacylglycerol produced/min per unit of lactate dehydrogenase. Results are given as means $\pm$ S.D. for rows I and II, for which there were four independent experiments, or means $\pm$ ranges for rows III-VIII, for which there were two independent experiments.

Additions	Relative activity of phosphatidate phosphohydrolase (%)
(I) None (19 h)	100
(II) + dexamethasone (19 h)	1102 $\pm$ 476
(III) None (42 h)	110 $\pm$ 8
(IV) + dexamethasone (42 h)	540 $\pm$ 90
(V) + insulin (42 h)	91 $\pm$ 14
(VI) + dexamethasone (42 h) + insulin (42 h)	349 $\pm$ 88
(VII) + insulin (23 h)	90 $\pm$ 6
(VIII) + dexamethasone (42 h) + insulin (23 h)	215 $\pm$ 11

radioactivities of the precursors in cells treated with different combinations of hormones. For example, insulin might have stimulated the synthesis of fatty acids from non-radioactive precursors which might have competed with exogenous oleate for incorporation in glycerolipids. This could have caused an underestimation of triacylglycerol synthesis when insulin was added.

The effects of dexamethasone that are described in Figs. 1 and 2 and Tables 1 and 2 were obtained after preincubating the hepatocytes in the presence of newborn-calf serum. Therefore these actions might be permissive and mediated through the effects of other hormones present in the serum. In subsequent experiments the hepatocytes were stabilized on the dishes in the presence of serum and then incubated in medium containing 0.2% fatty-acid-poor bovine serum albumin (Pittner *et al.*, 1985a,b). The hepatocytes were then preincubated with combinations of dexamethasone and insulin in a similar way to that described in Table 1, and the synthesis and secretion of lipids were measured. The secretion of [¹⁴C]oleate in triacylglycerol was increased by 3.4- and 8.8-fold in hepatocytes from male rats and by 2.0- and 2.6-fold in hepatocytes from female rats by preincubation with dexamethasone in the four independent experiments. These increases were also antagonized by insulin, and changes similar to those observed with triacylglycerol were obtained for phosphatidylcholine secretion. The overall rates of glycerolipid synthesis were similar in male hepatocytes that were preincubated with hormones in the presence of serum or albumin. The results from the latter incubations indicate that dexamethasone has a direct effect in stimulating the secretion of very-low-density lipoproteins.

This conclusion is essentially compatible with other work with glucocorticoids *in vitro* with perfused liver (Klausner & Heimberg, 1967; Cole *et al.*, 1982) or *in vivo* (Reaven *et al.*, 1974; Krausz *et al.*, 1981; Taskinen *et al.*, 1983). The increased secretion of triacylglycerol in our experiments was balanced by a decrease in the accumulation of triacylglycerol within the hepatocytes (Table 1). An increase in the concentration of circulating glucocorticoids *in vivo* is often accompanied by the development of a fatty liver (Hill & Droke, 1963; Ožegović *et al.*, 1975), but this effect is probably paralleled by an increased fatty acid supply from adipose tissue. Dexamethasone has also been reported to increase the accumulation of triacylglycerols in isolated hepatocytes (Dich *et al.*, 1983), but in this case the total turnover of triacylglycerols, including the quantity of very-low-density lipoprotein secreted, was not measured.

The results in Table 1 show that insulin decreased the amount of triacylglycerol that was recovered in the secreted lipids of the medium and that it counteracted the effects of dexamethasone in stimulating this secretion. The effects of insulin alone were particularly obvious when the cells were preincubated with insulin for 19 h before addition of the [¹⁴C]oleate and [³H]glycerol. The general conclusion that insulin can decrease the rate of secretion of very-low-density lipoproteins is essentially compatible with those of Nikkilä (1974), Durrington *et al.* (1982), Patsch *et al.* (1983a) and Pullinger & Gibbons (1985). Other workers have concluded that insulin has little direct action on the secretion of very-low-density lipoproteins (Woodside & Heimberg, 1976; Beynen & Geelen, 1982; Haagsman & van Golde, 1984). However, it can antagonize the action of other hormones such as

glucagon, and therefore its action in stimulating the synthesis of fatty acids could provide additional substrate for subsequent secretion. Insulin has been reported to stimulate the secretion of very-low-density lipoproteins by perfused liver (Topping & Mayes, 1972; Laker & Mayes, 1984). Reaven & Mondon (1984) have also shown that the secretion of very-low-density lipoproteins by perfused livers depended on the insulin concentration that existed in animals before the experiments. Furthermore, long-term treatment of rats with insulin produced an increased secretion of very-low-density lipoproteins from the liver *in vivo* (Steiner *et al.*, 1984). However, it is difficult to establish the direct importance of the differences in insulin concentrations that occur *in vivo*, relative to the inevitable changes that take place in the concentrations of other hormones.

In our work the preincubation of hepatocytes for 19 h with insulin before the incubation with radioactive substrates decreased the total amount of triacylglycerol that was recovered within the cells as well as in the medium (Table 1). We do not know the reason for this long-term effect of exposure to insulin. The effect of insulin on the cell triacylglycerol was not seen when insulin was added only during incubation with the radioactive substrates. In other work, insulin added at this stage increased the content of triacylglycerol within the cells (Durrington *et al.*, 1982). Dich *et al.* (1983) reported that insulin increased the intracellular content of triacylglycerol in isolated hepatocytes. It is difficult to relate this to the overall rate of synthesis, since the rate of secretion was not recorded.

The mechanisms by which insulin and dexamethasone altered the rate of secretion of triacylglycerol and phosphatidylcholine by the hepatocytes (Table 1) is not known. We have not yet examined the role of these hormones on the formation of the apoproteins that are needed for the secretion of very-low-density lipoproteins. However, the increased secretion of these lipoproteins that occurs in response to increasing the availability of oleate can occur in the absence of changes in the rate of apoprotein synthesis (Davis & Boogaerts, 1982; Patsch *et al.*, 1983b).

The action of insulin in stimulating the synthesis of glycogen (Whitton & Hems, 1976) and fatty acids (Salmon & Hems, 1973; Diamant & Shafir, 1975; Kirk *et al.*, 1976; Amatruda *et al.*, 1983) can be increased by the permissive action of glucocorticoids and their abilities *in vivo* to produce hyperinsulinism. Conversely, when added alone to hepatocytes glucocorticoids appear to enhance insulin-insensitivity (Amatruda *et al.*, 1983). Our results on the synthesis and secretion of phosphatidylcholine and triacylglycerol indicate an antagonism between insulin and dexamethasone rather than a permissive action (Table 1). An antagonism is also seen between the long-term effects of these hormones on the activity of phosphatidate phosphohydrolase (Table 3; Lawson *et al.*, 1982a,b; Pittner *et al.*, 1985a,b), and on the activities of certain regulatory enzymes involved in amino acid breakdown and gluconeogenesis (see Pittner *et al.*, 1985a).

The changes in the total phosphatidate phosphohydrolase activity that are shown in Table 3 also establish that the hepatocytes were sensitive to insulin and glucocorticoids. However, the dexamethasone-induced increases in the total phosphohydrolase activity were not matched by increases in the rate of triacylglycerol synthesis (Fig. 1,

Table 1). Nevertheless, it should be remembered that the glucocorticoid-induced increases in phosphatidate phosphohydrolase activity reflect an enhanced reserve capacity of the liver to synthesize triacylglycerols. This protects the liver from the potentially toxic effects of high concentrations of fatty acids and acyl-CoA esters that could occur during a severe stress reaction in which insulin concentrations are very low compared with those of glucagon, the catecholamines and glucocorticoids. The expression of this reserve capacity depends on the availability of fatty acids and acyl-CoA esters, which cause the phosphohydrolase to be translocated from the cytosol to the endoplasmic reticulum (Cascales *et al.*, 1984; Brindley, 1984; Martin-Sanz *et al.*, 1984, 1985; Hopewell *et al.*, 1985). The effects of the balance between the availability of insulin, glucagon, cyclic AMP and glucocorticoids to modify the ability of fatty acids to activate the phosphohydrolase through its translocation are discussed elsewhere (Butterwith *et al.*, 1984; Pittner *et al.*, 1985b). The increase in the total phosphohydrolase activity that is produced by glucagon and glucocorticoids can be expressed when the synthesis of glycerolipids is measured at optimum concentrations of substrates (Pittner *et al.*, 1985b). In the present work the initial oleate concentration in all incubations was 0.72 mM, and this fell rapidly (Fig. 4). Consequently, the total capacity of the phosphohydrolase would not have been expressed.

It is clear that the rates of triacylglycerol synthesis and secretion by the liver depend very largely on substrate supply. The sources of the substrates and their routes of metabolism are in turn controlled by hormonal status. In the present experiments the concentration of oleate was not maintained throughout the incubation period (Fig. 4). However, the main purpose of this work was to determine the subsequent effects of hormones on the rates of secretion of triacylglycerol, phosphatidylcholine and lysophosphatidylcholine that had initially been synthesized. These rates remained relatively constant for 23 h (Figs. 1–3), and the effects of dexamethasone were also observed at 4 h, where maximum rates of glycerolipid synthesis were maintained (Figs 1 and 2). Furthermore, when the initial concentration of oleate was increased to 1.44 mM, there was about a 2-fold increase in the secretion of [14 C]triacylglycerol at 23 h. However, a stimulation of this secretion by dexamethasone was still observed (results not shown). An alternative approach to see how different fatty acid concentrations might modify the synthesis of glycerolipids, their subsequent secretion and the effects of hormones would be to supplement the medium with oleate continuously.

It is likely that some of the discrepancies in the literature, particularly concerning the observed effects of insulin on glycerolipid metabolism, depend on the experimental system used, and on the availability of substrates and their alternative routes of metabolism. For example, insulin can provide more fatty acid for esterification by stimulating their synthesis *de novo* and by inhibiting β -oxidation. Conversely, a lack of insulin *in vivo* can increase the net availability of fatty acid to the liver because of increased lipolysis in adipose tissue even though β -oxidation in the liver may also be stimulated. In the perfused liver it is possible to increase the secretion of triacylglycerols by increasing fatty acid availability through an inhibition of β -oxidation (Ide & Ontko, 1981). Conversely, an increased rate of β -oxidation is accompanied by a decreased rate of triacylglycerol secretion

(Fukuda & Ontko, 1984). In the hepatocyte system used in the present work, the rate of β -oxidation was low relative to that of esterification.

The origin of the lysophosphatidylcholine that was detected in the medium (Fig. 3) is not fully understood. It could have been derived by the action of hepatic lipases, phospholipases or acyltransferases on the phosphatidylcholine that had been secreted from the hepatocytes. If so, then it might have been expected that the formation of lysophosphatidylcholine would have been stimulated by dexamethasone (Table 1) and decreased by colchicine (Table 2) in parallel with the appearance of phosphatidylcholine in the medium. This was not the case. However, the combination of insulin and dexamethasone did decrease the labelling of lysophosphatidylcholine in the medium (Table 1).

It has been proposed that lysophosphatidylcholine is secreted directly from the liver in rats (Sekas *et al.*, 1985). Those authors used perfused rat livers and found that lysophosphatidylcholine accumulated in the perfusate at about twice the rate of phosphatidylcholine. The lysophosphatidylcholine was relatively unsaturated, and they also concluded that it was not derived from the hydrolysis of phosphatidylcholine in the perfusate. They suggested that this secretion could be largely responsible for the lysophosphatidylcholine that is found in rat plasma bound to albumin (Sekas *et al.*, 1985). This lipid is often the second most prevalent phospholipid of plasma after phosphatidylcholine (Nelson, 1967). It seems likely that the lysophosphatidylcholine isolated in the medium from the hepatocyte cultures (Table 1, Fig. 3) was associated with albumin, and this is consistent with its distribution on the density gradients.

The hepatocyte culture system used in the present studies could be very useful in studying the mechanisms and control of the secretion of lysophosphatidylcholine from the liver. The secreted lysophosphatidylcholine is probably taken up by other tissues, including brain, and used as a source of choline for the synthesis of phosphatidylcholine or acetylcholine (Stein & Stein, 1966; Illingworth & Portman, 1972; Savard & Choy, 1982; Esko & Matsuoka, 1982; Ansell & Spanner, 1982).

The present work demonstrates that the secretion of very-low-density lipoproteins by cultured hepatocytes can be stimulated by a glucocorticoid and inhibited by insulin. It is hoped that this system can be used further to determine the mechanisms that bring about these effects. Such work should improve our knowledge of the control of glycerolipid synthesis and secretion by the liver, especially in stress conditions and in diabetes, where the control of metabolism by glucocorticoids is increased relative to that by insulin. The accumulation of triacylglycerol in the liver increases in these conditions (Murthy & Shipp, 1979; Woods *et al.*, 1981) and the secretion of very-low-density lipoproteins can increase in ketotic diabetes (Nikkilä & Kekki, 1973). Conversely the injection of insulin can partially decrease the accumulation of triacylglycerol in the liver (Murthy & Shipp, 1979; Woods *et al.*, 1981).

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