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Effect of Adrenaline on ^{32}P Incorporation into Rat Fat-Cell Phospholipids

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1. The phospholipid composition of fat-cells prepared from rat epididymal fat-pad was determined. 2. The incorporation of [^{32}P]P_i into the phospholipids of fat-cells incubated in glucose-free medium, and the effect of adrenaline and of α- and β-adrenergic blocking agents, were studied. 3. Incorporation of [^{32}P]P_i into fat-cell phospholipid increased with time; incubation with adrenaline resulted in increased incorporation that was related to the concentration of adrenaline. 4. The pattern of incorporation of [^{32}P]P_i into the individual phospholipids of fat-cells after incubation for 1 h was determined; adrenaline (5.4μM) resulted in increased incorporation into phosphatidylcholine. 5. Incubation of fat-cells with propranolol (34μM) and adrenaline (5.4μM) resulted in abolition of adrenaline-stimulated lipolysis; there was a decrease in the specific radioactivity of phosphatidylcholine and an increase in the specific radioactivity of phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol and cardiolipin compared with cells incubated with adrenaline alone. 6. Incubation of fat-cells with phenoxybenzamine (0.1mM) and adrenaline (5.4μM) resulted in stimulation of lipolysis, and in diminished specific radioactivities of phosphatidylcholine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol and choline plasmalogen compared with cells stimulated with adrenaline alone.

There is substantial evidence that the actions of adrenaline and insulin on the fat-cell are mediated via effects on the cell membrane (Klainer *et al.*, 1962; Rodbell *et al.*, 1968; Jarett *et al.*, 1971; Cuatrecasas, 1969). Indirect evidence that a cell-membrane effect could mediate the action of insulin has come from the observation that the action of phospholipases C and A on fat-cells leads to a number of changes in metabolism similar to those produced by insulin (Rodbell, 1966; Rodbell & Jones, 1966; Blecher, 1967). It has been postulated that a change in the disposition of the phospholipids of the fat-cell membrane may be involved in the mechanism of insulin action (Rodbell *et al.*, 1968). Very little information is available about the effects of hormones on phospholipid synthesis in isolated fat-cells.

A variety of hormones increase incorporation of [^{32}P]P_i into the phospholipids of their respective target tissues. Growth hormone injected into rats increased the incorporation of [^{32}P]P_i and [^{32}P]phosphorylcholine incubated with rat liver slices (Leal & Greenbaum, 1961). Insulin increased incorporation of [^{32}P]P_i into phospholipids of rat adipose tissue (de Torrontegui & Berthet, 1966*b*) and into phospholipids of rat diaphragm (Manchester, 1963), and adrenaline increased incorporation of [^{32}P]P_i into liver slices (de Torrontegui & Berthet, 1966*a*). Thyrotrophin increased incorporation of [^{32}P]P_i into the phospholipids of dog thyroid slices (Scott *et al.*, 1966); investigations of the effect of thyrotrophin on stimulation of [^{32}P]P_i incorporation into phospholipids of pig thyroid slices (Kerkof & Tata, 1969) and of sheep thyroid slices (Burke, 1969) have led to the conclusion that the effects of thyrotrophin on phospholipogenesis may not be mediated by the adenylate cyclase system.

Little or no work has been done to determine whether hormones can affect phospholipid synthesis in the subcellular fraction of tissue. Effects of adrenaline can be obtained on the plasma-membrane fraction of a fat-cell homogenate (Jarett *et al.*, 1971), but attempts to obtain effects of insulin directly on such a fraction have been unsuccessful. The object of the present studies was therefore twofold: first, to characterize precisely the effect of hormones on phospholipid synthesis in the subcellular fractions of fat-cells, and, secondly, to determine whether any of these effects could be obtained directly in subcellular fractions. If the latter could be achieved, it would provide a very useful system in which to analyse the mechanism of such hormone action.

This work was begun by an investigation of the effects of hormones on [^{32}P]P_i incorporation into the phospholipids of intact fat-cells. During the course of this work effects of adrenaline were discovered, which could be differentiated by the use of α- and β-adrenergic blocking agents.

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It is generally accepted that adrenaline stimulates lipolysis in adipose tissue via the β-receptors and the adenylate cyclase system (Butcher *et al.*, 1965). α-Adrenergic receptors also appear to be present in isolated rat fat-cells (Turtle & Kipnis, 1967; Perry & Hales, 1970), and Burns & Langley (1970), in studies of lipolytic responses of human fat-cells to various hormones, have concluded that both α- and β-adrenergic receptors are present.

The present paper gives the results of our investigations into the effect of adrenaline and of α- and β-adrenergic blocking agents on incorporation of $[^{32}P]P_i$ into the phospholipids of rat fat-cells.

Materials and Methods

Materials

Adrenaline (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) was made up in 0.1M-ascorbic acid (BDH Chemicals Ltd., Poole, Dorset, U.K.) and was used on the same day. Propranolol hydrochloride [1-isopropylamino-3-(1-naphthyloxy)propan-2-ol hydrochloride; Inderal] was from I.C.I. Ltd., Pharmaceuticals Division, Macclesfield, Cheshire, U.K. Phentolamine mesylate [2-*N*-(3-hydroxyphenyl)-*p*-toluidinomethyl-2-imidazoline mesylate; Rogitine] was from Ciba Laboratories Ltd., Horsham, Sussex, U.K. Phenoxybenzamine hydrochloride [2-(*N*-benzyl-2-chloroethylamino)-1-phenoxypropane hydrochloride; Dibenyline] was from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts., U.K. Glycerol kinase (EC 2.7.1.30), pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), NADH (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt), ATP (disodium salt) and triethanolamine hydrochloride were from Boehringer Corp. (London) Ltd., London W.5, U.K. Silicic acid (Mallinckrodt, 100 mesh) was obtained from Camlab Ltd., Cambridge, U.K., and Hyflo Supercel was from Hopkin and Williams Ltd. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Chloroform, methanol, diethyl ether and all chemicals were A.R. grade, or the purest grade available.

Animals

Male Wistar rats (120–140g), fed on a stock laboratory diet, were used without previous starvation. The rats were killed by a sharp blow on the head, followed by decapitation.

Incubation medium

Krebs–Ringer bicarbonate buffer (1.3mM-Ca^{2+}), pH7.4 (Cohen, 1957), containing 4% (w/v) bovine serum albumin was used in all experiments. The albumin was previously dialysed overnight at 4°C against the Krebs–Ringer bicarbonate buffer. The buffer was gassed with O_2+CO_2 (95:5) before use, and was maintained at 37°C. There was no glucose in the incubation medium for these experiments. For incubations of fat-cells with ^{32}P, the radioisotope was added to the gassed Krebs–Ringer bicarbonate buffer just before use. Portions were then dispensed into the vials or flasks used for the experiment and the fat-cells were added last. Concentrations of 5, 8 or 10μCi/ml of incubation medium were used in the experiments, and these corresponded to 136mCi/mg of P, 218mCi/mg of P and 272mCi/mg of P respectively. Details are given with each experiment of the concentration of ^{32}P in the incubation medium.

Methods

Preparation of fat-cells. Fat-cells were prepared from rat epididymal fat-pads by the method of Rodbell (1964), except that glucose was not present in the buffer at any stage of the procedure. The fat-cells were washed three times with Krebs–Ringer bicarbonate buffer, then resuspended in the appropriate incubation medium, gassed with O_2+CO_2 (95:5) and incubated in a Dubnoff metabolic shaker at 37°C. Incubations were for 60min unless otherwise stated. Glassware with which the fat-cells came into contact during the procedure was previously silicone-treated by immersing in Repelcote (Hopkin and Williams Ltd.), rinsing with water and drying overnight at 70°C.

Incorporation of ^{32}P into total phospholipid. Fat-cells (20–50mg/ml) were incubated in capped polythene specimen tubes (76mm×24mm) (Gallenkamp) with incubation medium containing ^{32}P (10μCi/ml). Total volume in each tube was 2ml. The incubation was stopped by the addition of methanol (5ml) and chloroform (2.5ml) to the contents of each tube, which was then shaken. The samples were transferred to glass-stoppered test tubes, and the extraction was completed by the addition of chloroform (2.5ml) and water (2.5ml) by the method of Bligh & Dyer (1959). The two phases were allowed to separate, and the upper phase (water+methanol) was discarded. The lower phase, containing lipid, was filtered through a pad of glass wool into a round-bottomed flask (50ml) and was evaporated to dryness on a rotary evaporator, below 50°C. The lipid residue was dissolved in chloroform–methanol (2:1, v/v) (6ml), and 0.9% NaCl (1.5ml) was added to separate the phases (Folch *et al.*, 1957). The upper aqueous layer was discarded, and the lower lipid-containing layer was washed six times with Folch upper phase (3ml). Following the modification of McMurray & Dawson (1969), the first three washes were with chloroform–methanol–10mM-KH_2PO_4 (3:48:47, by vol.) and the last three washes were with chloroform–

methanol – 0.9% NaCl (3:48:47, by vol.). Radioactivity in the upper phase was at background values by the fifth wash. Duplicate samples of the washed lipid extracts were transferred to boiling tubes (150mm × 25mm) and were evaporated to dryness on a boiling-water bath. Then 1% ammonium molybdate (0.05ml) and 72% (w/w) $HClO_4$ (1.2ml) were added to each sample, and the contents of the boiling tubes were digested (with caution) on a Kjeldahl rack at low heat until colourless. The large amount of triglyceride in the samples caused considerable charring, and digestion for 20–30min was usually necessary before the samples became clear. The volume of $HClO_4$ in each tube was finally decreased by strong heating to 0.5–0.8ml, and the contents were made up to 8ml with glass-distilled water. The radioactivity of each sample was measured in a Nuclear–Chicago Unilux liquid-scintillation counter by using Čerenkov radiation, with an efficiency of about 30% (Clausen, 1968). Phosphorus was subsequently determined in the same sample (Bartlett, 1959).

Incorporation of ^{32}P into individual phospholipids. Equal volumes of a suspension of fat-cells were dispensed into six 50ml conical flasks containing incubation medium with ^{32}P. Three flasks contained the substance to be tested and three flasks were controls. The total volume in each flask was 20ml, and the fat-cell concentration was about 100mg/ml. Details of the ^{32}P concentration and the fat-cell concentration used are given with each experiment. At the end of the incubation, the contents of each flask were transferred to a polypropylene centrifuge tube and were centrifuged at 300*g* for 1min. A measured volume of the radioactive incubation medium was removed and discarded, and the remaining fat-cells+medium were immediately extracted by the method of Folch *et al.* (1957), which also served to terminate the incubation. The samples were left with chloroform–methanol (2:1, v/v) (20vol.) overnight at 4°C, and the residues were re-extracted the next day with the same solvent. The pooled filtrates were washed to remove water-soluble radioactivity as described in the previous section. The washed lipid extract was evaporated below 50°C in a rotary evaporator and redissolved in chloroform. Preliminary experiments showed that the very large amounts of triglyceride present in the extracts interfered with the subsequent separation of the phospholipids. Triglyceride was therefore removed from the extracts by the method of Borgström (1952). Each sample, containing about 200μg of phospholipid P, was dissolved in chloroform and was added to a column of silicic acid (2g) and Hyflo Supercel (1g), previously dried overnight at 110°C. Neutral lipids were eluted with chloroform (80ml) and discarded. Phospholipids were eluted with methanol (100ml). Each phospholipid sample was analysed by the alkaline-degradation method (Dawson, 1967). The deacylated products were separated on paper chromatograms, and the individual spots were cut out from the paper and digested with 72% (w/w) $HClO_4$, together with appropriate paper blanks. The specific radioactivity of each spot was then determined (Jungalwala & Dawson, 1970). For each chromatogram, the glycerylphosphorylcholine spot was very large, and this enabled triplicate determinations of its specific radioactivity to be carried out. Similarly, duplicate measurements from a single chromatogram were performed on glycerylphosphorylethanolamine, glycerylphosphorylserine and bis(glycerylphosphoryl)glycerol. The specific radioactivity for each phospholipid in each flask was calculated and the results were expressed as the mean of the three flasks. The mean value for phosphatidylcholine was therefore a mean of nine determinations, and those for phosphatidylethanolamine, phosphatidylserine and cardiolipin were means of six determinations. Determination of P in the glycerophosphoric acid spot was unreliable if there was less than 0.8μg of P in the spot, and also in some chromatograms the glycerylphosphorylinositol spot overlapped on to part of the glycerylphosphorylserine spot. In these cases (Tables 2 and 5), values for P were calculated from the measured cardiolipin P on the appropriate chromatogram and the proportionate amount of phosphatidic acid and phosphatidylinositol, as calculated from the values for phospholipid composition (Table 1). A portion of the glycerylphosphorylserine spot that was not overlapped by the glycerylphosphorylinositol spot was then assayed. Results are expressed as mean specific radioactivity of each phospholipid, together with the mean counts recorded for this spot on a single chromatogram after subtraction of background counts. Counts were not adjusted for radioactive decay (half-life of ^{32}P is 14 days), and radioactivity counting of the phospholipids for the alkaline-degradation experiments was done 10–14 days after the start of the experiment.

Assay of glycerol

Fat-cell suspensions were deproteinized with an equal volume of 10% (w/v) trichloroacetic acid. After centrifugation, the supernatants were extracted three times with 5vol. of diethyl ether. Glycerol was assayed in the supernatants by the method of Garland & Randle (1962).

Results

Composition of fat-cell phospholipid

Phosphatidylcholine accounted for about 50% of the total phospholipid P, phosphatidylethanolamine for 10% and the remaining phospholipids each for less than 10% (Table 1). Minor components included

Table 1. *Phospholipid composition of fat-cells*

For experimental details see the text. Phospholipids are expressed as % of total recovered phospholipid P. Results are means±S.E.M. of eight analyses, with ranges given in parentheses.

Phosphatidylcholine	52.1±1.3 (45.3–57.4)
Phosphatidylethanolamine	10.2±0.8 (6.8–12.3)
Phosphatidic acid	2.0±0.4 (0.9–3.7)
Phosphatidylinositol	6.5±0.4 (5.3–8.3)
Phosphatidylserine	5.4±0.7 (2.7–8.8)
Cardiolipin	5.8±0.3 (4.4–7.2)
Phosphatidylglycerol	1.9±0.2 (1.0–2.9)
Choline plasmalogen	0.9±0.2 (0.4–1.6)
Ethanolamine plasmalogen	5.2±0.7 (2.3–8.2)
Sphingomyelin	7.4±0.6 (4.5–10.0)
Glycerol ethers	2.8±0.6 (0.8–5.8)

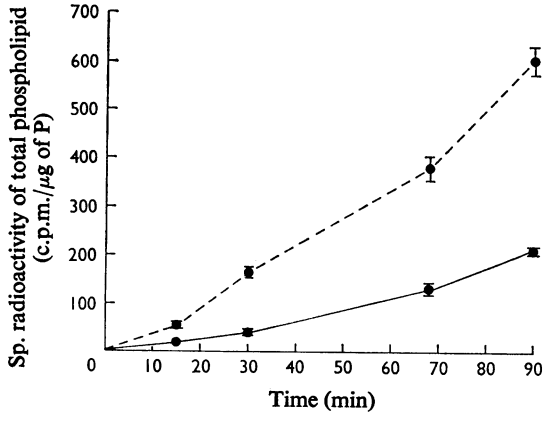

Fig. 1. *Incorporation of ^{32}P into fat-cell phospholipid with time*

Incubation medium (see the Materials and Methods section) contained ^{32}P (10μCi/ml). Concentration of fat-cells was 67mg/ml. ----, Cells incubated with adrenaline (5.4μM); ——, control cells. Results are expressed as means±S.E.M. of four observations.

plasmalogens of ethanolamine (5%) and of choline (<1%), but no inositol plasmalogen was detected. No change in phospholipid composition was detected after incubation of fat-cells for 1h in the presence or absence of adrenaline.

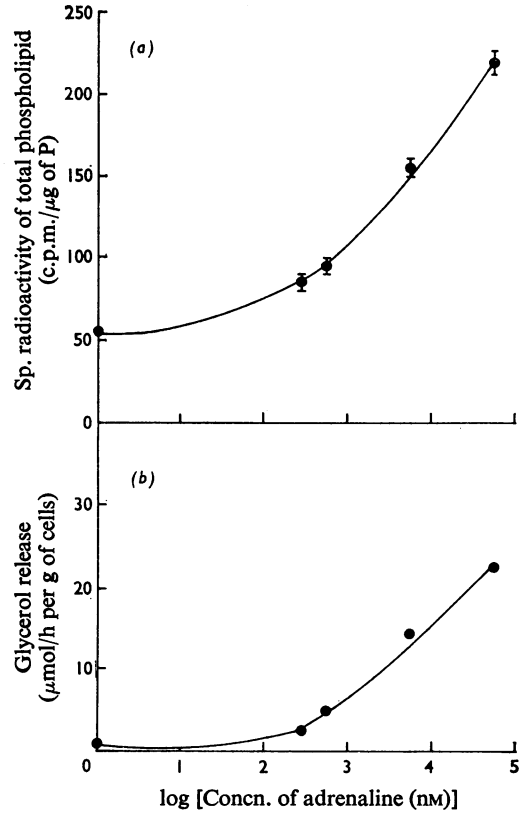

Fig. 2. *Effect of adrenaline concentration on incorporation of ^{32}P into fat-cell phospholipid and on glycerol release*

Fat-cells (21mg/ml) were incubated for 1h with various amounts of adrenaline. For ^{32}P incorporation, the incubation medium (see the Materials and Methods section) contained ^{32}P (10μCi/ml), and points are means of five incubations±S.E.M. Glycerol-release values are means of duplicate incubations. (*a*) Incorporation of ^{32}P into total lipid; (*b*) glycerol release.

Incorporation of ^{32}P into fat-cell phospholipid

Fat-cells were incubated for 90min in glucose-free buffer in the presence and absence of adrenaline (5.4μM). The incorporation of ^{32}P into total phospholipid, which was essentially linear in both cases over the first hour, was stimulated by adrenaline (Fig. 1). The response of fat-cells to various amounts of adrenaline is shown in Fig. 2. Incorporation of ^{32}P into total lipid and glycerol release were measured

Table 2. *Effect of adrenaline on ^{32}P incorporation into fat-cell phospholipids*

Three flasks were incubated with adrenaline (5.4μM) and were compared with three control flasks incubated without hormone. Incubation medium (see the Materials and Methods section) contained ^{32}P (8μCi/ml). Concentration of fat-cells was 102mg/ml; incubation time was 1h. Results, each a mean of three measurements, are expressed as specific radioactivities (c.p.m./μg of P), with total radioactivities incorporated into each spot (c.p.m.) in parentheses.

	Specific and total radioactivity			
	Control		+Adrenaline	
Phosphatidylcholine	121	(2727)	339	(7692)
Phosphatidylethanolamine	7	(23)	5	(10)
Phosphatidic acid*	309	(455)	262	(380)
Phosphatidylinositol*	110	(387)	107	(373)
Phosphatidylserine	22	(25)	12	(20)
Cardiolipin	12	(55)	5	(18)
Phosphatidylglycerol	26	(32)†	19	(11)†
Choline plasmalogen	54	(58)	33	(38)
Ethanolamine plasmalogen	8	(12)	5	(11)
Sphingomyelin	10	(52)	3	(6)
Glycerol ethers	9	(41)	5	(27)
Total phospholipid	55	—	121	—
	Glycerol release (μmol/h per g of cells)			
Basal	0.22			
+Adrenaline	16.0			

* Specific radioactivities from calculated P values (see the Materials and Methods section).

† Mean of two determinations.

in the same experiment. Incorporation of ^{32}P was related to the concentration of adrenaline, and the response was similar to that for glycerol release.

The increased incorporation of ^{32}P into fat-cell phospholipid caused by adrenaline was investigated in greater detail by separating the phospholipids by the alkaline-degradation method and determining the specific radioactivities of the isolated glycerylphosphoryl derivatives. Six samples of washed fat-cells were incubated for 1h. Three samples were incubated with adrenaline (5.4μM) and three control samples were incubated without hormone. The phospholipids of each of these six samples were isolated and their specific radioactivities determined. Mean values for adrenaline-stimulated and control cells are listed in Table 2.

Phosphatidic acid showed the highest specific radioactivity, with phosphatidylinositol and phosphatidylcholine next. Choline plasmalogen showed considerable incorporation of ^{32}P, and cardiolipin, phosphatidylethanolamine, phosphatidylserine, ethanolamine plasmalogen, sphingomyelin and glycerol ethers all showed low specific radioactivities.

Incorporation of ^{32}P into phosphatidylcholine was approximately doubled as a result of adrenaline stimulation, but incorporation into all other phospholipids showed no difference from the control values. The small amounts of P in the choline plasmalogen fraction made accurate determinations of its specific radioactivity difficult. However, in three separate experiments, no increase was found in the incorporation of ^{32}P into choline plasmalogen as a result of adrenaline stimulation.

Effect of α- and β-adrenergic blocking agents on uptake of ^{32}P into total lipid

The effect of incubation of fat-cells with adrenaline and α- and β-adrenergic blocking agents was investigated. Incorporation of ^{32}P into total lipid and also glycerol release were measured in the same preparation (Table 3). Expts. 1 and 2 show that propranolol (34μM) did not impair the adrenaline stimulation of uptake of ^{32}P into total phospholipid. In the same experiment, propranolol abolished adrenaline-stimulated glycerol release.

Phentolamine (36μM) together with adrenaline produced no significant diminution in the adrenaline stimulation of ^{32}P uptake (Table 3, Expts. 1 and 2). Phentolamine alone, when incubated with fat-cells,

Table 3. *Effect of adrenaline and of α- and β-adrenergic blocking agents on ^{32}P incorporation into fat-cell phospholipid and on glycerol release*

Fat-cells were incubated with adrenaline (5.4 μM), propranolol (34 μM), phentolamine (36 μM) and phenoxybenzamine (33 μM in Expt. 3 and 0.1 mM in Expt. 4). In Expts. 3 and 4, phenoxybenzamine was preincubated with the fat-cells for 20 min before addition of the adrenaline. Incubation medium for incorporation experiments contained 10 μCi of ^{32}P/ml, and results from these experiments are expressed as means±S.E.M. with nos. of incubations in parentheses. Significance (*P*) of the difference from basal values was estimated by Student's *t* test: **P* <0.001; ***P* <0.05. Glycerol-release values are means of two incubations.

Expt. no.	Concn. of fat-cells (mg/ml)		Basal	Adrenaline	Adrenaline+ phentolamine	Phentolamine	Adrenaline+ propranolol	Propranolol
1	52	Incorporation (c.p.m./μg of P)	90±9 (6)	164±9 (6)*	153±10 (6)*	159±13 (4)**	197±16 (6)*	86±4 (4)
		Glycerol release (μmol/h per g of cells)	0.4	22.4	25.2	—	0.7	—
2	20	Incorporation (c.p.m./μg of P)	96±5 (6)	—	186±12 (6)*	143±15 (6)**	254±23 (5)*	130±16 (5)
		Glycerol release (μmol/h per g of cells)	2.6	—	36.9	2.4	2.4	2.6
					Adrenaline+ phenoxy-benzamine	Phenoxy-benzamine		
3	36	Incorporation (c.p.m./μg of P)	64±6 (5)	184±18 (6)*	168±8 (6)	—		
		Glycerol release (μmol/h per g of cells)	0.9	23.0	29.3	0.8		
4	21	Incorporation (c.p.m./μg of P)	137±11 (6)	331±37 (6)*	114±14 (6)	101±8 (6)		
		Glycerol release (μmol/h per g of cells)	0.6	39.8	43.5	0.7		

Table 4. *Effect of propranolol incubated with adrenaline and of propranolol alone on ^{32}P incorporation into fat-cell phospholipids*

Expt. 1. Three flasks were incubated with adrenaline (5.4μM) and three were incubated with adrenaline (5.4μM)+propranolol (34μM). Concentration of fat-cells was 118mg/ml. Expt. 2. Three flasks were incubated with propranolol (34μM) and were compared with three control flasks. Concentration of fat-cells was 104mg/ml. Incubation medium (see the Materials and Methods section) contained ^{32}P (10μCi in Expt. 1 and 5μCi in Expt. 2). Incubation time was 1h. Results, each a mean of three determinations, are expressed as specific radioactivities (c.p.m./μg of P) with total radioactivities incorporated (c.p.m) in parentheses. Glycerol-release values are means of two incubations.

Expt. 1.

	Specific and total radioactivity			
	Adrenaline		Adrenaline+ propranolol	
Phosphatidylcholine	185	(8198)	77	(2209)
Phosphatidylethanolamine	9	(65)	39	(198)
Phosphatidic acid	514	(1591)	2407	(4543)
Phosphatidylinositol	391	(936)	1770	(4923)
Phosphatidylserine	44	(68)	62	(51)
Cardiolipin	13	(64)	50	(267)
Phosphatidylglycerol	71*	(50)	97†	(85)
Choline plasmalogen	121	(104)	103	(114)
Ethanolamine plasmalogen	6	(16)	9	(31)
Sphingomyelin	2	(9)	4	(25)
Glycerol ethers	11	(49)	13	(49)
Total phospholipid	156	—	239	—

Glycerol release (μmol/h per g of cells)	
Basal	0.56
Adrenaline	12.6
Adrenaline+propranolol	0.58

Expt. 2.

	Specific and total radioactivity			
	Control		+Propranolol	
Phosphatidylcholine	72	(3357)	47	(2481)
Phosphatidylethanolamine	5	(61)	16	(72)
Phosphatidic acid	447	(689)	444	(1000)
Phosphatidylinositol	183	(830)	229	(1146)
Phosphatidylserine	8	(48)	8	(61)
Cardiolipin	12	(63)	13	(75)
Phosphatidylglycerol	27	(40)	34	(61)
Choline plasmalogen	282	(117)	200	(122)
Ethanolamine plasmalogen	6	(23)	5	(33)
Sphingomyelin	6	(57)	5	(48)
Glycerol ethers	17	(25)	26	(62)
Total phospholipid	49	—	52	—

Glycerol release (μmol/h per g of cells)	
Basal	0.8
Propranolol	0.8
Adrenaline	18.6

* Single determination.
† Two determinations.

Table 5. *Effect of phenoxybenzamine incubated with adrenaline and of phenoxybenzamine alone on ^{32}P incorporation into fat-cell phospholipids*

Expt. 1. Three flasks were incubated for 20min with phenoxybenzamine (0.1mM) and then adrenaline (5.4μM) was added and the incubation continued for 1h. Three control flasks were incubated for 20min and then adrenaline (5.4μM) was added and incubated for 1h. Concentration of fat-cells was 90mg/ml. Expt. 2. Three flasks were incubated with phenoxybenzamine (0.1mM) and were compared with three control flasks. Concentration of fat-cells was 108mg/ml; incubation time was 1h. Incubation medium (see the Materials and Methods section) contained ^{32}P (5μCi/ml). Results, each a mean of three determinations, are expressed as specific radioactivities (c.p.m./μg of P), with total radioactivities incorporated (c.p.m.) in parentheses. Glycerol-release results are means of two incubations.

Expt. 1.

	Specific and total radioactivity			
	Adrenaline		Adrenaline+ phenoxybenzamine	
Phosphatidylcholine	481	(14008)	172	(5101)
Phosphatidylethanolamine	5	(29)	3	(13)
Phosphatidic acid	504	(634)	82	(132)
Phosphatidylinositol	282	(446)	25	(83)
Phosphatidylserine	8	(28)	4	(12)
Cardiolipin	11	(33)	7	(23)
Phosphatidylglycerol	72	(87)	21	(23)
Choline plasmalogen	153	(73)	51	(24)
Ethanolamine plasmalogen	8	(10)	5	(5)
Sphingomyelin	4	(10)	2	(6)
Glycerol ethers	9	(42)	4	(14)
Total phospholipid	225	—	85	—
Glycerol release (μmol/h per g of cells)				
Basal	0.4			
Adrenaline	16.9			
Adrenaline+phenoxybenzamine	18.3			
Phenoxybenzamine	0.5			

Expt. 2.

	Specific and total radioactivity			
	Control		+Phenoxybenzamine	
Phosphatidylcholine	62	(2435)	50	(2102)
Phosphatidylethanolamine	9	(52)	12	(82)
Phosphatidic acid*	407	(585)	214	(282)
Phosphatidylinositol*	197	(656)	143	(447)
Phosphatidylserine	5	(15)	14	(28)
Cardiolipin	13	(56)	11	(45)
Phosphatidylglycerol	15	(18)	46	(50)
Choline plasmalogen	67	(57)	109	(166)
Ethanolamine plasmalogen	8	(18)	7	(15)
Sphingomyelin	2	(12)	2†	(8)
Glycerol ethers	19	(57)	20†	(54)
Total phospholipid	95	—	71	—
Glycerol release (μmol/h per g of cells)				
Basal	0.4			
Phenoxybenzamine	0.6			
Adrenaline (1μg/ml)	18.0			

* Calculated phosphorus values were used to determine specific radioactivities.
† Two determinations.

produced a significant increase in incorporation of ^{32}P into lipid. Since phentolamine has non-specific histamine-like effects (Goodman & Gilman, 1970), phenoxybenzamine, a drug that promotes irreversible blockade of the α-receptors, was investigated.

For these experiments the fat-cells were incubated for 20min with phenoxybenzamine before the addition of adrenaline. Control cells (without phenoxybenzamine) were simultaneously preincubated. When phenoxybenzamine (33μM) was incubated together with adrenaline (5.4μM), there was a decrease in incorporation of ^{32}P into lipid, although this was not statistically significant (Table 3, Expt. 3). However, phenoxybenzamine at a concentration of 0.1mM incubated together with adrenaline (5.4μM) abolished the adrenaline stimulation of ^{32}P uptake. In the same experiment the adrenaline stimulation of lipolysis was not diminished in the presence of phenoxybenzamine (Table 3, Expt. 4).

Effects of propranolol and phenoxybenzamine together with adrenaline on incorporation into individual phospholipids

The effect of propranolol on the adrenaline stimulation of ^{32}P incorporation into phospholipid was investigated by comparing three incubations carried out with fat-cells stimulated with adrenaline alone, with three incubations stimulated with adrenaline together with propranolol. Phospholipids were separated by alkaline degradation and mean specific radioactivities are listed in Table 4, Expt. 1. Glycerol release measured from fat-cells in this experiment confirmed that the preparation was responsive to adrenaline stimulation, and that adrenaline-stimulated lipolysis was abolished by propranolol. Incorporation of ^{32}P into phosphatidylcholine was diminished in the fat-cells incubated with propranolol together with adrenaline, compared with fat-cells incubated with adrenaline alone. In contrast, there was a greatly increased incorporation of ^{32}P into phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol and cardiolipin, compared with cells incubated with adrenaline alone. The net result of these changes in specific radioactivities was that uptake of ^{32}P into total phospholipid was not diminished in the presence of propranolol, and this explained the observation (Table 3, Expts. 1 and 2) that the adrenaline stimulation of ^{32}P uptake into total lipid was not diminished by propranolol.

The effect of propranolol incubated alone was investigated. Three incubations of fat-cells with propranolol were compared with three incubations under basal conditions. Mean specific radioactivities of the phospholipids are shown in Table 4, Expt. 2. There was a small decrease (35%) in the specific radioactivity of phosphatidylcholine in the cells incubated with propranolol compared with the control cells, but otherwise no differences were observed between the phospholipids under these conditions. The glycerol-release results show that propranolol caused no increase in lipolysis. The stimulation of glycerol release caused by adrenaline (5.4μM) confirmed the responsiveness of the cell preparation.

The effect of incubation of phenoxybenzamine together with adrenaline on the pattern of incorporation of ^{32}P into phospholipids was investigated. Three batches of cells were stimulated with adrenaline and were compared with three batches incubated with adrenaline and phenoxybenzamine (Table 5, Expt. 1). Adrenaline-stimulated glycerol release was measured and was not impaired in the presence of phenoxybenzamine. The fat-cells incubated with phenoxybenzamine together with adrenaline showed lower specific radioactivities of phosphatidylcholine (36%), phosphatidic acid (16%), phosphatidylinositol (9%), phosphatidylglycerol (29%) and choline plasmalogen (33%) compared with cells incubated with adrenaline alone.

The effect of phenoxybenzamine incubated with fat-cells under basal conditions is shown in Table 5, Expt. 2. Three flasks were incubated with phenoxybenzamine (0.1mM) and were compared with three control flasks. Table 5, Expt. 2 shows the specific radioactivities of the phospholipids. The fat-cells incubated with phenoxybenzamine showed lower specific radioactivities of phosphatidylcholine (81%), phosphatidic acid (53%) and phosphatidylinositol (73%) compared with control cells.

Discussion

The composition of the phospholipids of rat fat-cells was similar to that of rat liver (Rouser *et al.*, 1968) and of dog thyroid slices, calculated from the results of Scott *et al.* (1966). Phosphatidylcholine was the major component of the fat-cell phospholipids, contributing about 50% to the total phospholipid P, although this value must also include lysophosphatidylcholine, which is estimated together with phosphatidylcholine by the alkaline-degradation method.

There is little information about pathways of phospholipid biosynthesis in adipose tissue. Incubation of ^{32}P with rat fat-cells for 1h resulted in a reproducible pattern of incorporation into the phospholipids. Phosphatidic acid showed the highest specific radioactivity, with phosphatidylinositol next. These results are compatible with a biosynthetic pathway in which phosphatidic acid is in a central position, and phosphatidylinositol is synthesized from it, a pathway similar to that found in liver (Kennedy, 1961). There was moderate incorporation into phosphatidylcholine, but because of the relatively large proportion of phosphatidylcholine present, this accounted for about 70% of the total radioactivity incorporated into phospholipid. There was very little incorporation

of ^{32}P into phosphatidylethanolamine in fat-cells. This is in contrast to the incorporation in the liver (McMurray & Dawson, 1969), in which microsomal phosphatidylethanolamine was found to have a higher specific radioactivity than phosphatidylcholine. Insulin stimulation of fat-cells produced a moderate increase in incorporation of ^{32}P into phosphatidylethanolamine (J. M. Stein & C. N. Hales, unpublished work) showing that fat-cells were capable, under these conditions, of incorporating ^{32}P into phosphatidylethanolamine. The pattern of incorporation of ^{32}P into the other phospholipids of fat-cells was compatible with synthesis via patterns similar to those described in liver (Kennedy, 1961).

Stimulation of fat-cells with adrenaline caused increased incorporation of ^{32}P into phospholipid that was both rapid and also related to the concentration of adrenaline used. Comparison of the dose–response curve of this effect with that of lipolysis shows these to be closely parallel and therefore strongly suggests that the ^{32}P effect is equally physiologically significant. Phosphatidylcholine in adrenaline-stimulated cells showed increased specific radioactivity, but there was no increase in specific radioactivity of choline plasmalogen or of sphingomyelin. This was unexpected, as in liver preparations phosphatidylcholine, choline plasmalogen and sphingomyelin are synthesized from a common precursor, cytidine diphosphate choline (Kiyasu & Kennedy, 1960; Sribney & Kennedy, 1958). If the biosynthesis of phosphatidylcholine and of choline plasmalogen in fat-cells resembles that in liver, then the adrenaline-sensitive step in fat-cells may occur after cytidine diphosphate choline, and may be located in choline phosphotransferase (EC 2.7.8.2).

The investigations of the adrenergic receptors responsible for the effects on incorporation of ^{32}P produced some unexpected findings. In experiments with fat-cells with α-adrenergic blocking agents (Tables 3 and 4; Perry & Hales, 1970) higher lipolysis rates have invariably been obtained with adrenaline and α-blocking agent than with adrenaline alone. As α-adrenergic blocking agents possess no intrinsic lipolytic activity, this suggested that α-receptors may be present in fat-cells. β-Adrenergic blocking agents have been shown to abolish the increase in cyclic AMP (adenosine 3′:5′-cyclic monophosphate) concentrations promoted by adrenaline (Butcher *et al.*, 1965; Butcher & Sutherland, 1967) and to inhibit the lipolytic response. Fat-cells incubated with adrenaline together with the β-adrenergic blocking agent, propranolol, showed inhibition of lipolysis and greatly changed patterns of ^{32}P incorporation compared with fat-cells incubated with adrenaline alone. There was a 400% increase of incorporation into phosphatidic acid, phosphatidylinositol, phosphatidylethanolamine and cardiolipin, and a decrease in the specific radioactivity of phosphatidylcholine. The intracellular localization of these changes is not known. In rat liver mitochondria, cardiolipin is located almost exclusively in the inner membrane and phosphatidylinositol in the outer membrane (McMurray & Dawson, 1969). Some phosphatidic acid synthesis has been shown to take place in the outer mitochondrial membrane (Shephard & Hübscher, 1969) as well as in the endoplasmic reticulum. The results of α-receptor stimulation therefore may be associated with changes in phospholipid synthesis in the mitochondria. It seems likely that the β-blocked isolated rat fat-cell preparation will prove useful for the investigation of the mechanism of the α-adrenergic effect.

Adrenaline stimulation in the presence of the α-adrenergic blocking agent phenoxybenzamine showed that the fat-cells were capable of a normal lipolytic response to adrenaline, but the adrenaline stimulation of ^{32}P incorporation into phospholipids was drastically decreased. Phenoxybenzamine is believed to exert its effect by irreversible alkylation of the α-adrenergic receptors (Belleau & Triggle, 1962), and this may explain the greatly decreased specific radioactivities that were observed under these conditions.

ATP concentrations under conditions of adrenaline stimulation of fat-cells with phenoxybenzamine were not determined. Glucose was not present in the medium, and results from experiments with fat-cells from the rat (Hepp *et al.*, 1968) and the mouse (Bihler & Jeanrenaud, 1970) have shown that in adrenaline-stimulated fat-cells ATP concentrations decrease. This change is thought to be related to the accumulation of free fatty acids within the cell when very high rates of lipolysis are produced. There is no report of ATP concentrations under conditions of adrenergic blockade.

To interpret the effect of adrenaline alone or of adrenaline in the presence of an α-adrenergic blocking agent further work is required to investigate the role of changes in ATP concentrations and specific activity. Only when conditions are achieved in which the β-adrenergic effect can be obtained in the absence of a decrease in ATP concentration will it be possible to speculate on the role of the β-receptor in regulatory phospholipid synthesis.

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