The Interaction of Insulin with Phospholipids

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(Received 30 April 1971)

1. A simple two-phase chloroform–aqueous buffer system was used to investigate the interaction of insulin with phospholipids and other amphipathic substances. 2. The distribution of ^{125}I -labelled insulin in this system was determined after incubation at 37°C . Phosphatidic acid, dicetylphosphoric acid and, to a lesser extent, phosphatidylcholine and cetyltrimethylammonium bromide solubilized ^{125}I -labelled insulin in the chloroform phase, indicating the formation of chloroform-soluble insulin–phospholipid or insulin–amphipath complexes. Phosphatidylethanolamine, sphingomyelin, cholesterol, stearylamine and Triton X-100 were without effect. 3. Formation of insulin–phospholipid complex was confirmed by paper chromatography. 4. The two-phase system was adapted to act as a simple functional system with which to investigate possible effects of insulin on the structural and functional properties of phospholipid micelles in chloroform, by using the distribution of $[^{14}\text{C}]$ glucose between the two phases as a monitor of phospholipid–insulin interactions. The ability of phospholipids to solubilize $[^{14}\text{C}]$ glucose in chloroform increased in the order phosphatidylcholine < sphingomyelin < phosphatidylethanolamine < phosphatidic acid. Insulin decreased the $[^{14}\text{C}]$ glucose solubilized by phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid, but not by sphingomyelin. 5. The significance of these results and the molecular requirements for the formation of insulin–phospholipid complexes in chloroform are discussed.

Phospholipid–protein interactions are currently of considerable interest as a molecular basis for the structure of biological membranes. It is probable also that the control of the transport and other functions of biological membranes depends to a large extent on these interactions. Enzymes that hydrolyse the structural elements of cell membranes and hence probably modify the phospholipid–protein interactions in the membrane, phospholipases A (Blecher, 1965) and C (Rodbell, 1966) and various proteases (Rieser & Rieser, 1964; Kuo, Holmlund & Dill, 1966; Kuo, 1968), have been shown to modify several membrane functions, including glucose transport. From studies on the effects of insulin and trypsin on the kinetics of entry of 3-*O*-methyl glucose into muscle cells, Weis & Narahara (1969) have postulated that insulin and trypsin may alter the structure of the plasma membrane in a manner that permits carrier molecules to move more rapidly within the membrane. Cuatrecasas (1969) has attached insulin to large particles (60–300 μm) of agarose polymers

that are larger than most fat-cells. This bound insulin increased the utilization of glucose and suppressed the hormone-stimulated lipolysis of isolated fat-cells. Since the bound insulin did not appear to enter the fat-cells, these findings were considered to support the view that the interaction of insulin with the cell surface may initiate a variety of changes in metabolism. Comparable observations have been reported by Turkington (1970), who found a threefold stimulation of nuclear RNA synthesis by insulin bound to Sepharose in preparations of mammary epithelial cells.

It has been suggested that the biochemical and physiological effects of protein and polypeptide hormones, particularly those concerned with the control of membrane permeability, such as insulin, may depend on transitions from a bimolecular leaflet to a micellar organization for membrane lipids, and on the properties of micellar areas of membrane (Lucy, 1964). From studies on the effects of phospholipase C and of insulin on the metabolism of isolated fat-cells, Rodbell (1966) has suggested that insulin may favour the micellar configuration for the lipids of membranes. It has also been postulated that

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insulin binds to a protein in the insulin receptor site by a thiol-disulphide interchange reaction, but the evidence for this is not strong (Rieser, 1967).

The possibility that insulin might interact directly with phospholipids in the cell membrane appears to have received little attention. We have therefore investigated the interaction *in vitro* of insulin with phospholipids in the absence of other proteins, and we report here the interaction of insulin with certain phospholipids in a relatively simple system. Under appropriate conditions, insulin is found to interact with some but not all of the membrane lipids studied. Although these interactions are not specific for insulin, they nevertheless result in an altered distribution of glucose in a two-phase solvent system: the possible relevance of these findings to the actions of insulin *in vivo* are discussed. A preliminary report of this work has been presented (Perry, Tampion & Lucy, 1970).

MATERIALS AND METHODS

Phospholipids. Phosphatidylcholine was prepared from fresh hen's egg by the method of Papahadjopoulos & Miller (1967). Phosphatidic acid was prepared from this phosphatidylcholine by digestion with phospholipase D from cabbage (Papahadjopoulos & Miller, 1967). Replicate small samples of phosphatidylcholine and phosphatidic acid in chloroform, containing known quantities of P, were sealed individually in ampoules under nitrogen. Phosphatidylethanolamine and sphingomyelin were from Lipid Products Ltd., Epsom, Surrey, U.K. Cholesterol was the British Drug Houses Ltd. (Poole, Dorset, U.K.) standard for chromatography. Cetyltrimethylammonium bromide was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Dicaprylate and stearylamine were from K. and K. Laboratories, Liverpool, U.K. Triton X-100 was from British Drug Houses Ltd.

The purity of all phospholipids was checked before use by t.l.c. on silica gel H (E. Merck A.-G., Darmstadt, Germany) in chloroform-methanol-aq. 7M-NH₃ (46:18:3, by vol.). Phospholipids were detected by the Molybdenum Blue spray reagent as modified by Vaskovsky & Kostetsky (1968). Other lipids were detected by spraying with 10% (v/v) H₂SO₄ or 20% (w/v) ammonium hydrogen sulphate and heating at 110°C for 30 min. The phospholipid preparations used were greater than 98% pure. Cholesterol purity was checked before use by t.l.c. (Smith, Matthews, Price, Bachmann & Reynolds, 1967).

Proteins. Crystalline zinc insulin from bovine pancreas (24.1 i.u./mg) from Sigma (London) Chemical Co. Ltd. was dissolved in 3 mM-HCl before use. Bovine serum albumin (fraction V; Sigma), cytochrome c from horse heart (a gift from Dr T. Hallinan, Department of Biochemistry, Royal Free Hospital School of Medicine) and ribonuclease from bovine pancreas (Koch-Light Ltd., Colnbrook, Bucks., U.K.) were dissolved in water.

Radiochemicals. D-[U-¹⁴C]Glucose (2.6–3.1 mCi/mmol) and L-[U-¹⁴C]serine (14.5 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

¹²⁵I-labelled insulin and insulin analogues labelled with ¹²⁵I were a gift from Dr K. L. Manchester, University College London. The three iodinated insulin analogues were as follows: 1. ¹²⁵I-labelled desasparaginodesalanino-insulin (specific radioactivities 0.664 and 1.8 mCi/mg), i.e. insulin from which the C-terminal amino acids of the A and B chains have been removed; 2. ¹²⁵I-labelled desglycino-desphenylalanino-insulin (specific radioactivities 0.46 and 2.0 mCi/mg), i.e. insulin from which the N-terminal amino acids of the A and B chains have been removed; 3. ¹²⁵I-labelled desoctapeptide-insulin (specific radioactivity 2.7 mCi/mg), i.e. insulin from which the terminal eight amino acid residues have been removed from the B chain.

¹²⁵I-labelled insulin (specific radioactivities 0.7 and 2.5 mCi/mg) and analogues were stored in water containing human serum albumin (1%, w/v) and benzyl alcohol (1%, v/v).

Other chemicals. Chloroform, either AnalaR or spectroscopic grade, was from British Drug Houses Ltd. The 2% (v/v) ethanol added as a preservative was removed by shaking the chloroform six times with an equal volume of water. The chloroform was used without subsequent drying. Ethanol-free chloroform was prepared fresh before each experiment.

Sephadex G-50 (coarse) was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. Tris (Trizma base), 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)-benzene were from Sigma (London) Chemical Co.

Other chemicals and solvents were AnalaR grade or the purest grade available and were used without further purification.

All glassware was silicone-treated (Silicone Repelcote; Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) before each experiment to minimize binding of phospholipids to glass (Green, 1969).

Methods. The two-phase experimental system used was adapted from that of Jung, Chaney & LeFevre (1968). A portion (1 ml) of an aqueous tris-buffered salts medium (125 mM-NaCl–5 mM-KCl–35 mM-tris, adjusted to pH 7.4 with conc. HCl), containing either 400 ng of ¹²⁵I-labelled insulin or 0.1–0.3 μmol (0.3–1.0 μCi) of [¹⁴C]-glucose, was layered over 1 ml of ethanol-free chloroform in a silicone-treated screw-top glass specimen tube (58 mm × 17 mm) (Griffin & George Ltd., Wembley, Middx., U.K.). After incubation at 37°C for 72 h, the phases were separated and the radioactivity in each phase was determined. Chloroform was removed before radioactivity counting by evaporation under a stream of nitrogen. ¹²⁵I was determined with a Wallach GTL Gamma Counter. ¹⁴C was determined by liquid-scintillation counting in a Nuclear-Chicago L725 liquid-scintillation spectrometer. The scintillation medium contained 2,4-dioxan (1 litre), 2-methoxyethanol (200 ml), naphthalene (80 g), 2,5-diphenyloxazole (3 g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.2 g). Quenching was corrected by the channels-ratio technique. Counting efficiency was 70–80%.

Incubations were carried out under strictly static conditions. Shaking caused severe emulsification and was not employed to assist equilibration.

Chromatography of ¹²⁵I-labelled insulin. Paper chromatography of ¹²⁵I-labelled insulin was carried out by the method of Fenton (1959), with butan-2-ol saturated

with 1% (v/v) acetic acid. The distribution of ^{125}I -labelled insulin was determined on strips ($3\text{cm} \times 1\text{cm}$) of the chromatograms in the Wallach GTL Gamma Counter. Marker insulin spots were detected by Bromocresol Green stain (Fenton, 1959). Phospholipids were detected by dipping the chromatogram in a 0.2% (w/v) solution of iodine in diethyl ether.

Chromatography of ^{125}I -labelled insulin ($1\mu\text{g}$) showed a major peak containing $88.6 \pm 3.0\%$ (mean $\pm$ s.e.m. of four determinations) of the ^{125}I , with a smaller faster-running shoulder. A minor radioactive peak ($6.2 \pm 1.8\%$ of the ^{125}I ; mean $\pm$ s.e.m. of four determinations) remained at the origin. Recoveries of ^{125}I were $98.0 \pm 1.7\%$ (mean $\pm$ s.e.m. of four determinations). Less than 1% of the ^{125}I radioactivity was not precipitated by 20% (w/v) trichloroacetic acid.

RESULTS

Solubilization of ^{125}I -labelled insulin in chloroform by phospholipids. Incubation of ^{125}I -labelled insulin in the aqueous phase of the chloroform-tris buffer system for up to 200h resulted in very little radioactivity entering the chloroform (Table 1; Fig. 1). Phosphatidylcholine ($1\mu\text{mol}$) in the chloroform increased the amount of radioactivity solubilized (Fig. 1). The amount of ^{125}I -labelled insulin solubilized reached a maximum after about 100h and was constant thereafter up to 200h. The use of the term 'solubilization' in this context implies the appearance of ^{125}I -labelled insulin in the chloroform in a form not sedimented by centrifuging at $5000g$ for 15min. In most other experiments the incubation period was 72h.

Various phospholipids and other amphipathic substances were tested for their ability to solubilize

^{125}I -labelled insulin in this system (Table 1). The most effective were phosphatidic acid and dicetyl phosphate. In some experiments these compounds solubilized more than half the insulin added (see dicetyl phosphate in Table 1). Phosphatidylcholine and cetyltrimethylammonium bromide supported solubilization of insulin, but to a markedly lower extent. Some variation in the effectiveness of different preparations of phosphatidylcholine and phosphatidic acid was noted, but the results with a single preparation were very consistent. The reason for this variation is not clear. The other amphipaths in Table 1 failed to effect solubilization of insulin.

Recoveries of ^{125}I -labelled insulin added to this system were $90.8 \pm 4.6\%$ (mean $\pm$ s.e.m. of 30 determinations) in the absence of phospholipid, $94.2 \pm 2.0\%$ (mean $\pm$ s.e.m. of 20 experiments) in the presence of phosphatidylcholine and $98.1 \pm 0.8\%$ (mean $\pm$ s.e.m. of 12 experiments) in the presence of phosphatidic acid. Recoveries of less than 100% may be the result of adsorption of insulin to glass, although this was considerably decreased by silicone treatment, or, since insulin is surface-active (Arnold & Pak, 1968), of accumulation of the ^{125}I -labelled insulin at the chloroform-water interface, which was not sampled. These recoveries do not affect the results obtained, as the critical test is the appearance of ^{125}I -labelled insulin in the bulk chloroform phase.

The amounts of ^{125}I -labelled insulin solubilized in these experiments were too small to be measured accurately with conventional protein assays. However, solubilization of the intact protein was demonstrated in a separate experiment. A sample of insulin (0.4mg) was added to 10ml of chloroform

Fig. 1. Time-course of solubilization of ^{125}I -labelled insulin by phosphatidylcholine in chloroform. Chloroform (1ml) alone (▲) or containing $1\mu\text{mol}$ of phosphatidylcholine (●) was incubated at 37°C with 1ml of tris-buffered medium containing 400ng of ^{125}I -labelled insulin. Each point is the mean of two determinations of the amount (ng) of ^{125}I -labelled insulin solubilized in chloroform.

Table 1. Solubilization of ^{125}I -labelled insulin in chloroform by phospholipids and other amphipaths

Chloroform (1ml) alone or containing $1\mu\text{mol}$ of the amphipath was incubated at 37°C for 72h with 1ml of tris-buffered medium containing 400ng of ^{125}I -labelled insulin. Results are expressed as ng of ^{125}I -labelled insulin solubilized in chloroform and are means $\pm$ s.e.m. of the numbers of determinations shown in parentheses.

Amphipath	^{125}I -labelled insulin solubilized (ng)
None	1.2 ± 0.1 (10)
Phosphatidic acid	187.6 ± 5.6 (4)
Dicetyl phosphate	228.1 ± 5.5 (4)
Phosphatidylcholine	10.0 ± 0.9 (10)
Cetyltrimethylammonium bromide	19.4 ± 0.2 (4)
Phosphatidylethanolamine	1.9 ± 0.1 (6)
Sphingomyelin	2.2 ± 0.1 (6)
Cholesterol	1.6 ± 0.2 (6)
Stearylamine	2.1 ± 0.1 (4)
Triton X-100	1.8 ± 0.1 (4)

containing 10 μ mol of phosphatidic acid or dicetyl phosphate, and the solvent removed by rotary evaporation. The dry phospholipid-insulin film was resuspended in 10 ml of chloroform and centrifuged at 5000g for 15 min. Portions (0.5 ml) of the chloroform extract were evaporated to dryness, resuspended in 1.5% (w/v) sodium deoxycholate and the insulin solubilized was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), with insulin as standard: 1 μ mol of dicetyl phosphate solubilized 29 μ g of protein, indicating that most of the intact insulin was in all probability being solubilized. Similar results were obtained with phosphatidic acid.

Paper chromatography of 125 I-labelled insulin in the presence of phospholipids. Further evidence for the formation of a complex between insulin and phosphatidylcholine or phosphatidic acid was obtained from a study of the chromatographic behaviour of 125 I-labelled insulin in the presence and absence of these phospholipids.

A sample (20 μ g) of 125 I-labelled insulin was added to 5 ml of chloroform containing 5 μ mol of phospholipid and the solvent removed by rotary evaporation. Chloroform (5 ml) was added to the dry phospholipid-insulin film and shaken briefly by hand. After centrifugation at 5000g for 15 min, the radioactivity remaining in solution was taken as a measure of the formation of a chloroform-soluble phospholipid-insulin complex. In this way 5 μ mol of phosphatidylcholine solubilized 4 μ g, and 5 μ mol of phosphatidic acid 18 μ g, of the added 20 μ g of 125 I-labelled insulin. Under these conditions, 125 I-labelled insulin was virtually insoluble in the absence of phospholipid. Similar experiments have shown that insulin-phosphatidic acid complexes form in hexane, trimethylpentane and, to a lesser extent, in ethanol and 2,4-dioxan.

The phospholipid-insulin complexes were concentrated to 0.5 ml by rotary evaporation and samples (25 μ l) were chromatographed on Whatman no. 1 paper (Fenton, 1959). The major radioactivity peak on chromatography of 125 I-labelled insulin alone had R_F 0.31, and the phospholipids, phosphatidylcholine and phosphatidic acid, had R_F values in the range 0.90–0.95. Chromatography of the insulin-phosphatidic acid and insulin-phosphatidylcholine complexes showed no radioactive peak of R_F 0.31. The major radioactive peak had R_F 0.90–0.95, characteristic of the phospholipids. These results suggest that the 125 I-labelled insulin-phospholipid complexes formed in chloroform are not dissociated in the chromatographic solvent (butan-2-ol saturated with 1% acetic acid) and that the behaviour of the complex in this system resembles that of the phospholipids alone rather than free insulin.

Solubilization of insulin analogues by phosphatidylcholine in chloroform. Three 125 I-labelled insulin analogues were compared with 125 I-labelled insulin with respect to their solubilization in chloroform by phosphatidylcholine (Table 2). This table contains the results obtained with two preparations of the analogues. Insulin and desglycinodesphenylalanino-insulin were solubilized to a similar extent. Solubilization was slightly but significantly greater with the one preparation of desoctapeptide-insulin investigated. Desasparaginodesalanino-insulin was solubilized to a significantly greater extent.

Effect of insulin on solubilization of [14 C]glucose in chloroform by phosphatidylcholine. Jung *et al.* (1968) reported that extracts of lipids from erythrocyte 'ghosts' promoted solubilization of [14 C]glucose in chloroform. We have found that a single phospholipid species, phosphatidylcholine, was similarly capable of solubilizing [14 C]glucose (Table 3). In

Table 2. *Solubilization of 125 I-labelled insulin and insulin analogues in chloroform by phosphatidylcholine*

Chloroform (1 ml) alone or containing 1 μ mol of phosphatidylcholine was incubated at 37°C for 72 h with 1 ml of tris-buffered medium containing 400 ng of 125 I-labelled insulin or insulin analogue. Results are expressed as ng of 125 I-labelled insulin or insulin analogue solubilized in chloroform and are means $\pm$ S.E.M. of six determinations. *, Significantly different ($P < 0.01$) by Student's *t* test from the amount of 125 I-labelled insulin solubilized in the presence of phosphatidylcholine.

	Phosphatidylcholine	125 I-labelled analogue solubilized (ng)	
		Preparation 1	Preparation 2
125 I-labelled insulin	—	0.8 $\pm$ 0.03	0.8 $\pm$ 0.02
	+	7.8 $\pm$ 1.2	6.5 $\pm$ 0.3
125 I-labelled desasparaginodesalanino-insulin	—	3.4 $\pm$ 0.2	0.4 $\pm$ 0.05
	+	16.5 $\pm$ 1.0*	14.7 $\pm$ 1.0*
125 I-labelled desglycinodesphenylalanino-insulin	—	1.1 $\pm$ 0.03	0.8 $\pm$ 0.2
	+	6.9 $\pm$ 0.4	6.2 $\pm$ 0.5
125 I-labelled desoctapeptide-insulin	—	—	0.7 $\pm$ 0.02
	+	—	8.6 $\pm$ 0.4*

Table 3. *Effect of insulin on solubilization of [¹⁴C]glucose in chloroform*

Chloroform (1 ml) alone or containing 1 μ mol of phosphatidylcholine was incubated at 37°C for 72 h with 1 ml of tris-buffered medium containing 0.3 μ Ci of [¹⁴C]glucose and, where indicated, 40 μ g of insulin. Results are expressed as pmol of [¹⁴C]glucose solubilized, and are means $\pm$ s.e.m. of six determinations. The decrease in [¹⁴C]glucose solubilized by phosphatidylcholine in the presence of insulin was significant ($P < 0.01$) by Student's *t* test.

	[¹⁴ C]Glucose solubilized (pmol)
Chloroform	4.2 $\pm$ 1.1
Phosphatidylcholine	33.5 $\pm$ 5.4
Chloroform + insulin	4.1 $\pm$ 0.5
Phosphatidylcholine + insulin	20.0 $\pm$ 1.3

the absence of phosphatidylcholine very little [¹⁴C]glucose entered the chloroform phase, and insulin was without effect on the distribution of radioactivity. In the presence of phosphatidylcholine insulin in the aqueous phase decreased the amount of [¹⁴C]glucose solubilized by the phospholipid in the chloroform. Equilibrium distribution of [¹⁴C]glucose across the chloroform–water interface in the presence of phosphatidylcholine was reached by 72 h whether insulin was present in the aqueous phase or not (Fig. 2). At equilibrium the [¹⁴C]glucose distribution ratio always favoured the aqueous phase by a factor of 1000 or more. At all time-intervals tested after the start of incubation insulin decreased the amount of [¹⁴C]glucose solubilized by the phosphatidylcholine. On the time-scale of this experiment insulin had no effect on the rate of equilibration of [¹⁴C]glucose between the chloroform and the aqueous phases. Insulin thus appeared to shift the final equilibrium position in favour of the aqueous phase without affecting the rate of attainment of equilibrium.

Similar results were obtained when the solubilization of [¹⁴C]serine by phosphatidylcholine in chloroform was investigated in the presence and absence of insulin (M. C. Perry, W. Tampion & J. A. Lucy, unpublished work). Insulin at 40 μ g/ml decreased the amount of [¹⁴C]serine solubilized by the phosphatidylcholine. The insulin effect was thus not specific to glucose.

The effects of insulin and three other proteins, which are known to interact with phospholipids under certain conditions, on the solubilization of [¹⁴C]glucose by a variety of phospholipids was investigated (Table 4). The ability of the phospholipids to solubilize [¹⁴C]glucose increased in order phosphatidylcholine < sphingomyelin < phosphatidylethanolamine < phosphatidic acid. Cholesterol did not support solubilization of [¹⁴C]glucose.

Fig. 2. Time-course of solubilization of [¹⁴C]glucose by phosphatidylcholine in chloroform in the presence and absence of insulin. Chloroform (1 ml) alone (▲) or containing 1 μ mol of phosphatidylcholine (●) was incubated at 37°C with 1 ml of tris-buffered medium containing 0.3 μ Ci of [¹⁴C]glucose and, where indicated, 40 μ g of insulin (■). Each point is the mean of two determinations.

Insulin significantly decreased the amount of [¹⁴C]glucose solubilized by phosphatidylcholine and phosphatidic acid, slightly but significantly decreased that solubilized by phosphatidylethanolamine and had no effect on solubilization by sphingomyelin. The response of phosphatidylcholine to the presence of insulin was observed at insulin concentrations of 4 and 40 μ g/ml, but not at lower concentrations. Control experiments showed that Zn²⁺, which was present in the insulin preparation, did not at the same concentration (2 μ M) affect solubilization of [¹⁴C]glucose by phosphatidylcholine. Of the phospholipids, sphingomyelin was inactive with all the proteins, although there was a slight, but not significant, depression of [¹⁴C]glucose solubilization by sphingomyelin in the presence of cytochrome *c*. Phosphatidic acid responded to insulin, bovine serum albumin and cytochrome *c* with a markedly decreased capacity to solubilize [¹⁴C]glucose. It did not respond significantly to ribonuclease.

As a control that the results obtained with phosphatidylcholine and insulin were not the result of a change in the distribution of the phospholipid between the two phases during incubation with insulin, the concentration of phospholipid P was measured in the chloroform and aqueous phases before and after incubation at 37°C for 72 h in the presence and absence of insulin. Portions (0.5 ml) of each phase were evaporated to dryness, digested with conc. nitric acid, and the phosphate liberated was determined by the method of Baginski, Foa & Zak (1967). There was no change in the distribu-

Table 4. *Effects of insulin and other proteins on solubilization of [¹⁴C]glucose in chloroform by four phospholipids*

Chloroform (1 ml) alone or containing 1 μ mol of phospholipid was incubated at 37°C for 72 h with 1 ml of tris-buffered medium containing 0.3 μ Ci of [¹⁴C]glucose and, where indicated, 40 μ g of protein. Results are expressed as pmol of [¹⁴C]glucose solubilized, and are means $\pm$ s.e.m. of six determinations. Significance of the effect of the protein on solubilization of [¹⁴C]glucose by the phospholipid was determined by Student's *t* test: * $P < 0.05$; ** $P < 0.01$.

Phospholipid ...	[¹⁴ C]Glucose solubilized (pmol)			
	Phosphatidyletholine	Sphingomyelin	Phosphatidyl-ethanolamine	Phosphatidic acid
Chloroform	7.0 $\pm$ 2.1	7.7 $\pm$ 2.5	4.9 $\pm$ 2.3	3.1 $\pm$ 0.2
Phospholipid alone	29.5 $\pm$ 1.4	45.0 $\pm$ 1.7	55.5 $\pm$ 2.0	76.9 $\pm$ 2.6
Phospholipid + insulin	16.5 $\pm$ 1.0**	46.2 $\pm$ 2.8	47.9 $\pm$ 1.1**	39.5 $\pm$ 2.3**
Phospholipid + bovine serum albumin	28.0 $\pm$ 3.5	48.9 $\pm$ 6.5	—	20.1 $\pm$ 1.4**
Phospholipid + cytochrome <i>c</i>	13.6 $\pm$ 1.1**	38.8 $\pm$ 2.1	47.3 $\pm$ 2.6*	16.5 $\pm$ 0.4**
Phospholipid + ribonuclease	18.2 $\pm$ 2.1**	46.8 $\pm$ 3.6	57.0 $\pm$ 2.3	64.8 $\pm$ 8.5

tion of either phosphatidyletholine or phosphatidyl-ethanolamine during the incubation whether insulin was present in the aqueous phase or not. There was no indication that phospholipid left the chloroform phase during the incubation.

Efflux of [¹⁴C]glucose from 'liposomes'. The swollen phospholipid vesicle (liposome) preparation described by Bangham, Standish & Watkins (1965) was used to investigate possible effects of insulin on the flux of [¹⁴C]glucose from one aqueous phase, the buffer trapped between the phospholipid lamellae of the liposomes, across a phospholipid membrane, to another aqueous phase. The liposomes were prepared from 40 μ mol of phosphatidyletholine, 8 μ mol of cholesterol and 4 μ mol of a long-chain anion (dicetylphosphoric acid) or cation (stearylamine or cetyltrimethylammonium bromide). The swelling solution was 3 ml of 300 mM-glucose containing 3 μ Ci of [¹⁴C]glucose. Untrapped [¹⁴C]glucose was separated from the [¹⁴C]glucose trapped within the liposomes by gel filtration on Sephadex G-50. The tris-buffered medium used in the two-phase system was the eluting buffer. The efflux of [¹⁴C]glucose from the liposomes was followed by dialysis against tris-buffered medium in the presence and absence of insulin (40 μ g/ml).

We have been unable to detect a consistent significant effect of insulin on efflux of [¹⁴C]glucose from either positively or negatively charged liposomes, whether insulin was incorporated into the liposomes during their preparation or added subsequently to the liposome suspension.

DISCUSSION

At concentrations greater than their critical micelle concentration, phospholipids exist in non-polar solvents as inverted micelles with their hydro-

philic head-groups orientated towards the centre of the micelle. Blei & Lee (1963) determined the true critical micelle concentration of phosphatidyletholine in benzene at 25°C by a dye solubilization method to be 3.3 mg/l (approx. 4 μ M). Above this concentration the phosphatidyletholine monomers exist in small aggregates. There is a second association limit at 0.73 g/l (approx. 1 mM), at which aggregation of small micelles into larger ones occurs (Elworthy, 1959). There appear to be no similar studies of the aggregation state of phosphatidyletholine and other phospholipids in chloroform in the literature, and so it is not possible to define accurately the aggregation state of the phospholipids in the chloroform phase of the two-phase system used in this work. It is, however, reasonable to assume, by analogy with the studies in benzene, that the phospholipid monomers exist in the form of micelles. Assuming a cross-sectional area of 64 Å for an egg phosphatidyletholine molecule in a monolayer at its collapse pressure (Deamer & Cornwell, 1966), it can be calculated that less than 0.1% of the phosphatidyletholine in the system that we have studied exists in the form of a monolayer at the chloroform-water interface. This is confirmed by our results, which showed a 100% recovery of phosphatidyletholine from the bulk chloroform solution after incubation.

Interactions of phospholipids with insulin. Phosphatidyletholine supported solubilization of ¹²⁵I-labelled insulin in the chloroform phase, the insulin having been added to the system in the aqueous phase (Fig. 1; Table 1). The cationic amphipath, cetyltrimethylammonium bromide, which, like phosphatidyletholine, contains a positively charged trimethylammonium group, solubilized ¹²⁵I-labelled insulin to a similar extent. Phosphatidic acid and dicetyl phosphate, both characterized by the presence of a free phosphate group, were the most

effective of the agents tested (Table 1) for their ability to solubilize insulin, and, in some experiments, proved capable of solubilizing more than half the added insulin, thus reversing the insulin concentration gradient across the chloroform-water interface. By comparison, in the [^{14}C]glucose system, the same concentration of phosphatidic acid solubilized a maximum of only 0.1% of the [^{14}C]glucose present in the aqueous phase, the [^{14}C]glucose distribution always greatly favouring the aqueous phase. Thus simple entrapment of bulk aqueous phase containing insulin by phosphatidic acid micelles in the chloroform is not the underlying mechanism for solubilization of insulin by the phospholipid. Interaction must occur between the phosphatidic acid and the insulin, resulting in the formation of a complex which is soluble in the bulk chloroform phase.

Insulin adsorbs to droplets of *n*-decane stabilized by phosphatidylcholine and to black lipid membranes prepared from phosphatidylcholine (Hanai, Haydon & Taylor, 1965). Insulin has been shown to adsorb to and penetrate monolayers of stearic acid and cholesterol (Eley & Hedge, 1956a), distearin, dipalmitoyl phosphatidylethanolamine and distearyl phosphatidylcholine (Eley & Hedge, 1956b) and cholesterol (Vilallonga, Altschul & Fernandez, 1967). Penetration of protein into lipid monolayers occurs at low surface pressures (2 dyn/cm) but not at surface pressures above about 20 dyn/cm (Quinn & Dawson, 1970). Thus it is unlikely that insulin would penetrate the phospholipid monolayer at a chloroform-water interface. However, the appearance of a phospholipid-insulin complex in the bulk chloroform phase suggests that initial adsorption of insulin to the monolayer is followed by detachment of an insulin-phospholipid complex from this monolayer in a form that is soluble in chloroform.

Chloroform, being of low dielectric constant, would be expected to stabilize ionic interactions between the phospholipid and the protein, and it is possible that the interaction between insulin and the phospholipids in this system is primarily electrostatic in character. The phosphatidic acid and phosphatidylcholine used in these experiments have the same fatty acid composition, the phosphatidic acid having been prepared by digestion of the phosphatidylcholine with phospholipase D. As these phospholipids differ markedly in their ability to solubilize insulin, it is probable that the interaction with insulin depends in large part on the nature of the charged head-group of the phospholipid. The results with dicetyl phosphate and phosphatidic acid suggest that a negatively charged phosphate group not substituted with a base is a prerequisite for strong interaction with insulin. Kafka & Pak (1969a,b) have shown that insulin

competes with Ca^{2+} for the free phosphate groups of a stearyl phosphate monolayer at an air-water interface. The present studies suggest that if the phosphate is substituted with a base, as in phosphatidylcholine, phosphatidylethanolamine or sphingomyelin the interaction of insulin with the phosphate is much decreased. With phosphatidylcholine alternative interactions could occur, through the trimethylammonium group, with negative sites on the protein. This seems probable, since a similar degree of interaction was observed with cetyltrimethylammonium bromide. Eley & Hedge (1956b) have similarly suggested that the interaction between phosphatidylcholine and insulin is predominantly ionic, occurring between positive groups on the phospholipid and negative sites on the protein. However, this is unlikely to be the full explanation, since phosphatidylcholine and sphingomyelin have identical head-groups and yet only the former was effective in solubilizing insulin. Possibly the ability of the phospholipid to aggregate in chloroform, and the structure of the phospholipid micelles when they have formed, are important in the second and third stages of insulin solubilization, when the protein appears in the chloroform as a phospholipid-protein complex.

The two-phase system was not shaken but allowed to reach equilibrium under static conditions, when it is probable that turnover of phospholipid between the chloroform-water interface and the bulk chloroform solution is the rate-limiting step in the process of insulin solubilization. Thus the length of the incubation period should not be taken as an indication of the rate of interaction between insulin and phospholipid. To obtain such information it would be necessary to follow their interaction at the interface, which is not possible in this system.

A phosphatidic acid- or phosphatidylcholine-insulin complex with a higher ratio of insulin to phospholipid than those attained in the two-phase system was prepared by shaking a dry phospholipid-insulin film with chloroform. We have not attempted to determine the maximum solubilization of insulin by the phospholipid or the stoichiometry of the interaction. The chloroform-soluble complexes formed were stable to paper chromatography in butan-2-ol saturated with 1% acetic acid. In this system the complexes have chromatographic properties similar to those of the phospholipids rather than the insulin, indicating an arrangement in which the insulin molecule(s) occupy the centre of a phospholipid micelle and are effectively screened from the chromatographic environment.

Effects of insulin on distribution of glucose. The two-phase system used in this study of interactions between insulin and phospholipids has a marked advantage over simpler systems designed

to study molecular interactions, such as the paper-chromatographic technique, in that it can be used as a simple model in which to determine a functional effect, in this case effects of interactions between insulin and membrane phospholipids on the distribution of a hydrophilic compound (e.g. glucose or serine) between aqueous and organic phases. Insulin added in the aqueous phase interacted with phosphatidylcholine or phosphatidic acid so as to decrease the ability of the phospholipid to solubilize [^{14}C]glucose. This was not the result of a decrease in phospholipid concentration in the chloroform or of a decrease in the rate of equilibration of [^{14}C]glucose between the two phases in the presence of insulin (Fig. 2). Jung *et al.* (1968) have presented evidence that migration of [^{14}C]glucose into chloroform in the presence of phospholipids was not accompanied by a parallel migration of water, and suggest an association between the sugar and the phospholipid. We therefore infer from our experiments that the result of insulin-phospholipid interactions in the present system was either a change in the structure of the phospholipid micelle or a masking of sites in the micelle that may bind glucose, leading to a decrease in the ability of the phospholipid to effect solubilization of glucose. Although phosphatidylethanolamine did not solubilize ^{125}I -labelled insulin, its ability to solubilize [^{14}C]glucose was decreased in the presence of insulin to a small, but consistent, extent. The reason for this is not clear. Sphingomyelin gave no indication of interaction with insulin in either system, although it supported solubilization of glucose.

Three other proteins (cytochrome *c*, bovine serum albumin and ribonuclease) were investigated for their effects on the distribution of [^{14}C]glucose in this system. Cytochrome *c*, a basic protein, forms ionic complexes with acidic phospholipids (phosphatidylinositol and cardiolipin) in water (Green & Fleischer, 1963; Das, Haak & Crane, 1965) and binds to cardiolipin-phosphatidylcholine liquid crystals (Kimelberg, Lee, Claude & Mrena, 1970). Phospholipid-cytochrome *c* complexes formed from mixed phospholipid (Das & Crane, 1964), cardiolipin (Green & Fleischer, 1963), ethanolamine phospholipid (Das, Hiratsuka, Machinist & Crane, 1962) and mixtures of phosphatidylethanolamine and phosphatidylcholine (Das *et al.* 1965) and phosphatidylserine and phosphatidylcholine (Hart, Leslie, Davis & Lawrence, 1969), are extractable into iso-octane, but pure phosphatidylcholine does not form iso-octane-soluble complexes with cytochrome *c* (Das *et al.* 1965). Cytochrome *c*, however, can penetrate phosphatidylcholine monolayers, although the penetration is much weaker than with phosphatidic acid monolayers (Quinn & Dawson, 1969). In the present work cytochrome *c* decreased

solubilization of glucose by two acidic phospholipids, phosphatidylethanolamine and phosphatidic acid, and also by the zwitterionic phosphatidylcholine. Although the formation of chloroform-soluble cytochrome *c*-phospholipid complexes is likely to underlie these results, further work is necessary to confirm the formation of such complexes.

The interaction of cytochrome *c* with acidic phospholipids is explicable in terms of an electrostatic attraction of the positively charged protein with the negatively charged phospholipid, possibly with secondary hydrophobic interactions stabilizing the complexes. However, such electrostatic attraction between oppositely charged proteins and phospholipids is unlikely to underlie all the results shown in Table 4. Thus one basic protein, cytochrome *c*, interacts strongly with phosphatidic acid whereas another, ribonuclease, interacts slightly, if at all. Similarly, insulin interacts with phosphatidic acid when both the phospholipid and the protein carry a net negative charge. It is noteworthy in this context that Eley & Hedge (1956*a*) demonstrated that penetration of insulin into stearic acid monolayers occurred most readily when both lipid and protein carried a negative charge. It should, however, be remembered that the ability of the protein, once solubilized, to change the glucose-solubilizing properties of the phospholipid micelle may influence these results. Thus a protein may interact with and be solubilized by a phospholipid, and yet, because it does not modify the glucose-binding or structural properties of the micelle, is unable to affect the solubilization of glucose.

It is not surprising, in view of the simplicity of the physicochemical system studied in this work, that other proteins that are capable of interacting with phospholipids produce similar functional changes. Further, the failure of insulin to affect the permeability of glucose through liposomal membranes indicates that no direct extrapolation can be made from the present two-phase system to the movement of glucose from one aqueous phase, across a lipid membrane, to a second aqueous phase.

The present experiments demonstrate, nonetheless, that under appropriate conditions insulin is capable of interaction with phosphatidic acid and phosphatidylcholine, but not sphingomyelin or cholesterol, to form chloroform-soluble complexes. The interaction is probably such that the macromolecular organization of the phospholipid micelles is altered. As a result, the distribution of small polar molecules, such as glucose or serine, between the aqueous and organic phases is altered in favour of the aqueous phase. The present results indicate that, in addition to possible interactions with membrane proteins, the direct binding of insulin to,

and its interactions with, specific membrane phospholipids are factors of potential importance in the mode of action of insulin *in vivo*. However, any extrapolation of these results to intact cell membranes must be treated with caution since the cell membrane, both structurally and functionally, is several orders of magnitude more complex than these simple model systems.

We thank Mr Q. F. Ahkong for skilled technical assistance. M.C.P. was a Junior Research Fellow of the Medical Research Council during this work.

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