nutrients had been adsorbed by the active carbon from the medium conditioned for 240 h. Furthermore, medium conditioned for 240 h was dialyzed in membrane tubing No 132678 (Spectrum Medical Industries, Inc., Los Angeles) against distilled water at high internal pressure. After dialysis, the medium exhibited a considerably longer lag phase, as shown by curve IV. This suggests that the inhibitor was nondialyzable, having a mol. wt higher than about 14,000. It was also found to be thermostable, since after autoclaving the 240-h conditioned medium for 1 h at 120 °C and 2 at, the growth response of ciliates inoculated into the autoclaved medium was essentially unchanged from that with unautoclaved medium.

In our recent work, the growth response of *T. pyriformis* in the presence of glass beads similar in size and weight to ciliates was compared with that in the absence of glass beads. The growth response of the ciliates inoculated into fresh medium containing suspended glass beads was almost the same as that in the absence of the beads. On the other hand, when the ciliates were inoculated into 240-h conditioned medium containing the glass beads, the growth response was considerably inhibited. It is possible that inhibitory substance(s) were produced as a result of collisions between the ciliates and the glass beads. Furthermore, collision of a ciliate possessing an inhibitory substance with other ciliates or glass beads may suppress further multiplication of the ciliates. As mentioned previously, when stock ciliates are inoculated into fresh medium, they show a lag time. This may represent the period required for diluting or removing inhibitory substances bound to the inoculated ciliates. The results will be reported elsewhere.

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The effect of insulin on the electrophoretic mobility of rat hepatocytes

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Summary. The addition of insulin (10 μU) to a suspension of isolated hepatocytes in Krebs-Ringer bicarbonate solution, causes an increase in the negative electrophoretic mobility of the cells from - 1.68 μm sec^{-1} V^{-1} cm to 2.26 μm sec^{-1} V^{-1} cm. This observation supports the findings by other workers that the binding of insulin to its receptor leads to a marked change in the membrane.

The initial step in insulin action is the binding of the hormone to receptors in the cell membrane of its target cells[2]. This triggers, by an unknown mechanism, the metabolic changes in the cells that typify insulin action. Many of these changes are intracellular, but there are also several changes in the functions and properties of the cell membrane itself. Functional changes include the stimulation of the passage of substrates into cells such as the transport of glucose into adipocytes and muscle cells[3-5], and the transport of amino acids into a variety of cell types[6,7]. Insulin also modifies ion transport, promoting Ca^{2+} efflux from adipocytes and muscle cells[8]. It stimulates the transport of K^+ ions into adipocytes[9] and Na^+ efflux from frog skeletal muscle[10], probably as a result of stimulation of the membrane $Na^+ + K^+$-activated adenosine triphosphatase of the sodium pump[11]. These changes in ionic fluxes may also relate to the hyperpolarisation of adipocytes[12] and muscle cells[13] which occurs following treatment with the hormone.

Other observations imply that the physical properties of the membrane may be changed when insulin binds to its receptor. For example, the insulin receptor has been shown to be mobile in the plane of the membrane of cultured fibroblasts, and the receptors appear to aggregate and subsequently be internalized after binding the hormone[14,15]. Electron microscopic examination of freeze-fractured plasma membranes from adipocytes has shown an increase in the number of intramembranous particles following treatment with insulin[16]. Insulin also affects the cooperativity shown by the membrane-bound enzymes, acetylcholinesterase and the $Na^+ + K^+$-activated adenosine triphosphatase, in erythrocytes from rats fed a diet high in corn oil[17,18], indicating a possible decrease in membrane fluidity.

The wide range of these observations implies that a marked change may occur in the structure and physical properties of cell membranes following interaction of insulin with its receptor. Support for such a change has come from studies involving fluorescent probe molecules[19] but the validity of the results has been challenged by some authors[20,21] and such experiments have not always been successful[5]. However, a recent paper showed that insulin markedly decreased the translational diffusion coefficient of a probe molecule in plasma membranes isolated from rat liver[22] which further supports the idea that the binding of insulin to its receptor leads to a change in the membrane. In this present study we have used cell microelectrophoresis as a means to examine possible changes in the surface charge on hepatocytes following treatment of these cells with insulin in vitro. Microelectrophoresis is a technique that has been successfully used to examine perturbations in the ionizable groups present on the surface of a range of cells and organelles[23,24]. It can detect changes in the surface following minimal chemical and physical manipulation of the membrane and would thus seem to be a useful technique for examining the possible effects of insulin at the membrane level. The rationale for these experiments has been strengthened by the report that exceptionally low concentrations of conca-

Changes in the electrophoretic mobility of isolated hepatocytes following the addition of insulin and concanavalin A. The results show the average mobility value ± SEM from measurements made on 30 cells

Treatment	Electrophoretic mobility (μm sec^{-1} V^{-1} cm)	
	Control cells	Test cells
Insulin (100 μU/ml)	−1.76±0.09	−2.40±0.15
Insulin (10 μU/ml)	−1.68±0.09	−2.26±0.12
Concanavalin A (15 μg/ml)	−1.65±0.08	−2.37±0.11

navalin A, a plant lectin that binds to glyco-proteins including the insulin receptor[25], can cause a marked change in the surface charge of cells[26].

Materials and methods. Hepatocytes were prepared from rat livers using a technique based on the method of Berry and Friend[27]. The liver from a Wistar rat (250 g) was removed, sliced and incubated with shaking for 1 h at 37 °C with 5 mg of collagenase and 10 mg of hyaluronidase in sealed vessels containing 10 ml of calcium-free Krebs-Ringer bicarbonate medium (pH 7.4) that had previously been gassed with a mixture of 95% O_2 and 5% CO_2[28]. After incubation, tissue debris was removed by filtration through 2 layers of nylon mesh and the cell suspension gently centrifuged at $50 \times g$ for 2 min. The supernatant was discarded and the cells carefully suspended in Krebs-Ringer bicarbonate containing 1.3 mM calcium. The hepatocytes were washed twice by centrifugation and finally resuspended in Krebs-Ringer bicarbonate containing calcium. The integrity of the cells was checked by their ability to exclude uptake of the dye trypan blue. In all the preparations used more than 75% of the cells excluded trypan blue. All glass vessels used were siliconized to prevent sticking of the cells to the walls.

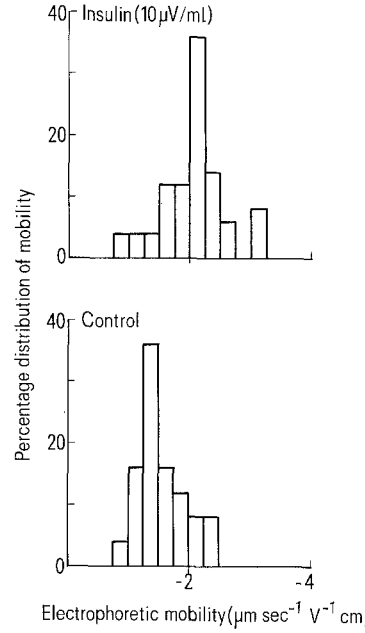
Electrophoretic measurements were carried out in a rectangular chamber supported on the stage of a microscope and mounted between 2 electrodes (Rank Brothers, Bottisham, Cambridge, England). A known field strength was applied and hepatocytes in focus at the stationary level were timed across a fixed distance. Generally about 20 readings were taken in alternate directions to minimise electrode polarization. From the velocity and the applied potential, the electrophoretic mobility was calculated as $\mu m\ sec^{-1}\ V^{-1}\ cm$. The results are expressed as the mean value ($\pm$SEM) with the number of observations given in parenthesis. The mobility is directly proportional to the electrokinetic or ζ potential and is related by an exponential function to the surface charge density and hence the charge carried by the hepatocyte. All electrophoretic measurements were carried out at 25 °C and pH 7.4 on hepatocytes suspended in Krebs-Ringer bicarbonate medium containing calcium in the absence and presence of hormones or concanavalin A.

Results. Control experiments showed that hepatocytes can be prepared which have a reproducible surface and that a standard preparation can be obtained by washing the cells twice and taking all the measurements within 4 h of the rat being killed. Under these conditions, the mean electrophoretic mobility of normal hepatocytes was -1.76 ± 0.05 (200). Concentrations of insulin in the physiological range (10 μU–100 μU/ml i.e. 0.4–4 ng/ml) caused an increase in the negative electrophoretic mobility immediately following the addition of the hormone to the values shown in the table. A typical distribution of mobility values before and after the addition of insulin (10 μU) is shown in the figure. The effect was not permanent and by 30 min after the addition of insulin, the mobility had returned to that of untreated cells. Therefore, all measurements were made during the 10 min immediately following the addition of the hormone.

The specificity of the effect for insulin has been investigated. Concanavalin A also reacts with the insulin receptor[25] and in our own experiments, the negative electrophoretic mobility of hepatocytes was increased to -2.37 ± 0.11[26] (table). In contrast to this, the addition of the hormones adrenaline or glucagon did not increase the negative electrophoretic mobility. Furthermore, the separate A and B chains of insulin (1 μg/ml) had no effect on the electrophoretic mobility. Guinea-pig anti-insulin serum (Wellcome Reagents Ltd., Beckenham, England) at a final dilution of 1:10,000 was used without effect on the hepatocytes (mobility, -1.76 ± 0.08 (13) without antibody and -1.75 ± 0.13 (18) with antibody) but blocked the effect of insulin when preincubated with the hormone. Mobilities were -1.64 ± 0.07 (14) for untreated cells; -2.55 ± 0.14 (16) for cells treated with insulin alone; and -1.86 ± 0.11 (11) for cells treated with insulin + antibody.

Discussion. We have demonstrated that insulin causes a marked increase in the negative electrophoretic mobility of rat hepatocytes. The increase was seen with insulin and concanavalin A, but not with adrenaline, glucagon or the separate chains of insulin. Furthermore, it was blocked by anti-insulin serum. The effect was rapid in onset being maximal at 5–10 min after addition of the hormone to the cells. The rapidity of this response is consistent with the time-courses reported for several of the rapid metabolic and transport effects of insulin e.g. suppression of the glucagon-stimulated glucose and cyclic AMP release from perfused rat liver[29], stimulation of monosaccharide transport in fat cells[30], elevation of cyclic GMP levels in fat cells[31], activation of pyruvate dehydrogenase in adipose tissue[32] and ribosomal activity in muscle[33]. However, the relationships between the observed change in the surface charge, the change in the membrane properties that produces it and the metabolic and transport effects of insulin remain to be determined. It is not yet clear for instance, whether the change in the membrane properties that leads to the alteration in the surface charge is an essential step in the mechanism by which insulin controls transport and metabolism in the cell or whether it simply represents yet another secondary effect of the hormone.

Nevertheless these results and those from other laboratories[14–18,22] suggest that insulin causes a significant change in the physical properties of the cell membrane. One explanation is that divalent cations are displaced from membrane binding sites but this seems unlikely since insulin has been reported to increase the number of Ca^{++} binding sites on plasma membranes from fat cells[34]. Another possible mechanism is a change in membrane phospholipid metabolism leading to the exposure of fresh ionogenic groups at the cell surface and indeed phospholipid turnover is known to be stimulated by several hormones in hepatocytes[35] and



The effect of insulin on the electrophoretic mobility of isolated hepatocytes. The results show the percentage distribution of mobility values of 25 cells before and after the addition of insulin to a final dose of 10 μU/ml.

by insulin in adipose tissue[36]. The precise nature of the alteration in the surface charge and the mechanism by which it is brought about are not yet known but are currently under investigation.

1 Acknowledgment. We wish to thank the Medical Research Council for the provision of the microelectrophoresis apparatus and initial running costs of the project.
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Interaction between fluorescent-labeled $ACTH_{1-24}$, isolated fat cells, and serum albumin[1]

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Summary. Part or all of the difference in the ability of $ACTH_{1-24}$ and a fluorescent-labeled $ACTH_{1-24}$ to activate lipolysis in fat cells can be accounted for by label-related binding to albumin present in the assay medium.

Considerable interest has been expressed in the development of molecular probes for use in evaluating hormone/cell-receptor interactions. One such potential probe is DNS-$ACTH_{1-24}$[5], a derivative of adrenocorticotropin-(1-24)-tetracosapeptide in which the sole modification is the introduction of the fluorescent DNS group at the ε-nitrogen atom of lysine-21. According to the available data[6,7], the conformation of DNS-$ACTH_{1-24}$ is the same as that of its parent hormone $ACTH_{1-24}$, i.e., a random coil. DNS-$ACTH_{1-24}$ stimulates activity in adrenal cells and in isolated fat cells to the same extent as the unsubstituted hormone[6,8]. However, the concentrations required to stimulate lipolysis in the fat cell system were considerably higher for the modified hormone in comparison to unlabeled $ACTH_{1-24}$[8]. Here we investigate the possibility that serum albumin, which is necessarily included in the fat cell assay medium as a physiological means of binding the fatty acids released during lipolysis[9], could also bind the labeled hormone and thus reduce its effective concentration in the medium. DNS-Lys was used as a control to estimate the binding due to the DNS group alone.

Materials and methods. DNS-$ACTH_{1-24}$ was a gift from Dr Peter Schiller, and $ACTH_{1-24}$ (Synactin®) was obtained through the courtesy of Dr W. Rittel, CIBA-Geigy Ltd, Basel (Switzerland). DNS-Lys lot 517 was purchased from Fox Chemical Co., and fraction V fatty acid-free bovine serum albumin lot 14 was obtained from Miles Laboratories. Human serum albumin fraction V was from the Swiss Red Cross, Bern (Switzerland), and defatted according to Chen[10]. Both albumins gave similar results. Titrations were made by changing the concentration of albumin in the presence of a fixed quantity of DNS-$ACTH_{1-24}$ or DNS-Lys. Fluorescence intensity and anisotropy measurements were carried out under N_2 on a Weber-type double beam instrument which simultaneously measures the parallel and perpendicular components of the emission and returns the computed values of the anisotropy and the total intensity[11]. The results were corrected for light scattering due to albumin in duplicate experiments with solutions which did not contain fluorescent ligand. Concentrations were determined from absorbance measurements using molar decadic extinction coefficients of 43,600 cm^2/mmole for (bovine)