EFFECTS OF LOW TEMPERATURE, UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION, AND RESPIRATORY INHIBITORS*  

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When isolated rat epididymal fat cells were incubated with [125I]iodoinsulin for 5 min at 37°, radioactivity accumulated in the plasma membrane fraction (Peak 1) and an unidentified particulate fraction (Peak 2) as reported previously (Kono, T., Robinson, F. W., and Sarver, J. A. [1975] J. Biol. Chem. 250, 7826-7835). This accumulation of radioactivity in Peak 2 (but not that in Peak 1) was greatly impaired when cells were incubated with toadinsulin in the presence of a variety of metabolic inhibitors that reduce the cellular content of ATP.

The reduction in the ATP level coincided with a disappearance of the stimulatory effects of insulin on sugar transport and the hormone-sensitive phosphodiesterase. In contrast, ATP depletion had no significant effects, at least during a 5- to 15-min incubation, on the intracellular water space and on the basal sugar transport and phosphodiesterase activities. When cells once depleted of ATP by treatment with 2,4-dinitrophenol (1 mM; 10 min) were washed and suspended in fresh buffer, the ATP level was recovered almost fully in 10 min. This recovery coincided with the restoration of responsiveness to insulin.

When cells were incubated with [125I]iodoinsulin or insulin for 5 min at 15° instead of 37°, a negligible quantity of radioactivity accumulated in Peak 2 and insulin failed to activate sugar transport. In contrast, under the same conditions, radioactivity accumulated in Peak 1 and insulin stimulated phosphodiesterase considerably.

These results suggest that ATP, or some other compound metabolically related to ATP, may be necessary for the actions of insulin on sugar transport and phosphodiesterase. ATP, or some other related compound, may also be necessary in the formation of the radioactive Peak 2, although the physiological function and cellular location of this peak are yet to be ascertained.

Insulin stimulates sugar transport across the plasma membranes of certain cell types (1) and activates phosphodiesterase in fat cells (2, 3). The initial step in these and other physiological actions of insulin is probably the interaction of the hormone with its specific receptors on the plasma membrane (4, 5). It has yet to be determined how this interaction modifies certain enzyme activities (6). Recently, we found that when fat cells were incubated with [125I]iodoinsulin, washed, and homogenized, radioactivity was recovered in an unidentified subcellular fraction as well as in the plasma membrane fraction (3). The work reported here was initiated to determine whether the accumulation of insulin (or its derivative) in this unidentified fraction is essential for activation of sugar transport and phosphodiesterase. A preliminary account of this work has been published (7).

MATERIALS AND METHODS

Crude bovine serum albumin (Fraction V, Lot 53C0670), crude bacterial collagenase (type II, Lot 15C0075), dried firefly lanterns, and carbonyl cyanide m-chlorophenylhydrazone were obtained from Sigma. Cyclic 3'-[3H]AMP was purchased from Schwarz Mann and purified by chromatography on AG 50W-X2 (3). Labeled [125I]iodoinsulin was obtained from Cambridge Nuclear and purified on a column (1.2 x 100 cm) of Sephadex G-50 using a solution containing 1 mM HCl, 50 mM NaCl, and 1 mg/ml of Fraction V albumin. Labeled [3H]inulin, [14C]urea, and [14C]mannitol (in 70% ethanol), and 3-O-methyl-D-[14C]glucose (in 90% ethanol) were purchased from New England Nuclear. The latter two preparations were dried in vacuo and redissolved in 0.145 M NaCl solution. All the labeled solutions were divided into small fractions and kept at -20°.

Insulin, 10 times recrystallized, was kindly supplied by Dr. J. Schlichtkrull of Novo Laboratories. Other reagents were either reagent grade or the best grade available.

Isolated fat cells were prepared by the collagenase method (8) from epididymal adipose tissue of Sprague-Dawley rats weighing 169 to 216 g. Unless stated otherwise, the binding of [125I]iodoinsulin, hormonal stimulation of sugar transport, and hormonal activation of phosphodiesterase were studied under the following conditions.

Binding of [125I]iodoinsulin—Approximately 400 mg of fat cells were incubated with 0.8 nm [125I]iodoinsulin (approximately 0.5 μCi/ml) for 5 min at 37° in 4 ml of Krebs-Henseleit Hepes buffer, pH 7.4, containing 20 mg/ml of Fraction V albumin (4). The incubated cells were fractionated essentially as described previously (3). Briefly, cells were washed twice with 0.25 M sucrose, 10 mM Tris/HCl (Buffer A) and homogenized in the same buffer with a Dounce tissue grinder (type R, 8 strokes). The homogenate was centrifuged for 9 min at 0° in a Beckman J-21B centrifuge (with a JA-20 rotor) set at 10,000 rpm. The supernatant, free of fat, was further centrifuged for 30 min at approximately 200,000 g (50,000 rpm in a Beckman No. 65 rotor). The second supernatant was discarded, and the pellet was suspended in 2 ml of cold Buffer A containing 1 mM EDTA. This suspension of

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crude microsomes) was well homogenized with a Warner apparatus (9), placed on top of a linear sucrose density gradient (13 × 100 mm; see below), and centrifuged in a Beckman SW 41 rotor for 35 min at 40,000 rpm. The sucrose density gradient solution was 15 to 45% w/w, with respect to the sucrose concentration, containing 1 mM EDTA (Na), and was buffered with 10 mM Tris/HC1, pH 7.4. After centrifugation, the sucrose solution was fractionated (0.7 ml/fraction) and analyzed for 31P and 5'-AMPase (3) activities.

All the tests to be compared were performed with portions of a pooled cell preparation; the samples, including an appropriate control, were centrifuged at the same time. The overall recovery of the plasma membrane was assessed from the height of the peak of 5'-AMPase activity in the sucrose density gradient profile. The difference in recoveries observed among samples centrifuged at the same time was usually less than 20%. In some experiments, the recovery of NADH-ferricyanide oxidoreductase (a marker enzyme for endoplasmic reticulum (3)) was also monitored. The distribution of protein, a commonly used reference standard, was not determined since its concentration in the sucrose solution was usually too low to be measured accurately.

Hormonal Stimulation of Sugar Transport — Approximately 50 mg of cells were preincubated, or "conditioned," in 5 ml of Krebs-Henoch-Leit Hepes buffer with albumin (see above) at 37° for 30 min (i.e. until the basal rate of transport was stabilized at a low level).2 Subsequently, the cell suspension was supplemented with 1 mM insulin or indicated reagents, centrifuged 10 s, and concentrated to approximately 50 mg/ml by removing excess infranatant solution. Several samples of the concentrated cell suspension, 0.2 ml each, were transferred to small test tubes and kept at 37°. The total time for insulin treatment, including the time for centrifugation, concentration, and dividing, was 5 to 10 min. In some experiments, insulin was added to the 0.2-ml cell suspension. The rate of sugar transport was determined by modification of the "oil flotation" method described by Glimann et al. (10). Briefly, approximately 10 mg of cells in 0.2 ml of buffer were mixed at 0 s with 0.05 ml of a sugar solution consisting of 5 mM 3-O-methyl-[14C]glucose (0.2 μCi), 0.9 mg/ml of [3H]linulin (0.2 mCi), and 0.154 M NaCl. The mixture was shaken manually at 37°. After 10 s, 0.2 ml of this incubation mixture was transferred to a small plastic centrifuge tube containing 0.1 ml of dinonylphthalate and placed in a Beckman microfuge (model 12B). The centrifuge was turned on at 20 s and turned off at 50 s. Subsequently, the centrifuge tube was cut at the middle of the dinonylphthalate layer, and the cells packed in the upper half were dispersed into a mixture of 1 ml of water and 10 ml of scintillation fluid containing Triton X-100. In each frame of this figure presents the results of a representative single experiment. Approximate specific gravities of sucrose solutions were determined by a modification of the double antibody method (18). All the experiments with fat cells were repeated on at least two separate occasions (3).

RESULTS

On Binding of Iodoinsulin — In confirmation of our previous results (3), when fat cells were incubated with [125I]Iodoinsulin for several minutes, washed, homogenized, and fractionated on a sucrose density gradient, radioactivity was recovered in the plasma membrane fraction (Fractions 7 to 9 in Fig. 1A, referred to as Peak 1) and in an unidentified particulate fraction (Fractions 13 and 14, referred to as Peak 2). When the incubation was terminated at 30 or 60 s, only Peak 1 was clearly distinguishable. This suggested that there might be a short lag in the formation of Peak 2.

Both peaks reached their maxima in 5 min (Peak 1 > Peak 2), which were maintained during the next 25 min (data not shown). When 1 nM native insulin was added to the system in this steady state, the peaks declined almost in parallel during the subsequent 10 min (Fig. 1B), presumably due to displacement of iodoinsulin by native hormone. The apparent competition between the two compounds was also seen when cells were incubated with 0.8 nM [125I]Iodoinsulin in the presence of graded concentrations of native insulin (Fig. 1C). Peaks 1 and

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Footnotes:
1 Similar results were obtained when the gradient was centrifuged for 45 min at 30,000 rpm or for 35 min at 35,000 rpm. However, the separation of the plasma membrane (5'-AMPase) and endoplasmic reticulum (NADH-ferricyanide oxidoreductase) was not good when the centrifugation was carried out for more than 40,000 rpm.
2 After the conclusion of the present work, it was found that the basal transport activity stabilized at a lower level (e.g. 10 s = 0.5 μl/g) and a relatively larger plus insulin activity (e.g. 45 s = 0.5 μl/g or 0.5 fold of the basal activity) was observed when cells were "aged" for 30 min at 37° in the same medium, as compared with those aged for 10 to 50 mg of cells in 0.2 ml rather than in a large volume (approximately 50 mg in 5 ml) as described here. The reason for this was unknown.

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Fig. 1. Distribution of [125I] activity in a crude microsomal fraction of fat cells incubated with [125I]Iodoinsulin. Fat cells were incubated with [125I]Iodoinsulin, and the cell homogenates fractionated through the sucrose density centrifugation step. The experiments were carried out as described under "Materials and Methods" with some modifications as specified below. A, cells were incubated with 0.8 nM [125I]Iodoinsulin at 37° for 0 (O), 2.5 (△), 10 (○), or 5 (□) min. After cells had been incubated with [125I]Iodoinsulin as above for 5 min, unlabeled insulin was added to a final concentration of 1 μM, and incubation continued at 37° for 0 (O), 5 (△), or 10 (○) min longer. C, cells were incubated for 5 min with 0.8 nM [125I]Iodoinsulin in the presence of native insulin at a level of 70 (O), 2.5 (△), or 10 (○) nM. D, cells were incubated with 0.8 nM [125I]Iodoinsulin for 5 min at 37° (O), 25 (△), or 15° (○). Activity of [122I]cpm (in each fraction is shown as a percentage with respect to the peak activity, or the height, of Peak 1 in the control sample plotted with □. Each frame of this figure presents the results of a representative single experiment. Approximate specific gravities of sucrose solutions were determined by a modification of the double antibody method (18). All the experiments with fat cells were repeated on at least two separate occasions (3).
were both suppressed slightly more than 50% by 10 nm
native insulin and almost eliminated by 1 pm hormone. These
data indicated that the apparent affinities of insulin for the
hypothetical binding sites in Peaks 1 and 2 were similar and
that the binding of radioactivity to both sites was almost fully
disposable by the native hormone.

The formation of Peak 2, but not that of Peak 1, was greatly
impaired when incubation of cells with [125I]iodoinsulin was
carried out at 15° instead of 37° (Fig. 1D). Similar results were
obtained when the incubation was performed (at 37°) in the
presence of 1 mm 2,4-dinitrophenol, 1 mm carbonyl cyanide
m-chlorophenylhydrazone (uncouplers of oxidative phosphoryla-
tion), 10 mm KCN, 10 mm NaN3 (reagents that inhibit
respiration (21), Fig. 2B), or 1 mm dicumarol (another
uncoupler of oxidative phosphorylation (20), data not shown).
In contrast, neither 0.1 mm 2,4-dinitrophenol nor 1 mm NaN3
had any detectable effect (data not shown).

Peak 2, once formed, was partially lowered in 10 min when 1
mm 2,4-dinitrophenol was added to cells that had been treated
with [125I]iodoinsulin (Fig. 2C). The rate of this lowering was
apparently slower than that observed in the displacement
experiment (Fig. 1B), possibly because the latter process was
facilitated by a high concentration of insulin as discussed by
De Meyts et al. (22). Fig. 2C also implies that 2,4-dinitrophenol
removal (1 mm, 10 min) had no detectable effect on the
sedimentation characteristics of the subcellular particles in
Peak 2 as well as those in Peak 1.

When incubated in a cell-free system, the subcellular parti-
cles corresponding to Peak 2, but prepared from cells that had
not been exposed to [125I]iodoinsulin, took up only a small
quantity of [125I]iodoinsulin (Figs. 2D). This observation was
in agreement with our previous data that Peak 2 was not
formed in a cell homogenate (3). Peak 2 was distinct from the
peaks of either N-acetyl-β-glucosaminidase or "insulinase"
(Fig. 3). The former is generally considered to be a marker
enzyme for lysosomes (15, 16). Although a part of this enzyme
was apparently fractionated into the soluble fraction (Fraction
15), most of its activity was found in Fracton 3 to 5. It should
be noted that these fractions (Fractions 3 to 5) exhibited negli-
gible 125I activity. Insulinase was thought to bind some iodoin-
sulin. However, it was not clear from this experiment (Fig. 3)
whether there was a special insulinase corresponding to Peak
2. The radioactive substances solubilized from Peaks 1 and 2
were still reactive with anti-insulin serum, but to lesser ex-
tents than the original iodoinsulin (Table I). No further infor-
mation was obtained regarding the homogeneity and identity
of these solubilized substances.

On Hormonal Stimulation of Sugar Transport—Similar to
the finding of Livingston and Lockwood (23), our preliminary
data (not shown) indicated that the transport of a nonmetabol-
able sugar across the plasma membrane of fat cells could be
measured with reasonable accuracy by the method used in the
present study. Table II shows that the transport of 3-O-
methyl-D-glucose was stimulated approximately 2-fold by in-
sulin treatment of fat cells and that this stimulation was
reduced to a large extent when cells were incubated with
insulin in the presence of 2,4-dinitrophenol, dicumarol, KCN,
or NaN3, at appropriate concentrations. At the same concentra-
tions, these inhibitors greatly depressed the ATP level. In

* 2,4-Dinitrophenol was used as Tris salt, which is highly water-
soluble. When 2,4-dinitrophenol in ethanol solution was added to a
cell suspension (the final ethanol concentration, 1%), recovery of
5'-AMPase and 125I activity in Peak 1 were considerably lower than
the controls.
insulin at 15° and their transport activity was determined at the same temperature, a negligible hormonal effect was recorded (Table V, Experiment B). Since the insulin-stimulated transport activity was clearly detectable at 15° (Experiment C), these data imply that the stimulation process, rather than the transport itself, was particularly sensitive to the low temperature. As noted before, the formation of Peak 2 was also sensitive to low temperature (Fig. 1D).

On Hormonal Stimulation of Phosphodiesterase When cells were incubated with graded concentrations of 2,4-dinitrophenol, the level of ATP and the hormonal stimulation of phosphodiesterase were depressed almost in parallel (Fig. 5). The stimulation was also depressed by dicumarol, carbonyl cyanide m-chlorophenylhydrazone, KCN, or NaN₃, at appropriate concentrations (Table VI, Experiment A). The effective concentrations of inhibitors were the same as those observed in the transport experiment (Table II). Arsenate, an uncoupler of certain nonoxidative phosphorylation (20), also reduced the transport itself, was particularly sensitive to the low temperature. As noted before, the formation of Peak 2 was also sensitive to low temperature (Fig. 1D).

![Fig. 3. Distribution of N-acetyl-β-glucosaminidase, "insulinase," and [3H]activity in sucrose density gradient. Cells were incubated with 0.8 nm [3H]iodoinsulin for 5 min at 37°. The cell homogenate was fractionated by sucrose density gradient centrifugation as described under "Materials and Methods." Samples obtained were analyzed for N-acetyl-β-glucosaminidase (Δ), "insulinase" (X), and [3H]activity (○). Results of assay (activity per fraction) are shown as a percentage of the maximum activity recorded in the sucrose density profile. This figure presents the data obtained in a single representative experiment. Although the data are not shown, the peaks for mitochondria, plasma membranes, and soluble materials are in Fractions 4, 8, and 15, respectively.

**Table I**

| Samples | Immunoreactivity* (%) |
|---------|-----------------------|
| [3H]iodoinsulin | 97.7 ± 2.4 (6)* |
| Peak 1 | 84.2 ± 0.7 (6) |
| Peak 2 | 70.2 ± 1.2 (6) |

* Immunoreactivity = ([3H] activity in precipitate) × (total [3H] activity)⁻¹ × 100.

**Table II**

| Inhibitors | Intracellular space of 2-O-methyl-d-glucose |
|------------|------------------------------------------|
|            | Basal + Insulin | ATP level pmol/mg cells |
| No inhibitor (control) | 17.9 ± 1.1* | 37.8 ± 1.0 |
| 0.1 mm DNPb,c | 19.7 ± 1.3 | 38.0 ± 2.3 |
| 1.0 mm DNP | 14.9 ± 0.9 | 17.0 ± 0.6 |
| 0.1 mm dicumarol | 20.7 ± 1.8 | 42.4 ± 1.8 |
| 1.0 mm dicumarol | 18.3 ± 0.3 | 19.3 ± 0.3 |
| 0.1 mm KCN | 18.6 ± 2.5 | 34.5 ± 1.6 |
| 1.0 mm KCN | 19.9 ± 0.9 | 16.6 ± 0.9 |
| 10 mm KCN | 19.6 ± 0.7 | 19.6 ± 2.3 |
| 1.0 mm NaN₃ | 14.8 ± 0.6 | 33.7 ± 0.0 |
| 10 mm NaN₃ | 18.3 ± 0.9 | 15.1 ± 1.2 |

* Mean value ± S.E. (n = 3, except control, where n = 6 to 9).

b DNP, 2,4-dinitrophenol.
c Dinitrophenol, dicumarol, and KCN were neutralized with Tris, NaOH, and KH₂PO₄, respectively.

**Table III**

| Inhibitor | Urea – insulin space* µg cells | Insulin space* µg cells | Urea – mannitol space* µg cells | Mannitol space* µg cells |
|-----------|--------------------------------|------------------------|--------------------------------|------------------------|
| None | 46.2 ± 1.4* | 17.8 ± 1.0 | 44.8 ± 4.5 | 21.0 ± 2.5 |
| 1 mm DNPb | 47.0 ± 1.9 | 17.8 ± 1.3 | 44.8 ± 0.6 | 20.4 ± 1.4 |
| 10 mm NaN₃ | 43.7 ± 2.7 | 16.5 ± 1.7 | 45.4 ± 1.6 | 18.3 ± 0.8 |

* Mean value ± S.E. (n = 6).
b DNP, 2,4-dinitrophenol.
Their responsiveness to insulin in 10 min during the subsequent incubation (Experiment Cl. As noted earlier, a parallel only (Experiment B). When 2,4-dinitrophenol-treated cells were washed and suspended in fresh buffer, cells regained their responsiveness to insulin in 10 min during the subsequent incubation (Experiment C). As noted earlier, a parallel

**TABLE IV**

Reversibility of 2,4-dinitrophenol effect on stimulation of sugar transport by insulin and effect of dinitrophenol added to cells after insulin

The sugar transport activity was determined by standard methods after cells had been treated at 37\(^\circ\)C as specified below. In Experiment A, aliquots of cells were incubated with buffer alone for 10 min (untreated cells). Other portions of cells had 1 mM 2,4-dinitrophenol for 10 min (DNP-treated cells). A third set of cells was incubated with 1 mM dinitrophenol and insulin for 10 min as above; the cells were then washed and incubated in a fresh buffer for 10 min (recovered cells). The buffer used in this experiment was supplemented with 2 mM glucose. All the cell preparations were incubated with or without 1 mM insulin for an additional 5 min before their transport activity was determined. In Experiment B, samples of cells were incubated with or without 1 mM insulin for an additional 5 min before their transport activity was determined. In Experiment C, cells were incubated with or without insulin for 5 min at 37\(^\circ\)C and 5 more min in a 15\(^\circ\)C water bath. During this second incubation, the temperature of the cell suspension (0.2 ml in volume) was lowered to 15\(^\circ\)C. The transport activity was determined at 15\(^\circ\)C.

**FIG. 4.** Reversibility of dinitrophenol (DNP) effect on the ATP level. Cells were equilibrated for 10 min at 37\(^\circ\)C and mixed with dinitrophenol (Tris salt) to a final concentration of 1 mM. After incubation for 10 min at 37\(^\circ\)C, cells were washed free of dinitrophenol and further incubated in fresh buffers at 37\(^\circ\)C. The buffer used in this experiment was supplemented with 2 mM glucose. ATP was assayed in samples taken at the indicated time intervals. Results from two separate experiments (△ and □) are reported.

| Experimental conditions | Intracellular space of 3-O-methyl-\(\alpha\)-glucose µg cells |
|------------------------|----------------------------------------------------------|
| A. Reversibility of DNP\(^*\) effect | | |
| Untreated cells | 13.6 ± 0.7\(^b\) | 34.9 ± 0.4 |
| DNP-treated cells | 11.7 ± 0.6 | 15.1 ± 2.1 |
| Recovered cells | 18.6 ± 0.7 | 37.1 ± 2.1 |
| B. Effects of DNP added after insulin | | |
| No DNP (control) | 10.8 ± 1.0 | 30.5 ± 1.6 |
| DNP added at 0 min | 11.2 ± 0.4 | 16.4 ± 1.7 |
| DNP added at 5 min | 9.5 ± 0.9 | 24.7 ± 0.8 |

\(^*\)DNP, 2,4-dinitrophenol.

\(^b\)Mean value ± S.E. (n = 3).

**FIG. 5.** Effects of different concentrations of dinitrophenol (DNP) on the ATP level and the stimulation of phosphodiesterase by insulin. Cells were incubated for 15 min at 37\(^\circ\)C with or without 2 mM insulin in the presence or absence of dinitrophenol at the indicated concentrations. Insulin and dinitrophenol were added at the same time. Standard procedures described under "Materials and Methods" were used for determination of ATP (△) and phosphodiesterase activities in the basal (○) and insulin-stimulated (●) states. The length of the small vertical bar indicates twice the standard error (n values for ATP and phosphodiesterase determinations are 6 and 3, respectively).

**TABLE V**

Effects of reduced temperature on stimulation of sugar transport by insulin

Sugar transport was determined as described under "Materials and Methods" at the indicated temperatures after cells had been incubated without or with insulin at the specified temperatures. In Experiment A, cells were incubated with or without insulin for 5 min at 37\(^\circ\)C, and the transport activity was determined at the same temperature. In Experiment B, cells were incubated with or without insulin for 5 min at 15\(^\circ\)C, and the transport activity was determined at the same temperature. In Experiment C, cells were incubated with or without insulin for 5 min at 37\(^\circ\)C and 5 more min in a 15\(^\circ\)C water bath. During this second incubation, the temperature of the cell suspension (0.2 ml in volume) was lowered to 15\(^\circ\)C. The transport activity was determined at 15\(^\circ\)C.

| Experimental group | Insulin concentration (nM) | Intracellular space of 3-O-methyl-\(\alpha\)-glucose µg cells |
|--------------------|---------------------------|----------------------------------------------------------|
| A. Insulin treatment at 37\(^\circ\); transport at 37\(^\circ\) | 0.0 | 12.0 ± 0.4* |
| B. Insulin treatment at 15\(^\circ\); transport at 15\(^\circ\) | 0.0 | 11.7 ± 0.6 |
| C. insulin treatment at 37\(^\circ\) and 15\(^\circ\); transport at 15\(^\circ\) | 0.0 | 10.3 ± 1.1 |

*Mean value ± S.E. (n = 3 in Experiments A and B; n = 6 in Experiment C).
**TABLE VI**

**Effects of some metabolic inhibitors and low temperature on hormonal stimulation of phosphodiesterase**

Cells were incubated with or without 2 mM insulin in the presence or absence of a metabolic inhibitor as indicated. The treated cells were washed and homogenized. Phosphodiesterase was assayed in Fraction P-2 (crude microsomal fraction) as described under "Materials and Methods." In Experiment A, cells were incubated with or without the indicated metabolic inhibitors for 10 min at 37°C. The inhibitors and insulin were added at the same time. In Experiment B, samples of insulin (Table IV) were added to the homogenates. Other aliquots of cells were incubated with or without insulin for 10 min at 37°C and, subsequently, washed and homogenized. Other cell samples were incubated with or without insulin for 10 min at 37°C and homogenized in the presence of 1 mM dinitrophenol. In Experiment C, aliquots of cells were incubated with or without 1 mM dinitrophenol for 10 min at 37°C (dinitrophenol-treated cells). A third set of aliquots of cells were incubated with 1 mM dinitrophenol and 10 mM sodium arsenate for an additional 5 min prior to washing followed by homogenization. The buffer used in this experiment was supplemented with 2 mM glucose. In Experiment D, cells were incubated with or without 2 mM insulin for 5 min at either 37°C or 15°C as indicated.

| Experiments                          | Phosphodiesterase activity (pmol/mg protein) |
|-------------------------------------|---------------------------------------------|
|                                     | Basal +Insulin                              |
| A. Effects of inhibitors added to the cell suspension |
| No inhibitors (control)             | 70 ± 1* 197 ± 2                             |
| 0.1 mM dicumarol†                   | 66 ± 4 192 ± 2                              |
| 1.0 mM dicumarol                    | 54 ± 2 63 ± 5                               |
| 1.0 mM CCC'                         | 66 ± 2 70 ± 3                               |
| 1.0 mM KCN                          | 67 ± 3 64 ± 4                               |
| 10 mM KCN                           | 63 ± 4 104 ± 13                             |
| 1.0 mM NaNO₃                       | 66 ± 8 51 ± 2                               |
| 10 mM NaNO₃                         | 55 ± 6 54 ± 3                               |
| 10 mM sodium arsenate               | 104 ± 6 157 ± 6                             |
| B. Effects of dinitrophenol added to the homogenization buffer only |
| 1.0 mM dinitrophenol was added      | 61 ± 10 65 ± 4                               |
| 1.0 mM dinitrophenol was added      | 77 ± 6 185 ± 8                               |
| C. Reversibility of dinitrophenol effect |
| Untreated cells                     | 65 ± 4 197 ± 3                               |
| Dinitrophenol-treated cells         | 72 ± 2 64 ± 2                               |
| Recovered cells                     | 70 ± 3 189 ± 7                               |
| D. Effect of temperature on the hormonal stimulation |
| 37°C                                | 66 ± 1 193 ± 7                               |
| 15°C                                | 75 ± 1 140 ± 7                               |

* Mean value ± S.E. (n = 3 to 6).
† Dicumarol, carbonyl cyanide m-chlorophenylhydrazone, KCN, and 2,4-dinitrophenol were neutralized with NaOH, NaOH, KH₂PO₄, and Tris, respectively.
‡ Carbonyl cyanide m-chlorophenylhydrazone.

**DISCUSSION**

The present data show that the level of ATP and stimulation of sugar transport and phosphodiesterase by insulin are affected almost in parallel by graded concentrations of several metabolic inhibitors (Tables II and VI and Fig. 5). The data also indicate that the level of ATP and the cellular responsiveness to insulin are both restored rapidly and almost completely in cells that have been treated with dinitrophenol, washed, and resuspended in fresh buffer (Tables IV and VI and Fig. 5). These results suggest that ATP may be necessary, either directly or indirectly, for the actions of insulin on sugar transport and phosphodiesterase. The inhibitors used in these experiments depress the ATP level by different mechanisms: dinitrophenol and dicumarol by uncoupling oxidative phosphorylation (20), and KCN and NaNO₃ by blocking the respiratory chain at the level of cytochrome c (31). Although these inhibitors may have additional effects on other metabolic processes, each of the inhibitors lowers the ATP level and the effect of insulin at the same concentration range. This implies that the fall in the ATP level is the common denominator in the processes, each of the inhibitors lowers the ATP level and the effect of insulin at the same concentration range. This implies that the fall in the ATP level is the common denominator in their effects on the actions of insulin. The results of the recovery experiment indicate that no irreversible effects are produced by dinitrophenol treatment on the cellular mechanisms that are involved in the generation of ATP and in the actions of insulin. It is of interest that a number of cellular...
activities and characteristics survive unimpared the low levels of ATP induced by the above mentioned inhibitors. For example, no disturbance was noted in (a) the basal activities of sugar transport and phosphodiesterase (Tables IV and VI and Fig. 5), (b) the formation of Peak 1, or the interaction of insulin with the plasma membrane (Fig. 2, A and D), and (c) the quantities of oil droplets (from broken cells) in the incubation mixtures (data not shown).

The present results are apparently consistent with previous observations that (a) oligomycin (a respiratory inhibitor) blocked the insulin stimulation of phosphodiesterase in fat cells (24), (b) uncouplers of oxidative phosphorylation inhibited the insulin activation of sugar transport in muscle, although the same agents partially stimulated the basal transport activity (12), and (c) insulin stimulated the turnover of 32P (25) and facilitated the incorporation of 32P into phospholipids (26) and proteins (27, 28) in fat cells. On the other hand, it has been repeatedly suggested in the past that insulin might stimulate dephosphorylation. This line of reasoning is supported by the observations that sugar transport in muscle is stimulated by anoxia (29) or uncouplers of oxidative phosphorylation (Table II). In addition, the earlier suggestion does not exclude a possibility that ATP, or some other compound metabolically related to ATP, might be necessary in some step(s) in the mechanism of insulin actions.

As noted earlier, the original aim of the present work was to examine whether the accumulation of insulin (or its derivative) in an unidentified subcellular fraction (Peak 2) is essential for the physiological actions of insulin. Our data show that, at 15°C, insulin apparently stimulates phosphodiesterase before Peak 2 is formed (Table VI and Fig. 1D) and that, at 37°C, 2,4-dinitrophenol seems to deactivate the enzyme before Peak 2 has disappeared (Figs. 6 and 2C). These results imply that the accumulation of insulin in the unidentified fraction may not be involved in the stimulation of phosphodiesterase. On the other hand, both the formation of Peak 2 and the hormonal activation of sugar transport are (a) apparently dependent on ATP or some other related compound (Fig. 2, A and B and Table II), (b) highly sensitive to low temperature (Fig. 1D and Table V), and (c) slowly lowered by dinitrophenol added after insulin or iodoinsulin (Fig. 2C and Table IV). Therefore, a correlation can still be postulated between these two processes.

As for the mechanism of formation of Peak 2, we think it highly unlikely that this peak is formed as an artifact of homogenization. In fact, no Peak 2 is detectable in homogenates prepared by our standard method after cells have been incubated with iodoinsulin at a low temperature or in the presence of certain metabolic inhibitors, such as 2,4-dinitrophenol (Figs. 1D, 2A, and 2B). As noted earlier, dinitrophenol has no apparent effects on the formation of Peak 1 and on the sedimentation characteristics of Peaks 1 and 2 (Fig. 2, A and C). It is also doubtful that Peak 2 represents [32]iodoinsulin incorporated by fat cells. This view is supported by the observations that Peak 2 disappears as rapidly as Peak 1 with the addition of native insulin to fat cells (Fig. 1B) and that very little [32] activity is found in the lysosomal fraction (Fig. 3). Therefore, it is tentatively considered that Peak 2 corresponds to [32]iodoinsulin (or its derivative) localized in some specific regions on the cell surface. A mosaic nature of the plasma membrane has been described in the liver cell system (31-33). The effects of low temperature and metabolic inhibitors (Figs. 1D, 2A, and 2B) as well as the apparent short delay in the formation of Peak 2 (Fig. 1A) may be explained if this peak is formed as a result of a lateral migration of insulin or the insulin:receptor complex on the cell surface. It was previously reported that the lateral translocation of immunoglobulin observed on the lymphocyte surface was blocked by low temperature, 2,4-dinitrophenol, or NaN3 (34, 35).

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Actions of Insulin in Fat Cells

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T Kono, F W Robinson, J A Sarver, F V Vega and R H Pointer

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