The effects of lowered O₂ tension on insulin secretion and changes in cellular energy parameters were investigated in isolated rat pancreatic islets perfused with buffers equilibrated with 21, 9, 5, and 1% oxygen and containing 5 mM glucose. Decreasing the external [O₂] reduced the amount of insulin released in response to 16 mM glucose, 20 mM α-ketoisocaproic acid, and 40 mM KCl. Secretion elicited by high glucose or KCl had declined significantly at 9% oxygen, whereas that caused by α-ketoisocaproic acid became inhibited below 5% O₂. Lowering the oxygen tension also decreased the ability of islets to respond with a rise in [ATP]/[ADP] upon stimulation with metabolic secretagogues. This reduction in the evoked increase in the nucleotide ratios paralleled the inhibition of stimulated insulin secretion. Addition of 2 mM amytal markedly decreased the islet energy level and eliminated the secretory response to 16 mM glucose. The results suggest that enhancement of B-cell energy production and a consequent rise in [ATP] (or [ATP]/[ADP]) are a necessary event for the hormone release elicited by high glucose and α-ketoisocaproic acid.

A decrease in temperature inhibited insulin secretion with all three secretagogues tested. The energies of activation were similar for high glucose and KCl-induced secretion, about 20 kcal/mol, but were higher for α-ketoisocaproic acid, about 35 kcal/mol. At 28 °C, the [ATP]/[ADP] was larger than that at 38 °C (8 versus 5) and was not increased further upon addition of 16 mM glucose. It is suggested that a decrease in the rate of energy production at lowered temperatures may contribute to the inhibition of insulin release caused by metabolic secretagogues.

Stimulation of insulin secretion by glucose is generally considered to involve an increase in the metabolism of the sugar, although the nature of the signal that links the two events remains unknown (1, 2). One of the key factors that has provided a new impetus to the field during the past 6 years has been the discovery that pancreatic B-cells contain an inward rectifying K⁺ channel that is controlled by ATP (3, 4). It has been postulated that a rise in the intracellular level of this nucleotide closes the channel and thus causes membrane depolarization, which in turn activates voltage-dependent Ca²⁺ channels with consequent influx of the cation into the cell (3-5).

The suggestion that ATP is the essential link between the stimulus and insulin secretion requires validation by demonstrating that the concentration of the nucleotide increases upon addition of physiologic concentrations of glucose and other metabolic secretagogues. Although there is some indication that this may be the case (6, 7), experimental studies have failed to show any consistent correlation between [ATP] (or [ATP]/[ADP]) and insulin release (8-11). In all the studies, the islet [ATP]/[ADP] ratios were found to be very low, generally not more than 3, which suggests that technical problems may have contributed to an inability to detect reliable alterations in the cellular energy state. In agreement with this, we have shown that when an improved quenching technique was used, a glucose concentration-dependent rise in [ATP]/[ADP] was observed in perfused pancreatic islets and that this occurred prior to, or coincident with, insulin secretion (12). A similar rise in [ATP]/[ADP] was observed with a powerful secretagogue α-ketoisocaproic acid, but not with lactate, which does not stimulate hormone release (12).

In this work, we have used alterations in O₂ tension to manipulate the cellular energy state. It was reasoned that if secretion is mandatorily linked to a rise in [ATP], it should correlate with changes in [O₂], since these are known to affect the ability of cells to generate energy. We have employed lowered temperatures in some of the perifusion experiments in order to influence the rates of energy synthesis and utilization. This technique should also help distinguish the various mechanisms responsible for insulin release as they relate to different secretagogues.

**MATERIALS AND METHODS**

*Islet Isolation and Perfusion—Fed male Wistar rats (200 to 250 g; Hilltop Laboratory Animals, Scottsdale, PA) were anesthetized with sodium pentobarbital (50 mg/kg injected intraperitoneally). Pancreatic islets were isolated using collagenase (Serva, New York) (13) and separated from the digest on Ficoll gradients (Sigma) (14).

Perfusion was carried out using a modification of the method of Weaver et al. (15) as described previously (12). The apparatus was modified to allow rapid removal of islets and minimize the chamber dead space. The upper portion of the Swinnex 13-mm filter holder (Millipore, Bedford, MA) was used to secure a Nitex filter (10 μm mesh; Tefko, Elmford, NY) was replaced with a retaining ring (OD 13 x 3 mm, ID 10 x 3 mm; nominal upstream dead volume 0.24 ml), which was secured to the lower portion of the filter holder by a single threaded turn so that it could be rapidly removed by a 360° rotation. The lower portion of the filter holder was unmodified, giving a nominal downstream space of 2.35 ml.

Oxygen and Temperature Dependence of Stimulated Insulin Secretion in Isolated Rat Islets of Langerhans*

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*This work was supported by National Institutes of Health Grant DK-35808. Insulin assays were performed by the University of Pennsylvania Diabetic Research Center Radioimmunoassay Core which is supported by National Institutes of Health Grant DK-19525. The section of the filter holder was unmodified, giving a nominal downstream space of 2.35 ml.
In experiments involving alterations in O2 tension, 100 freshly isolated islets were placed in paired chambers and perfused at 2 ml/min with Krebs bicarbonate buffer, at pH 7.4, supplemented with 0.25% bovine serum albumin. One of paired chambers was equilibrated at 37°C with a gas mixture of 21% O2, 5% CO2, and 74% N2 as the other was equilibrated with one of the following mixtures: 1) 95% O2, 5% CO2; 2) 90% O2, 5% CO2, and 9% N2; 3) 5% O2, 5% CO2, and 90% N2; 4) 90% O2, 5% CO2, and 94% N2. After 40 min of perfusion with a buffer containing 5 mM glucose, each solution of glucose (2 mM), sodium α-ketoisocaproic acid (2 mM) or KCl (4 m) were added into the medium to attain a final concentration of 16, 20, and 40 mM of each secretagogue, respectively. Control experiments (n = 6) in which 40 mM saline chloride was added into the perifusion medium showed that insulin secretion was not significantly affected by an 80% increase in osmolality.

In experiments involving changes in temperature, isolated islets were cultured for 1 h in RPMI 1640 medium containing 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in an atmosphere of humidified 95% air and 5% CO2 at 37°C. Batches of 100 islets were loaded into paired chambers. One of the chambers was maintained at 38°C as a control, whereas the other was perfused with medium equilibrated to a desired temperature. The perfusion technique, with a buffer equilibrated with 95% O2, 5% CO2, were the same as those used in the O2 experiments explained above.

After perfusion, the filters containing adhering islets were removed from the chambers and stored at 70°C. These islets were used for determining DNA and insulin content. Both DNA and insulin were extracted at room temperature in a saline solution (2 mM NaCl, 50 mM sodium phosphate, 1.6 mM EDTA, pH 7.4) by sonicating with 50 pulses from a Heat Systems Sonicator model W-385 (Plainview, NY) with a power setting of 3 and a 50% duty cycle. This procedure gave 30% larger amounts of insulin than acid-ethanol extraction.

For measurement of nucleotide levels 200 islets were used and perfused as described above. The preparations were quenched either after 45-min perfusion or after 1- and 5-min stimulations using a slight modification of the previous method (12). Islets were snap-frozen on dry ice and then were sonicated in 2 ml HClO4 at -10°C with 2 bursts of 10 pulses and then extracted for 10 min. The power setting and duty cycle of the sonicator were the same as those for the extraction of DNA and insulin. The HClO4 extracts were centrifuged at 9,000 x G for 5 min at 4°C, and the supernatant was neutralized by addition of 2.5 M K2CO3. The potassium perchlorate precipitate was suspended in a dispersion in a Beckman microfuge B for 2 min at 4°C. Supernatants were stored at -70°C.

Analytic Methods—Nucleotides were analyzed by high performance liquid chromatography using an Alltech Associates C18 Adsorbosphere HS column (100 x 4 mm, 3 μm particle size; Deerfield, IL). The pairing reagent was 20 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4) by sonicating with 80% ethanol.

The Effect of O2 Tension on Insulin Release—Insulin release was measured at five different O2 tensions: 95, 21, 9, 5, and 1%. Measurements with an O2 electrode in a solution equilibrated with the appropriate mixtures gave the following values: 21%, 189 μM; 5%, 41.4 μM; and 1%, 8 μM.

The dependence of insulin secretion on oxygen was measured with two metabolic secretagogues, glucose and α-ketoisocaproic acid, and also with a depolarizing agent, potassium chloride. When the latter was used as a secretory stimulus, 5 mM glucose was present as fuel. The combined data from all experiments are shown in Fig. 1. Results obtained at 95% oxygen were omitted because there were no statistically significant differences with those at 21% O2 for any of the three secretagogues (Table I). It can be seen that insulin secretion was markedly dependent on O2 tension and declined when the latter was lowered. The least oxygen sensitive was the release caused by α-ketoisocaproic acid.

To evaluate the influence of lowered O2 more accurately, the two phases of hormone secretion, the early (acute, 40–45 min) and the late (45–70 min), were quantified separately. Moreover, to minimize errors arising from daily variations, only paired experiments (i.e. carried out simultaneously) were used for analysis and the results were expressed as percentages of controls, i.e. islets perfused with 21% O2 (Table I). It can be seen that while release with 16 mM glucose and with 40 mM KCl was already reduced at 9% O2, with α-ketoisocaproic acid did not decline until O2 was decreased below 5%. Basal release did not change until O2 was decreased below 5% (not shown).

There were no statistically significant effects of oxygen on insulin content in the islets perfused before stimulation with lowered O2. In controls (21% O2), the value obtained was 914.0 ± 70.0 (n = 6, means ± S.E.) ng/μg DNA and in samples perfused with 1% oxygen, it was 1080.1 ± 64.4 (n = 6) ng/μg DNA. This means that the decrease of insulin secretion at low oxygen is not due to the depletion of the hormone stores.

The Effect of O2 Tension on ATP Production—Perifusion of islets with 2 mM amytal (a known inhibitor of mitochondrial phosphorylation site I) in the presence of 5 mM glucose caused a time-dependent decrease in ATP. The [ADP] initially tended to rise but did
FIG. 1. Secretion profiles for insulin in pancreatic islets perfused at various oxygen tensions. Islets were isolated and perfused as described under "Materials and Methods." A, 16 mM glucose; B, 20 mM α-ketoisocaproic acid; C, 40 mM KCl. Results are means ± S.E. for the number of experiments indicated (n) in the figure.

TABLE I

Effect of oxygen concentration expressed as percentage of 21% oxygen (control) on insulin release in islets stimulated with 16 mM glucose, 20 mM α-ketoisocaproic acid, and 40 mM KCl

<table>
<thead>
<tr>
<th>Conditions of perifusion</th>
<th>Glucose</th>
<th>α-Ketoisocaproic acid</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Late</td>
<td>Acute</td>
</tr>
<tr>
<td>95% oxygen</td>
<td>93 ± 9</td>
<td>92 ± 8</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>9% oxygen</td>
<td>63 ± 7°</td>
<td>74 ± 2°</td>
<td>102 ± 14</td>
</tr>
<tr>
<td>5% oxygen</td>
<td>36 ± 12°</td>
<td>58 ± 15°</td>
<td>33 ± 9°</td>
</tr>
<tr>
<td>1% oxygen</td>
<td>17 ± 4°</td>
<td>15 ± 2°</td>
<td>29 ± 2°</td>
</tr>
</tbody>
</table>

* Statistical significance compared with control: p < 0.01.
* Statistical significance compared with control: p < 0.05.

not show any continued increase, whereas the [ATP]/[ADP] fell to 1.64 in 5 min and to a limiting value of 1±1.20 in 15 min (Table IV). Addition of 16 mM glucose failed to increase the adenine nucleotide ratio.

Consistent with its inability to increase the [ATP]/[ADP] in islets pretreated for 5 min with 2 mM amytal, the insulin secretory response to 16 mM glucose was almost completely eliminated (Fig. 2). Moreover, hormone secretion induced by further addition of 40 mM KCl was also very small, although in control experiments such treatment resulted in a release which was equal to the sum of the amounts obtained with each of the stimuli independently (Fig. 1, A and C).

Temperature Dependence of Insulin Secretion—To avoid some of the variability in the data collected on freshly isolated islets, the preparations were cultured for 1 h as described in detail under "Materials and Methods." Fig. 3 shows that in such "stabilized" islets basal insulin secretion was generally lower and the responses to the three secretagogues were very reproducible. Moreover, the characteristic features of the curves obtained at both high (Fig. 3, A–C, 38°C) and low O2 (not shown) were the same as those in fresh preparations.

The dependence of insulin release on temperature is displayed in Fig. 3. It can be seen that as the temperature was lowered, there was a gradual decrease in the ability of B-cells to secrete the hormone with all three secretagogues tested. To assess the effect of temperature more accurately, Arrhenius analysis of the early (acute) and late phases of release were performed and the resulting curves are presented in Fig. 4. The Arrhenius plots of insulin secretion caused by high glucose and 40 mM KCl (in the presence of 5 mM glucose) were linear over the temperature range investigated (28–38°C). Data for α-ketoisocaproic acid could also be fitted to a straight line, although an apparent inflection point at about 35.5°C suggests that a transition temperature may exist in this range.

Activation energies ($E_a$) for each secretagogue were calculated using the slope of the Arrhenius plot and the Arrhenius equation (18). Table V shows that the values for stimulation of insulin secretion by 16 mM glucose and 40 mM KCl were about 20 kcal/mol, the same for both agents (within the limits of experimental error) and essentially identical for the two phases, whereas those for α-ketoisocaproic acid were higher, 31–37 kcal/mol. If the energy of activation for the latter secretagogue is calculated assuming the transition temperature at 35.5°C, the values obtained are 11–12 kcal/mol in the range 35–38°C and 42–43 kcal/mol in the range 28–35°C.

The Effect of Temperature on Insulin Contents in Cultured Islets—Attempts were made to measure the content of insulin in islets at various temperatures after 40 min of preliminary perfusion and after 30 min (total of 70 min) of stimulated perfusion. It was found that after 40 min of the initial
O2 and Temperature Dependence of Insulin Release

TABLE II

Effect of oxygen concentration on nucleotide levels ([ATP]/[ADP]), and insulin release in islets stimulated with 16 mM glucose (Glc) or 20 mM α-ketoisocaproic acid (α-KIC)

Conditions of perfusion and analytical techniques for measurement of both nucleotides and insulin are described in detail under "Materials and Methods." Controls refer to samples that were rapidly frozen after 45 min of perifusion with 5 mM glucose at the O2 tensions indicated. High glucose or α-ketoisocaproic acid was added at 40 min of perifusion. Islets were then snap-frozen after either 1 or 5 min of additional perifusion (41 or 45 min total).

TABLE II. Values represent mean ± S.E. for the number (n) of experiments indicated.

<table>
<thead>
<tr>
<th>Oxygen</th>
<th>Treatment</th>
<th>n</th>
<th>ATP (mM)</th>
<th>ADP (mM)</th>
<th>[ATP]/[ADP]</th>
<th>Insulin (ng/μg DNA/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21%</td>
<td>Control</td>
<td>15</td>
<td>1.43 ± 0.17</td>
<td>0.27 ± 0.03</td>
<td>5.29 ± 0.15</td>
<td>5.52 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>1 min Glc</td>
<td>4</td>
<td>1.35 ± 0.14</td>
<td>0.18 ± 0.02</td>
<td>7.58 ± 0.29*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 min α-KIC</td>
<td>5</td>
<td>1.63 ± 0.25</td>
<td>0.22 ± 0.03</td>
<td>7.44 ± 0.48*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 min Glc</td>
<td>4</td>
<td>2.59 ± 0.26</td>
<td>0.33 ± 0.04</td>
<td>7.94 ± 0.26*</td>
<td>26.05 ± 2.61</td>
</tr>
<tr>
<td></td>
<td>5 min α-KIC</td>
<td>5</td>
<td>2.25 ± 0.68</td>
<td>0.18 ± 0.02</td>
<td>8.35 ± 0.36*</td>
<td>21.54 ± 4.89</td>
</tr>
<tr>
<td>5%</td>
<td>5 min Glc</td>
<td>4</td>
<td>1.45 ± 0.18</td>
<td>0.22 ± 0.02</td>
<td>6.77 ± 0.38*</td>
<td>15.19 ± 4.48</td>
</tr>
<tr>
<td></td>
<td>5 min α-KIC</td>
<td>4</td>
<td>1.27 ± 0.12</td>
<td>0.14 ± 0.01</td>
<td>8.76 ± 0.07*</td>
<td>24.54 ± 2.28</td>
</tr>
<tr>
<td>1%</td>
<td>Control</td>
<td>7</td>
<td>1.12 ± 0.16</td>
<td>0.22 ± 0.03</td>
<td>5.20 ± 0.10</td>
<td>2.48 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>1 min Glc</td>
<td>4</td>
<td>0.73 ± 0.10</td>
<td>0.20 ± 0.05</td>
<td>3.87 ± 0.47*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 min α-KIC</td>
<td>5</td>
<td>1.39 ± 0.34</td>
<td>0.23 ± 0.06</td>
<td>6.01 ± 0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 min Glc</td>
<td>5</td>
<td>1.07 ± 0.18</td>
<td>0.18 ± 0.05</td>
<td>5.83 ± 0.40</td>
<td>4.87 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>5 min α-KIC</td>
<td>5</td>
<td>1.83 ± 0.48</td>
<td>0.28 ± 0.08</td>
<td>6.67 ± 0.51*</td>
<td>11.90 ± 1.96</td>
</tr>
</tbody>
</table>

*p < 0.001 compared with 21% control.

Table III

Effect of oxygen concentration on [ATP]/[ADP] and insulin release in islets stimulated with 40 mM KCl

Conditions of perfusion and analytical techniques for measurement of both nucleotides and insulin are described in detail under "Materials and Methods." Control (taken from Table II) and experimental samples have been obtained as described in the legend to Table II. Values represent mean ± S.E. for the number (n) of experiments indicated.

<table>
<thead>
<tr>
<th>Oxygen</th>
<th>Treatment</th>
<th>n</th>
<th>ATP (mM)</th>
<th>ADP (mM)</th>
<th>[ATP]/[ADP]</th>
<th>Insulin (ng/μg DNA/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21%</td>
<td>Control</td>
<td>15</td>
<td>5.29 ± 0.15</td>
<td>5.52 ± 1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 min KCl</td>
<td>3</td>
<td>3.87 ± 0.36*</td>
<td>18.73 ± 3.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 min KCl</td>
<td>3</td>
<td>3.25 ± 0.12*</td>
<td>18.73 ± 3.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>Control</td>
<td>7</td>
<td>5.20 ± 0.10</td>
<td>2.48 ± 0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 min KCl</td>
<td>2</td>
<td>4.11 ± 0.23*</td>
<td>2.48 ± 0.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.001 compared with 21% control.

TABLE IV

Nucleotide levels and [ATP]/[ADP] in islets treated with 2 mM amytal and 16 mM glucose

Conditions of perfusion and analytical techniques for measurement of both nucleotides and insulin are described in detail under "Materials and Methods." The perfusion medium was equilibrated with 21% oxygen. Amytal was added after 40 min of preliminary perifusion and the perifusion continued for either 5 or 15 min. High glucose was added after 5 min of perifusion with amyta, i.e. at 45 min. Control values were taken from Table II. Values represent mean ± S.E. for the number (n) of experiments indicated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ATP (mM)</th>
<th>ADP (mM)</th>
<th>[ATP]/[ADP]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>1.43 ± 0.17</td>
<td>0.27 ± 0.03</td>
<td>5.29 ± 0.15</td>
</tr>
<tr>
<td>5 min amytal</td>
<td>4</td>
<td>0.62 ± 0.07</td>
<td>0.39 ± 0.06</td>
<td>1.64 ± 0.15*</td>
</tr>
<tr>
<td>15 min amytal</td>
<td>1</td>
<td>0.49</td>
<td>0.38</td>
<td>1.29</td>
</tr>
<tr>
<td>15 min amytal + 10 min glucose</td>
<td>3</td>
<td>0.29 ± 0.07</td>
<td>0.22 ± 0.06</td>
<td>1.24 ± 0.07*</td>
</tr>
</tbody>
</table>

*p < 0.001 compared with control.
**Fig. 3.** The effect of temperature on insulin release in perfused islets. Procedures for perfusion and stimulation are outlined under "Materials and Methods." Data are means ± S.E. for the number of experiments indicated.

**Fig. 4.** Arrhenius plots of stimulated insulin release. The rates of insulin secretion in the acute and late phases were calculated for each secretagogue by integrating the amount of hormone released during the interval of interest and dividing it by its duration. The data points were fitted to straight lines and activation energies were calculated from their slopes ± S.E. (see Table V). Statistical significance of the regression was p < 0.001 for all three secretagogues in both phases.

**TABLE V**

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Acute</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>15.8 ± 1.9</td>
<td>23.4 ± 1.8</td>
</tr>
<tr>
<td>α-Ketoisocaproic acid</td>
<td>36.7 ± 2.9</td>
<td>30.8 ± 1.8</td>
</tr>
<tr>
<td>KCl</td>
<td>18.6 ± 1.8</td>
<td>20.5 ± 2.2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The results presented in this work demonstrate that a substantial correlation exists between the early rise in islet [ATP]/[ADP] and the stimulation of insulin secretion induced by high glucose and α-ketoisocaproic acid (Fig. 5). Since the increase in [ATP]/[ADP] inevitably means a gain in ATP (see below for the arguments), these findings could be taken as support for the postulate that metabolic secretagogues induce an augmentation of the energy level in B-cells, which then closes the ATP-dependent K⁺ channel and thus causes plasma membrane depolarization. On the other hand, it is also possible that there are other processes in islets that are sensitive to ATP. Although their nature remains to be identified, our data provide strong evidence that such ATP- or [ATP]/[ADP]-dependent processes are a necessary event in insulin secretion elicited by high glucose or α-ketoisocaproic acid.

One of the puzzling findings of the present work is that we could not find in islets stimulated by either high glucose or α-ketoisocaproic acid a consistent rise in [ATP] and a concomitant decline in [ADP] as compared with nonstimulated preparations. This may be due to at least two reasons. The first is that variations in the measured concentrations of these nucleotides were rather large. The variability, in part, arises from the inability to quantify the relevant parameters on the same islets before and after stimulation, which would be required in an ideally paired experiment. Parallel determinations on stimulated and nonstimulated preparations may help
to eliminate some of the differences in the perifusion technique, but they do not correct for variation in islet size and degree of intactness or other contributing properties of the cells. Moreover, although we measured DNA in each pair of methods of quantification (6-12, 19).

The second reason for the inability to detect consistent changes in [ATP] and [ADP] elicited by addition of metabolic secretagogues is that these are expected to occur in the cytosolic compartment (20-22), any rapid alteration in the ATP and ADP co-secreted with insulin during the first 1-5 min after stimulation is not large. The validity of this approach is substantiated by the data in Fig. 5 which show that the proportion of the hormone (and by inference that of the adenine nucleotides) that is released in this time interval is less than 5% of the total insulin content of the islets.

The second premise is that the sensitivity of the K+ channel to [ATP] / [ADP] that are seen experimentally provide only minimum values, because they are superimposed on a large and relatively constant background on unchanging vesicular ATP and ADP. The presumed constancy of this background relies on the assumption that the amount of ATP and ADP is sequestered with insulin during the first 1-5 min after stimulation is not large. The validity of this approach is substantiated by the data in Fig. 5 which show that the proportion of the hormone (and by inference that of the adenine nucleotides) that is released in this time interval is less than 5% of the total insulin content of the islets. The third premise is that the sensitivity of the K+ channel to [ATP] / [ADP] depends on [ADP] (5) and hence the true regulatory factor of its activity may be the [ATP] / [ADP].

Our results show that the [ATP] / [ADP] in nonstimulated islets is 5-5.5 which is within the range of values for metabolically competent cells that have adenine nucleotides partly bound or sequestered (23). This figure is, however, almost twice as large as earlier published estimates in islets capable of high rates of insulin secretion (7, 11, 19). Since the latter can be used as a sign of the metabolic intactness of these preparations, the simplest interpretation of the differences between our results and those of others is that special precautions must be taken to obtain reliable estimates of cellular energy parameters.

Administration of either high glucose concentration or α-ketoglutaric acid results in a rise of the [ATP] / [ADP] to 8-9. This large increase provides, in our opinion, the first clear indication that changes in [ATP] and [ADP] induced in the cytosol by the action of metabolic secretagogues must be substantial. Such a conclusion cannot be drawn on the basis of the earlier observations in the literature. The rise in the cytosolic [ATP] / [ADP] reported by Malaisse and Sener (7), in islets incubated for 45 min after their transfer from 4.8 to 16.7 mM glucose, was barely detectable, 2.7-3.8. Similarly, in B-cells, Kakei et al. (6) have noted a 2-fold increase (2.85 versus 5.52) when glucose concentration in the medium was changed from 0 to 20 mM. Since in the latter experiments, high glucose caused a 19% increase in A1P + ADP, it is likely that the effects of the metabolic substrate and not those of the secretagogue were being evaluated.

We have used alterations in O2 tension, which at appropriate levels affect mitochondrial ATP synthesis, to define the relationship between [ATP] / [ADP] and the ability of B-cells to secrete insulin. Our results lead to a number of interesting

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**TABLE VI**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Treatment</th>
<th>n</th>
<th>ATP (mM)</th>
<th>ADP (mM)</th>
<th>[ATP/ADP]</th>
<th>Insulin (ng/µg DNA/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38°C</td>
<td>Control</td>
<td>4</td>
<td>1.26 ± 0.06</td>
<td>0.24 ± 0.01</td>
<td>5.37 ± 0.10</td>
<td>1.57 ± 0.34</td>
</tr>
<tr>
<td>5 min Glc</td>
<td></td>
<td>5</td>
<td>0.84 ± 0.08</td>
<td>0.11 ± 0.01</td>
<td>7.47 ± 0.43*</td>
<td>7.53 ± 1.45</td>
</tr>
<tr>
<td>28°C</td>
<td>Control</td>
<td>4</td>
<td>1.30 ± 0.17</td>
<td>0.16 ± 0.02</td>
<td>8.18 ± 0.22</td>
<td>1.13 ± 0.22</td>
</tr>
<tr>
<td>5 min Glc</td>
<td></td>
<td>4</td>
<td>0.80 ± 0.09</td>
<td>0.10 ± 0.01</td>
<td>8.02 ± 0.42</td>
<td>2.78 ± 0.52</td>
</tr>
</tbody>
</table>

*p < 0.001 compared with 38°C control.
and important conclusions. (i) Lowering [O2] to 1% does not significantly decrease the [ATP]/[ADP] in nonstimulated islets, albeit it substantially limits their ability to respond to stimulation, by high glucose or α-ketoisocaproic acid, with a rise in the nucleotide ratio. This means that when addition of a metabolic secretagogue increases the oxidative activity of B-cells, i.e. their O2 consumption rate (24, 25), in order for this enhanced O2 utilization to occur, there must be a sufficient provision of oxygen. If the O2 tension falls below a critical level, oxygen diffusion becomes rate-limiting, and the mitochondrial respiratory chain fails to respond with increased activity. It might be interesting to speculate that in pathological states accompanied by high demand for insulin, such as non-insulin-dependent diabetes or obesity, the secretion of the hormone may be curtailed by an apparent hypoxia which results from an inadequate blood supply to the hyper trophyed islets (26). (ii) Comparison of the results obtained with high glucose and α-ketoisocaproic acid show that insulin secretion correlates with the rise in [ATP]/[ADP] and not with changes in [O2]. This indicates that oxygen exerts its influence through action on the respiratory chain and not in a more direct way, as for example on the K+-channel itself. Such an effect of oxygen, which does not require the presence of either ATP or Ca++, has been described recently in the carotid body (27). The data is also consistent with the suggestion that a rise in [ATP] or [ATP]/[ADP] is a necessary event in insulin release elicited by metabolic secretagogues (4). (iii) The high sensitivity to amytal of insulin release by 16 mM glucose indicates that activation of glycolysis and consequent enhancement of the α-glycerophosphate shuttle plays only a minor role in providing ATP for secretory events in intact stimulated islets. This is consistent with the earlier results obtained with rotenone (8, 24) and may mean that the high content of mitochondrial flavin-linked 3-glycerol phosphate dehydrogenase (28) is not a feature of B-cells but of other constituents of islets. Alternatively, the results might suggest that the demands for ATP during stimulated insulin secretion are so large that they cannot be met adequately by less energy-proficient glycolysis and α-glycerophosphate shuttle. (iv) The fourth conclusion is based on the findings that the rise in insulin secretion and [ATP]/[ADP] elicited by high [glucose] is more sensitive to decreases in [O2] than that caused by α-ketoisocaproic acid. It indicates that if the action of these secretagogues involves closure of the ATP-dependent K+-channels, glycolysis does not act as a preferential source of ATP for this event, as is the situation in the heart (29, 30). (v) Finally, the observation that insulin release stimulated by 40 mM KCl was nearly as dependent on [O2] as that caused by high glucose came as a surprise. Based on previous studies (31), one would expect that depolarization activates Ca++ influx via voltage-operated channels directly and thus stimulates insulin secretion without the need for an increased ATP level. The evidence of a rise in [ATP]/[ADP] (Table III) upon addition of 40 mM KCl at high O2 tension and the better preservation of the early phase of hormone release when oxygen is lowered (Table I) are consistent with this suggestion. However, the relatively large overall sensitivity of the depolarization-stimulated release to hypoxia also means that there is a component (step) in this secretory pathway which requires enhancement of oxidative metabolism for sustained (longer than 1–2 min) hormone release. The presence of 5 mM glucose in our experiments would allow such metabolic events to occur.

It should be pointed out that the effects of limitation in cellular energy production on insulin release and a rise in [ATP]/[ADP] become noticeable at a rather high medium [O2], in the case of 16 mM glucose and 40 mM KCl at 40–80 μM, and with α-ketoisocaproic acid below 40 μM. Although the relations between the external and the internal oxygen concentrations in islets are not known, it is likely that the intracellular tension is much lower than that in the perfusing buffer. Moreover, it is also possible that during increased respiratory activity which follows addition of secretagogues, [O2] falls even more and reaches a value that limits the function of cytochrome oxidase.

An interesting observation, which confirms earlier studies with metabolic inhibitors (8, 10, 24, 25) is that when total [ATP] declines to 0.8–1.0 mM, stimulated as well as nonstimulated insulin releases cease. This behavior, which is also manifested in other secretory systems such as chromaffin cells (32, 33) and isolated nerve endings (34), arises from the known energy demand for exocytosis.

In addition to alterations in [O2], we have used manipulations in the temperature as a means to gain insight into the relations between cellular energy synthesis and insulin secretion. Lowering temperature to 28 °C resulted in an increase in the [ATP]/[ADP] in a nonstimulated basal state to over 8. It was not possible to increase this further by addition of 16 mM glucose. Although the events that lead to readjustments in cellular energy level are rather complex, the simplest interpretation of the higher [ATP]/[ADP] at 28 °C is that lowering the temperature decreases energy utilization more than synthesis. The inability of metabolic secretagogues to increase the [ATP]/[ADP] may mean that at low temperatures, even in the presence of these molecules, the decrease in the rate of metabolic reactions is large enough to prevent energy-producing pathways from generating ATP at a rate which significantly raises its cellular concentration. Alternately, the results might suggest that the signals that cause increase in ATP synthesis are too weak to enhance energy production. Whether this failure to increase [ATP] contributes to inhibition of insulin release elicited by high glucose and α-ketoisocaproic acid remains to be established. However, it is also possible that a major effect of cooling is "distal" to ATP and that these distal steps become rate controlling in secretion. Our results provide no information on the identity of these reactions.

Independent of its effects on the energy level in islets, lowering the ambient temperature had a marked inhibitory effect on stimulated insulin secretion, consistent with earlier studies (35–37). However, in contrast to most previous investigations we used a series of temperatures in the range 28–38 °C so that energies of activation (Ea) could be calculated. The values obtained ranged from 16 to 37 kcal/mol (Table V), which corresponds to Q10 of 2.6–7.0, and were in good agreement with the figure of 5 for tolbutamide-stimulated insulin release measured by Curry and Curry (36). The Q10 of 2.6 for the early (5 min) phase of glucose-elicited secretion is also consistent with the behavior observed by the above authors in the temperature range 30–38 °C, although the precipitous drop at 28 °C observed in perfused pancreas was not seen in islets.

Our results show that Ea for stimulation by 16 mM glucose and 40 mM KCl were the same in both the acute and late phases. Since high [KCl] was added to islets perfused with 5 mM glucose, a metabolic effect is superimposed on the K+-induced depolarization. This means that the rate-determining step in the overall sequence of events leading to insulin secretion could be the same for both stimulants. Alternately the limiting reactions are different but have very similar energies of activation. By contrast, the different temperature profile for α-ketoisocaproic acid-elicited hormone secretion...
indicates that the rate-limiting step in this process is confined to a reaction(s) which is the property of this particular secretagogue and is not shared by either KCl or high glucose-activated pathways. Although the nature of the rate-determining steps has not been explored in this study, it is unlikely that ion fluxes through the channels are limiting, because these have been shown not to be very temperature-dependent (38).

An interesting observation is that stimulated insulin release exhibits a much larger temperature dependence than glucagon secretion by pancreatic A cells (36), catecholamine release from chromaffin cells (39, 40), or biogenic amine efflux from brain nerve endings (41). This indicates that in spite of their apparent mechanistic similarities, the various stimulus-coupled secretory events contain within their pathway a step, or steps, which have unique properties.

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