STUDIES ON THE RELATIONSHIPS BETWEEN INSULIN,
GLUCOSAMINE, AND GLUCOSE IN RAT
DIAPHRAGMS*

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One approach toward the elucidation of the mechanism of insulin action is to study the effect of insulin on glucose analogues. Studies of this type have been carried out in this laboratory by use of the eviscerated and nephrectomized rabbit. It has been shown (1), with the above test preparation, that insulin accelerates the transfer of D-glucosamine from the extracellular fluid into the cells. Concurrently, the demand for glucose by the maximally insulin-treated animal was lowered considerably after a single injection of D-glucosamine. Since the decrease in glucose requirements of these animals could not be explained, even assuming complete conversion of D-glucosamine to glucose, it was suggested that D-glucosamine acts in some manner as a metabolic block.

While the metabolic changes observed in the eviscerated animal are usually attributed to the muscle cells, it is desirable to test this directly. Consequently, we have examined the effect of insulin action on glucosamine, using the isolated rat diaphragm. Studies of this type can be carried out in the absence of glucose, in contrast to the intact animal, in which glucose is always available to the muscle tissues.

Methods

Tissue Preparation—Young fed rats (Slonaker strain) were decapitated, and the diaphragms were rapidly removed and chilled in cold Krebs-Ringer-phosphate buffer solution (pH 7.4). This medium was used in all diaphragm experiments. The tissues were trimmed, divided, blotted, and weighed. For studies on the effect of insulin on sugar uptake, the hemidiaphragms were divided between the control and insulin-containing flasks. When the effect of glucose competitors was studied, each of about six diaphragms was divided nearly equally among the six vessels in each series. The diaphragms were added to chilled Warburg vessels previously prepared

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1 The crystalline zinc insulin used in this work was contributed by the Lilly Research Laboratories.
with the necessary substrates and medium. The final volume in all experiments was 3 ml., and 0.2 ml. of KOH (20 per cent) was added to the center wells to absorb CO₂. The flasks were gassed for 5 minutes with oxygen, then incubated for 2 hours at 37°. When radioactive substrates were used, 0.2 ml. of 50 per cent H₂SO₄ was tipped in from the side arm to stop the reaction and to liberate CO₂. At least 10 minutes were allowed for absorption of the CO₂. The contents and rinses from the center well were transferred to small Erlenmeyer flasks, 0.4 mmole of carrier Na₂CO₃ added, and the carbonates precipitated as BaCO₃. The BaCO₃ was collected by filtration, dried, and assayed for radioactivity.

**Determination of Glucose and Glucosamine**—After incubation the flasks were removed, and the diaphragms were quickly separated from the medium and rinsed three times. The combined medium and rinses were made up to 20 ml., and aliquots were immediately pipetted into test-tubes containing the reagents for reducing sugar or glucosamine determinations. The controls with tissue incubated without substrate gave negligible values for reducing sugar or glucosamine. Reducing sugars were determined by the method of Miller and Van Slyke (2) and glucosamine by a modification of the Elson and Morgan colorimetric method (1).

When both glucose and glucosamine were present in the flasks, the glucose was determined after separation on a cation exchange column. In these experiments, 1 ml. of the medium was transferred immediately after incubation to Duolite C-3 columns (8 X 70 mm.) regenerated to the acid form with HCl. Glucose was washed out with water at a rate of approximately 2 ml. per minute until 25 ml. were collected. Glucose recoveries by this method were between 96 and 100 per cent. Control glucose determinations were run for added glucose and column glucose recovery with each experiment.

**Results**

As shown in Table I, d-glucosamine disappears from the medium when incubated with rat diaphragms, and this disappearance is accelerated on addition of insulin. The amino sugar uptake by the diaphragms was measured by difference in the quantity of sugar present at zero time and at the end of 2 hours of incubation. Determinations made by both a reducing method and the glucosamine color reaction of Elson and Morgan showed a close agreement between the two methods.

It was found that in the eviscerated rabbit d-glucosamine retarded the entry of glucose into the cell. This blocking action of glucose by glucosamine has also been demonstrated in the isolated diaphragm (Table II). At a glucose-glucosamine concentration of 0.01 to 0.005 M, respectively, glucose uptake was reduced to about one-half the value obtained in the absence of glucosamine.
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### Table I

*Effect of Insulin on Glucosamine and Glucose Disappearance by Rat Diaphragm*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Sugar disappearance, mg. per gm. wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without insulin</td>
</tr>
<tr>
<td>1</td>
<td>d-Glucosamine</td>
<td>0.801</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>1.54</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>1.97</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>0.963</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>1.30</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>2.09</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>1.67</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>2.10</td>
</tr>
<tr>
<td>9</td>
<td>d-Glucose</td>
<td>4.42</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>3.42</td>
</tr>
<tr>
<td>11</td>
<td>&quot;</td>
<td>4.47</td>
</tr>
</tbody>
</table>

Two flasks were used in each experiment, containing paired hemidiaphragms from two rats (trimmed to approximately 350 mg of wet weight). The final sugar concentration was 0.01 M, and insulin, when used, was 0.7 unit per flask. The flasks were incubated at 37° for 2 hours with O₂ in the gas phase.

### Table II

*Effect of Glucosamine on Glucose Uptake by Rat Diaphragm*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glucose disappearance, mg. per gm. wet tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No glucosamine</td>
</tr>
<tr>
<td>1</td>
<td>4.30</td>
</tr>
<tr>
<td>2</td>
<td>5.03</td>
</tr>
<tr>
<td>3</td>
<td>5.10</td>
</tr>
<tr>
<td>4</td>
<td>4.19</td>
</tr>
</tbody>
</table>

The two flasks in each experiment contained paired hemidiaphragms from two rats (trimmed to approximately 350 mg of wet weight) suspended in 3 ml of medium consisting of Krebs-Ringer-phosphate buffer, pH 7.4, and the substrates which were at a final concentration of 0.01 M for glucose, 0.005 M for glucosamine, and 0.7 unit of crystalline zinc insulin per flask. The flasks were incubated at 37° for 2 hours with O₂ in the gas phase. Glucose was determined after removal of glucosamine by passage through cation exchange columns.

This reduction of glucose uptake in the presence of glucosamine is reflected in the amount of glucose converted to CO₂. Using a constant concentration of C¹⁴ uniformly labeled glucose in the presence of increasing concentrations of d-glucosamine, we found that glucose oxidation by rat
diaphragms was decreased significantly (Fig. 1). This apparent inhibition of glucose oxidation is not due to ammonia formation or the increased chloride ion concentration, for ammonium chloride had relatively little effect on glucose oxidation by this system. Fructose was also tested as an inhibitor for glucose oxidation, but its effect is not as great, especially at lower concentrations, as that of glucosamine. It is noteworthy that, at a

![Graph](http://www.jbc.org/)

**Fig. 1.** Effect of added substances on glucose oxidation by rat diaphragm. For each experiment, the diaphragms from six rats were divided nearly equally among the six flasks. The weights of diaphragms in each vessel were adjusted to 350 ± 10 mg. Each flask contained radioactive uniformly labeled glucose (0.01 M) (specific activity 18,767 c.p.m. at infinite thickness as BaCO₃), 0.7 unit of insulin (except where noted), as well as the designated amounts of d-glucosamine hydrochloride, NH₄Cl, or d-fructose. Incubations were carried out at 37° for 2 hours with oxygen in the gas phase. Activity was calculated as (counts per minute of BaCO₃ at infinite thickness × micromoles of carbon)/(gm. of wet tissue).

glucose to glucosamine ratio of 2:1, the extent of inhibition is about the same for glucose uptake (Table II) and glucose oxidation (Fig. 1). The action of glucosamine in reducing the conversion of glucose to CO₂ in the absence of insulin is identical with its action in the presence of insulin, except for the correspondingly lower rates of glucose oxidation. The effect of preincubating glucosamine with the diaphragms for 10 minutes, then adding the radiocarbon glucose, resulted in respiratory CO₂ values that were indistinguishable from those in experiments in which both substrates were present from the start.
DISCUSSION

In these studies with the isolated rat diaphragm, glucosamine has been shown to behave in a manner similar to that reported for eviscerated rabbits (1). Glucosamine disappears from the incubation media, and this disappearance is definitely enhanced in the presence of insulin. The uptake of glucosamine was determined both by sugar reduction and by the glucosamine colorimetric method. The close agreement between the two methods indicates the simultaneous disappearance of the amino group and the aldehyde group of glucosamine and not merely the masking of the amino group or its removal by deamination.

Mackler and Guest (3) have reported evidence for the utilization of fructose by the rat diaphragm that is independent of the insulin-controlled glucose-utilizing system. The straight line oxidation curve obtained (Fig. 1) when fructose at various concentrations was superimposed on radioactive glucose shows that fructose was competing with glucose oxidation only to a small degree.

On the other hand, glucosamine was found to compete vigorously with glucose, especially at low concentrations of glucosamine. The site of this competition with glucose can occur at any of the glycolytic enzymes that act on both of these sugars. Harpur and Quastel (4) as well as other workers (5-7) have demonstrated the phosphorylation of glucosamine by hexokinase and adenosine triphosphate. Hoare and Kerly (6) have reported that retinal extracts could not metabolize glucosamine beyond the glucosamine-6-phosphate stage, while glucose or fructose could be metabolized further, and Brown (8) has shown that the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate is catalyzed by purified rabbit muscle phosphoglucomutase.

The fact that glucosamine not only depresses glucose oxidation, but also lowers glucose uptake and can respond to insulin, can be interpreted as a competitive action between glucosamine and glucose for entry into the cell, the mechanism suggested (9, 10) as the site of insulin action.

SUMMARY

1. Insulin increases the disappearance of glucosamine when incubated with rat diaphragm.
2. Glucosamine inhibits the uptake of glucose by the rat diaphragm.
3. Glucose oxidation is decreased more by glucosamine than by fructose or ammonium chloride when these are incubated with rat diaphragm.

These results suggest that glucosamine competes with glucose at the site of insulin action.
GLUCOSAMINE AND INSULIN

BIBLIOGRAPHY

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