The Effect of Trivalent Chromium on Galactose Entry in Rat Epididymal Fat Tissue

WALTER MERTZ* AND EDWARD F. ROGINSKI*

From the Section on Experimental Liver Diseases, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland, and the Division of Biochemistry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.

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Trivalent chromium, recently identified as the active ingredient of the glucose tolerance factor, appears to be a dietary agent required for maintenance of normal glucose tolerance in the rat. The element, applied in the form of certain coordination complexes, prevents impairment of removal rates of intravenously injected glucose in rats on rations deficient in glucose tolerance factor, and it cures the fully developed defect overnight with one dose of approximately 20 μg per 100 g of body weight administered by stomach tube (1). In epididymal fat tissue of glucose tolerance factor-deficient rats, 0.001 to 0.1 μg of chromium per 2 ml of medium and approximately 100 mg of tissue in vitro increase glucose uptake and incorporation of glucose carbon into fat by up to 94% (2). The presence of insulin was found to be an indispensable requirement for this effect. Response to chromium supplementation was obtained with 0.1 and 1.0 milliunit per flask. Furthermore, in experiments studying the incorporation of carbon 14 into lipids, the greatest effect of chromium was observed with glucose as substrate and in the presence of insulin. The effect diminished when acetate-C14 was used in the presence of unlabeled glucose and insulin, and it became insignificant when either glucose or insulin was omitted from the system. These observations suggested a site of action of chromium close to that of insulin.

The experiments reported here were designed to measure the effect of the element of the entry mechanism for sugar into the cell, and by the use of this system under well controlled chromium-free conditions, to determine the possible interdependence of insulin and chromium in their effect on entry rates. D-Galactose was used as a substrate; this sugar is poorly metabolized by peripheral tissue but its entry is regulated by insulin (3).

EXPERIMENTAL PROCEDURE

Methods—Since chromium is effective in very small amounts, exceedingly great care must be taken to avoid introduction of GTF-active chromium into diet, glassware, reagents, etc.

Animals—Male Sprague-Dawley rats from the National Institutes of Health colony and male rats from the Walter Reed inbred strain were raised after weaning in wire mesh cages. Only healthy, normal-appearing animals weighing from 250 to 300 g were used after being deprived of food for 18 hours.

Diets—For the basic experiments measuring glucose and galactose metabolism by fat tissue and for those comparing two methods for the estimation of extracellular space, rats on Purina laboratory chow were used. A 30% Torula yeast diet served as basal ration (4). This diet is of low glucose tolerance factor content and leads to impaired glucose removal rates in rats after several weeks (5). McCollum's wheat-casein ration was fed as control diet of optimal glucose tolerance factor content (5). Animals on this ration were supplemented with kale twice weekly.

Glucose and Containers—Polyethylene containers were used for storage of solutions and water. Glassware was washed in a mild detergent by ultrasonic vibrations (Disintegator, Ultrasonic Industries, Albertson, New York), rinsed, and treated with dilute hydrochloric acid. Flasks used for incubation were discarded after 8 to 10 uses.

Reagents—D-Galactose (“substantially glucose-free”) (Sigma Chemical Company) and d-galactose-1-C14 (Volk Radio-Chemical Company) were the substrates. The latter yielded one sharply defined peak of radioactivity on chromatography in butanol-acetic acid. Absence of fermentable sugars was demonstrated by incubation with bakers' yeast. Only 0.01% of the added label was recovered in the CO2, as compared to 11.3% for glucose-C14 in a control experiment with the same yeast. Various samples of Zn-insulin were obtained from Eli Lilly and Company. Dilutions in water were made daily from a stock solution prepared once every month. No protein was added to the solutions. The incubation medium was prepared daily from distilled, deionized water and reagents from Merck and Company, Inc., Rahway, New Jersey. Chromalum, Cr3 (SO4)3·K2SO4·24H2O (Baker Analytical Reagent Grade), was dissolved fresh daily, as described (2). The desired amount was added to the flasks in 0.05 ml of water.

Preparation of Tissue—The animals were killed by stunning and decapitation. The epididymal fat pads were quickly excised in toto, transferred to flasks containing 6 ml of incubation medium without galactose, and incubated for 30 minutes at 37°. At the end of this period, two pieces were cut from the distal end of each pad and immediately transferred to flasks containing 2 ml of warm medium, according to a randomization table. After

* Present address, Division of Biochemistry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.

1 The abbreviation used is: GTF, glucose tolerance factor.

2 This ration had been used since 1956 for the production of GTF deficiency. The use was discontinued because since late 1960 an increasing percentage of rats did not develop the deficiency symptom anymore. It appears possible that the diet has been improved with regard to GTF content.

3 The authors are indebted to Drs. Otto Behrens and Robert L. Mann.
addition of the tissue, the supplement of insulin and, after 1 minute of shaking, that of chromium was added, each in 0.05 ml of water. Incubation was carried out in oxygenated phosphate medium at pH 7.4 as described by Robinson (6), containing 1 mg of galactose per ml to which a sufficient amount of D-galactose-1-C\textsuperscript{14} had been added. The flasks were shaken in a Dubnoff apparatus for the desired period of time.

**Extraction of Galactose**—At the end of the incubation, the tissue was quickly removed, washed with ice-cold 0.9% solution of sodium chloride, blotted on filter paper, and weighed. It was then submersed for 15 seconds in ice-cold medium containing nonlabeled galactose, and transferred to graduated test tubes containing 2 ml of water. After 15 minutes of boiling, the volume was readjusted to 2 ml where necessary, and an aliquot (0.4 ml) was plated out for counting and dried under a heating lamp at a temperature not exceeding 100°. The samples were counted to at least 2000 counts on a thin window gas flow counter. Under these conditions no appreciable self-absorption was detectable. The extracted tissue residue contained insignificant amounts of radioactivity, as shown by subsequent homogenization and extraction.

**Determination of Total Water Content**—One piece of fat tissue from each animal was incubated for the experimental period, after which it was blotted and weighed. It was then transferred to a tarred beaker and dried for 18 to 20 hours at 100°. The loss of weight was determined.

**Determination of Extracellular Tissue Space**—The tissue was incubated for 30 to 60 minutes in phosphate medium at pH 7.4 containing 500 and 1000 mg per 100 ml of inulin. The tissue inulin was extracted as described above for galactose and determined photometrically in the deproteinized extract (7). The values for "inulin space" detected with this method were found to be very close to those obtained when the tissue galactose concentration after 7\textfrac{1}{2} minutes of incubation was measured. The latter method was therefore routinely applied in each animal for the estimation of extracellular galactose concentration. On the basis of these values, intracellular galactose concentration was calculated.

**Determination of C\textsuperscript{14}O\textsubscript{2}**—Fresh, washed bakers' yeast or epididymal fat tissue was incubated in water and phosphate buffer, respectively, for 30 to 90 minutes. Incubation was carried out in 15-ml Warburg flasks, containing a rolled piece of filter paper in the center well, and closed with a tightly fitting rubber cap. At the end of the incubation, 0.2 ml of 5% LiOH was injected through the cap into the center well, and 0.2 ml of 10 N H\textsubscript{2}SO\textsubscript{4} was introduced into the main compartment. After 15 minutes of shaking the contents of the center well were quantitatively transferred to a calibrated test tube and made to volume with water. An aliquot was counted for radioactivity.

**RESULTS**

The very low degree of galactose metabolism by epididymal fat tissue is evident from Table I. Whereas 2.9% of the added label appeared in the CO\textsubscript{2} with glucose as substrate, only 0.05% was detected when galactose was used. Radioactivity in the total lipids was not significantly above background. Furthermore, on paper chromatography in n-butanol, acetic acid, and water (8), the water extract of the tissue, after incubation with galactose, yielded only one peak of radioactivity with an \( R_f \) value of 0.14. Although this differed from that of nonincubated galactose (\( R_f \), 0.17), it was almost identical with that of galactose applied to the paper together with a corresponding amount of water extract from fat tissue (\( R_f \), 0.15). From these observations, it follows that galactose is neither significantly metabolized nor altered by epididymal fat tissue during 30 to 60 minutes of incubation.

**Total and Extracellular Water**—In nine rats on the basal ration, with weights from 150 to 200 g and deprived of food for 18 hours, the water content of epididymal fat tissue was 20.8 \((-0.9)\% \) of the wet weight. This value represents the total space in which, under optimal conditions, galactose could distribute itself. Determination of the extracellular portion of water was done with the assumption that the space measured with inulin corresponds to that occupied by extracellular galactose. This space was determined in seven rats with 5 mg of inulin per ml, and in four, with 10 mg per ml. The results were 8 \((\pm 0.9)\% \) and 8 \((\pm 0.3)\% \) of the wet weight, respectively. Thus, in the epididymal fat tissue under the conditions used, approximately 60% of the total water is inaccessible to inulin and can be considered intracellular. These findings are in agreement with the time course of tissue galactose concentration, to be shown below. It was assumed on the basis of previous findings (9) that the inulin space does not change significantly under the influence of insulin.

With these assumptions and with the knowledge that galactose does not accumulate against a concentration gradient (9), a theoretical saturation of tissue water should be reached when 21 \(\mu g\) of galactose are present per 100 mg of tissue, when the medium contains 1 mg of the sugar per ml. Of these, approximately 8 \(\mu g\) should be extracellular, i.e., this amount can be expected to appear in the tissue quite rapidly. Table II shows that in four rats the extracellular space was already saturated after 7\textfrac{1}{2} minutes. The amount of tissue galactose extracted at this time was only slightly above the theoretical value of 8 \(\mu g\) per 100 mg of tissue.

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Uniformly labeled glucose-C\textsuperscript{14}</th>
<th>Galactose-1-C\textsuperscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input(^a)</td>
<td>160,000</td>
<td>150,000</td>
</tr>
<tr>
<td>Recovered in CO\textsubscript{2}(^c)</td>
<td>4900 ± 1400</td>
<td>78 ± 19</td>
</tr>
</tbody>
</table>

\(^a\) Incubation in phosphate medium, at 38°, for 60 minutes, with 1 mg per ml of sugar.

\(^b\) Initial counts per minute per flask.

\(^c\) Counts per minute per 100 mg of tissue (mean ± standard error).

### Table II

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Tissue galactose</th>
<th>Saturation of extracellular space</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>(\mu g/100 \text{mg tissue} )</td>
<td>%</td>
</tr>
<tr>
<td>2.5</td>
<td>5.3 ± 0.4</td>
<td>66</td>
</tr>
<tr>
<td>5.0</td>
<td>6.7 ± 0.4</td>
<td>84</td>
</tr>
<tr>
<td>7.5</td>
<td>8.7 ± 0.8</td>
<td>109</td>
</tr>
<tr>
<td>10.0</td>
<td>8.6 ± 0.7</td>
<td>107</td>
</tr>
</tbody>
</table>

\(^a\) \( N = 4 \).
can enter into the cell without added insulin (Fig. 1). The ex-

-time course of entry of \( \beta \)-galactose in epididymal fat tissue. Unsupplemented controls, \( \cdots \); 1 milliunit of insulin per flask, \( \cdots \); 1 milliunit of insulin and 0.01 \( \mu \)g of chromium per flask, \( \Delta \)-\( \Delta \). Total and extracellular water, galactose concentrations without supplements, with insulin, and with insulin plus chromium were determined in each rat. Tissue from 10 rats was incubated for 10 minutes, from 10 other rats for 15 minutes, from 12 for 30 minutes, and from 10 for 45 minutes. (United States Army photograph.)

**Table III**

<table>
<thead>
<tr>
<th>Insulin</th>
<th>No. of rats</th>
<th>Intracellular galactose concentration*</th>
<th>mg/ml cell water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Insulin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg/ml cell water</td>
<td>mg/ml cell water</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>0.37 ± 0.07</td>
<td>0.21 ± 0.11</td>
</tr>
<tr>
<td>1.0</td>
<td>12</td>
<td>0.39 ± 0.08</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>10.0</td>
<td>30</td>
<td>0.60 ± 0.07</td>
<td>0.77 ± 0.05</td>
</tr>
<tr>
<td>10.0</td>
<td>15*</td>
<td>0.36 ± 0.03</td>
<td>0.64 ± 0.07</td>
</tr>
</tbody>
</table>

*Chromium, 0.01 \( \mu \)g per flask.  
*In incubation for 30 minutes; each rat served as its own control.  
*Mean ± standard error.

Because of the possibility that an insulin effect may have been masked by high nonspecific entry, the data of animals with low nonspecific entry are shown separately.

**Figure 1**

Time course of entry of \( \beta \)-galactose entry measured after 15 minutes of incubation. This period could be calculated: control, 1.46; with insulin, 3.7; with chromium and insulin, 0.8 mg of galactose per ml of cell water per hour. If this calculation was made from the 7.2, 10, and 15 minute values only, the resulting rates were 1.9, 3.5, and 13.2 mg of galactose per ml of cell water per hour, respectively. It appears from these data that the main effect of chromium takes place during the early phase of the incubation and that the element does not influence the final galactose space.

**Responses of GTF-deficient Tissue to Insulin and Chromium**

The effect of very high insulin doses (0.27 unit per ml) on galactose entry has been established (9). In the present studies, more nearly physiological concentrations of the hormone were used, during a 30-minute incubation period. The results (Table III) demonstrate that in GTF-deficient animals at least 1 milliunit of insulin is required to produce a significant effect. One-tenth of this dose, although found quite effective in other systems, did not elicit a significant response. That the rather small increase of intracellular galactose concentrations with 10 milliunits of insulin from 0.6 to 0.77 mg per ml was not due to the high rate of unspecific entry in those animals is shown by singling out those rats with low entry rates of the unsupplemented tissue. In those, the effect of 10 milliunits of insulin is not much greater than that of 1 milliunit. Supplementation with 0.01 \( \mu \)g of chromium resulted in a significantly higher galactose entry on each insulin level tested. In agreement with previous studies measuring glucose uptake (2), the effect of chromium was best observed with low concentrations of insulin.

**Response of Adipose Tissue to Varying Doses of Chromium**

The effect of chromium with and without insulin on galactose entry was measured after 15 minutes of incubation. This period had been chosen because it was found optimal for the demonstration of the chromium effect. In the absence of added insulin, chromium was ineffective (Table IV). With 1 milliunit of the hormone added per flask, the greatest chromium effect was observed with 0.01 \( \mu \)g per flask. With 10 and 100 times this dose, the effect diminished. This shape of the dose-response curve has been established for various systems although the optimal dose may vary with the various conditions of the experiment.

**Dependence of Tissue Response to Insulin on Dietary Conditions**

The results reported above were obtained with rats on a GTF-deficient diet, which, in intact rats, leads to an impairment of intravenous glucose tolerance (5). In Table V, experiments with GTF-deficient rats are compared with those performed on animals on a ration of sufficient GTF content. In the former, 0.1 milliunit of insulin was effective in
the rate of entry. It did not further increase the rate of entry.

Different. Insulin was now more effective by itself, with 3 out
obtained with GTF-sufficient rats on a complete diet were quite
significant in the present experiment; however, similar results
have been obtained in other tests measuring glucose uptake.

The content of GTF-active chromium in diets and other
biological materials cannot yet be determined by chemical
methods because such methods measure the total amount of the
element present in materials and give no information about that
part of the total which is GTF-active. On the other hand,
results obtained with GTF-sufficient rats on a complete diet were quite
different. Insulin was now more effective by itself, with 3 out
of 5 and 4 out of 5 rats responding to 0.1 and 1.0 milliunit,
respectively. Addition of chromium was no longer required and
it did not further increase the rate of entry.

**DISCUSSION**

It has been demonstrated in previous studies that GTF-active,
trivalent chromium increases impaired intravenous glucose
tolerance and low glucose uptake of epididymal fat tissue of
GTF-deficient rats (1, 2). The results presented here show that
small amounts of the element also increase significantly
the rate of entry of a nonutilizable sugar, n-galactose, into the
cell. Thus, the site of the chromium effect appears to be at the
entry mechanism of sugar into the cell. This finding does not
necessarily exclude other possible sites of action of chromium
in metabolism. Several such possible sites are known (10-12);
however, they appear to be of no importance in our systems
because of their much higher and rather unspecific chromium
requirements.

Previous experiments with intact rats have shown that this is
also true for the response of the whole animal to its endogenous
insulin. Under nondeficient conditions, for example in rats on a
natural diet, the response of tissue to the hormone is greater,
and chromium does not further increase it. The element does
not exert a pharmacodynamic action within the dose range used
in these experiments.

The content of GTF-active chromium in diets and other
biological materials cannot yet be determined by chemical
methods because such methods measure the total amount of the
element present in materials and give no information about that
part of the total which is GTF-active. On the other hand,
results obtained with such methods show that the
effective dose levels used in the present studies are well within
the biological range (13). When this range is exceeded with
high concentrations the effect is not only lost but a depression
of activity may be observed. Such modes of response have been
demonstrated for several elements in widely different systems
(14).

The mode of interaction between insulin and chromium is not
known. The data presented above show a close relation between
the two factors. Insulin must be present for the demonstration
of a chromium effect and, vice versa, to obtain an effect of small
concentrations of insulin in GTF-deficient animals, chromium is
required. The main effect of the element is very clear during
the first 15 minutes of the incubation when insulin has not yet
produced a significant effect. From these observations, the role
of chromium as an insulinase inhibitor appears unlikely. Such
a role would become apparent only during the later part of the

### Table IV

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Chromium</th>
<th>No. of rats</th>
<th>Intracellular galactose concentration</th>
<th>Increase due to chromium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Insulin</td>
</tr>
<tr>
<td>mg galactose/mi cell water&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.01</td>
<td>5</td>
<td>0.36 ± 0.06</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>1.0</td>
<td>0.01</td>
<td>5</td>
<td>0.14 ± 0.08</td>
<td>0.28 ± 0.47 ± 0.12</td>
</tr>
<tr>
<td>1.0</td>
<td>0.01</td>
<td>12</td>
<td>0.21 ± 0.28</td>
<td>0.57 ± 0.12 ± 0.04</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1</td>
<td>5</td>
<td>0.27 ± 0.06</td>
<td>0.46 ± 0.14 ± 0.02</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>5</td>
<td>0.07 ± 0.11</td>
<td>0.13 ± 0.45 ± 0.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> Incubation for 15 minutes after 30-minute preincubation.

<sup>b</sup> Mean ± standard error.

### Table V

<table>
<thead>
<tr>
<th>Ration</th>
<th>Insulin</th>
<th>No. of rats</th>
<th>Intracellular galactose concentration</th>
<th>Increase due to insulin</th>
<th>No. of rats responding to insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mg galactose/mi cell water&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Torula yeast diet, GTF-deficient</td>
<td>0.1</td>
<td>10</td>
<td>0.37 ± 0.07</td>
<td>−0.15 ± 0.07</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>12</td>
<td>0.39 ± 0.08</td>
<td>+0.2 ± 0.06</td>
<td>6</td>
</tr>
<tr>
<td>McCollum's wheat-casein diet, GTF-sufficient</td>
<td>0.1</td>
<td>5</td>
<td>0.17 ± 0.1</td>
<td>(−0.08 ± 0.24)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5</td>
<td>0.21 ± 0.1</td>
<td>+0.45 ± 0.2</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Incubation for 30 minutes.

<sup>b</sup> Mean ± standard error.
experiment. Two other possible modes of action, that on "insulin binding" and the interaction of chromium with tissue-bound insulin, are now being investigated.

SUMMARY

Trivalent chromium, previously identified as the active ingredient of the glucose tolerance factor (GTF), has been shown to potentiate the effect of insulin on glucose uptake by fat tissue of GTF-deficient rats. In the present study, the effect of chromium (III) and insulin on the penetration of a nonmetabolizable sugar, D-galactose-1-C\(^{14}\), in epididymal fat tissue was investigated. Addition of 0.01 \(\mu g\) of chromium \textit{in vitro}, ineffective by itself, increased entry rates in the presence of 1 milliunit of insulin by a factor of 3.8 after 15 minutes of incubation. Supplementation with 0.1 milliunit of insulin per flask failed to elevate entry rates in GTF-deficient animals. In those, trace amounts of chromium (0.01 \(\mu g\)) were required for the demonstration of an insulin effect \textit{in vitro}. The described effects were observed in GTF-deficient animals only, in which the impaired function was restored close to the levels found in rats on the GTF-sufficient wheat-casein ration. These results locate the site of action of chromium (III) at the sugar transport mechanism. It appears that the element may be an essential cofactor for insulin.

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