The glucose tolerance factor is a dietary agent required in rats for maintenance of normal removal rates of glucose from the blood (2). Recently, we have identified trivalent chromium as the active ingredient (3). GTF is present in natural diets, for instance table scraps or McCollum's wheat-casein diet, whereas Torula yeast diets, casein-sucrose diets, and even commercial laboratory chows were found to be GTF-deficient (4). Trivalent chromium thus appears to be a physiological dietary agent, in very small amounts essential for normal utilization of glucose. Certain, but not all, trivalent chromium complexes prevent the development of the impaired glucose tolerance on GTF-deficient diets; they also cure the fully developed defect. The technique of the intravenous glucose tolerance test has been modified for in vitro studies of additions to the others was governed by a randomization procedure. The sequence of additions to the others was governed by a randomization procedure. The sequence of additions to the others was governed by a randomization procedure.

**EXPERIMENTAL PROCEDURE**

**Animals**—Male Sprague-Dawley rats of the National Institutes of Health colony were raised in individual, wide mesh wire cages after weaning. They had free access to food and water. The technique of the intravenous glucose tolerance test has been described (6). Animals for the studies in *vivo* were killed by decapitation after 18 hours of fasting, after having been on the diets for approximately 4 to 5 weeks. Rats showing indications of disease or a less than normal weight gain were discarded.

**Diets**—As basal diet, Purina laboratory chow for rats, mice, and hamsters (Ralston Purina Company) was fed ad libitum. This diet is GTF-deficient (4). For one experiment, the diet was supplemented with 1 mg of chromium, as hexaurea chromium(III)trichloride, per 100 g of ration.

**Chromium Compounds**—Hexaurea chromium(III)trichloride, (Cr[CONHCONH]Cl), 100 mg, was dissolved in 10 to 20 ml of water and slowly added to 1 kg of finely ground Purina laboratory chow under constant stirring. For most assays in *vivo*, chromate, chromium(III) potassium sulfate, “CrO4 SO4 · 2H2O,” (Baker Analyzed reagents) was used. Of this, 100 mg were dissolved in 4 ml of water. NaOH, 2 ml, 1 N, were added. The clear green solution was immediately diluted to 50 ml with water, and further diluted to contain the desired concentration of chromium in 0.05 ml. This was added directly to the medium.

**Experiments in *vivo***—Epididymal fat tissue was prepared according to Winegrad and Renold (7). Four pieces of tissue, weighing approximately 100 mg, were easily obtained from each rat. One of these served as a control. The sequence of additions to the others was governed by a randomization procedure.

**Uptake** was calculated from the difference of glucose concentrations between individual flasks and that of medium incubated without tissue. It is expressed as micrograms per 100 milligrams of tissue per hour. The results of a series of preliminary experiments, based on measurements of glucose uptake of 4 pieces of epididymal fat each from 10 rats, were analyzed statistically. It was calculated that a 25% increase of uptake over the controls becomes significant at the 5% level when 8 animals are used. To obtain this significance for a 50% increase, only 3 animals were required. Most results reported here were obtained with 10 rats or more.

**Radioisotope Experiments**—Uniformly labeled glucose-Cl4 (Volk Radiochemical Company) and sodium acetate-1-C14 (Tracerlab, Waltham, Massachusetts) were used. Samples were counted in a windowless flow counter; corrections for background and self-absorption were applied. The procedures for determining the incorporation of label into fat were those described by Winegrad and Renold (7).

* The authors are indebted to Dr. Joan M. Gurian for the statistical analysis.
RESULTS

The effect of dietary chromium supplementation to the GTF-deficient Purina ration is shown in Table I. A dose level of 1 mg of chromium per 100 g of diet, added as the hexaurea complex, led to significantly higher glucose removal rates in the intravenous glucose tolerance test of 10 supplemented rats as compared to 9 controls after 5 weeks on the ration. Body weights of the two groups did not significantly differ, in accordance with previous observations (4). When these rats were killed and glucose uptake by epididymal fat tissue was measured, no difference could be detected between the two groups in the absence of insulin. In the presence of 1 milliunit of insulin per flask, however, glucose uptake was found to be 67% higher in the chromium-supplemented group than in the controls. The incorporation of lesser amounts of chromium (100 and 500 μg/100 g) into the diet produced neither an effect on glucose tolerance nor on glucose uptake in vitro, but it is not known to what extent the hexaurea complex is really absorbable and biologically effective under these circumstances.

When trivalent chromium, as a neutralized solution of chrome alum, was added in vitro to epididymal fat tissue of rats on the unsupplemented Purina diet, a pronounced increase of glucose uptake was obtained with 0.1 μg of the element (Table II). For this level of chromium, as chrome alum, the insulin level optimal for the effect was determined. Without chromium, fat tissue shows a progressively rising glucose uptake with increases of insulin levels. In the preceding, dietary experiments, the element did not stimulate glucose uptake in the absence of insulin. With rising insulin concentrations, the chromium effect increased until an optimal insulin concentration of approximately 1 milliunit per flask was reached. With further increases of the hormone, the response to chromium diminished. At 10 milliunits of insulin, the tissue without chromium showed an uptake of 92 μg of glucose per hour, and no significant increment by supplementations of 0.1 μg of chromium is seen at this or higher insulin levels. It appears as if chromium, under these conditions, does not increase further an already elevated glucose uptake (see below).

With the dose of 1 milliunit of insulin per flask, the response to different levels of chromium was then studied (Table III). In animals with a basal uptake of less than 50 μg per 100 mg per hour, the dose response shows an optimum observed between 0.01 and 0.1 μg of chromium per flask. These levels are 10⁻⁷ and 10⁻⁸ M, respectively, but it must not be assumed that all the added chromium is present in a biologically active form. Glucose uptake was almost doubled. With lower, but also with higher doses, the effect diminished.

The effect of chromium supplementation depends clearly on the state of the animal. It is most pronounced in rats with impaired low glucose uptake by epididymal fat tissue. As could be expected from the results described above, the addition of chromium brings the incorporation of glucose into fat to a certain optimal level which cannot be exceeded by higher doses of the element. A certain percentage of our rats has still normal or near normal intravenous glucose removal rates when pretested for the curative GTF assay, i.e. after 5 to 6 weeks on the GTF-deficient basal diet. In these, application of GTF-active chromium complexes by stomach tube does not increase glucose tolerance further. Neither does it, when added in vitro, further increase the incorporation of glucose into the epididymal fat tissue if the latter is at its peak of activity. From our results with large numbers of experiments at an insulin level of 1 milliunit it is evident that stimulation of uptake by the chromium diminishes with increasing basal glucose uptake; usually, no great effect is observed when the latter exceeds 60 μg/100 mg of tissue per hour. These results seem fully consistent with the concept that GTF-active chromium is a normal dietary require-
ment. It appears that animals with high basal glucose uptake are not sufficiently depleted of GTF-active chromium.

In the intravenous glucose tolerance test in GTF-deficient rats it has been shown that different chromium complexes possess widely different degrees of biological activity. The compounds had been given by stomach tube (3). A similar pattern of relative potencies has evolved for the effect in vivo on glucose uptake by epididymal fat tissue (to be published separately). Compounds of high structural stability, such as the ethylenediamine complexes or chromium-acetyladamantane, were ineffective. Others showed varying degrees of biopotency depending on the ligands contained in the shell. Neutralized solutions of chrome alum, and the bis-biuanide hydroxoaquo chromium sulfate were potent. These findings demonstrate that not all trivalent chromium complexes are GTF-active. The same conclusion has been drawn from the results with intact animals. The determination of chromium in nutrients and other materials does not furnish information about their GTF contents. The latter can be measured only by bioassay.

The effect of 13 other elements on glucose uptake in vivo was tested. Three rats were used for each compound. The following salts were added at levels supplying 0.1 μg of element per flask: TiCl₃, VO(SO₄)₂.2 H₂O, MnSO₄·H₂O, FeSO₄, Co(C₃H₅O₂)₂·4 H₂O, Ni(C₃H₅O₂)₂·4 H₂O, Y(NO₃)₃·6 H₂O, Zr(SO₄)₂·4 H₂O, MoO₃, RhCl₃, PdCl₂·2.5 H₂O, CdSO₄. Of these, only manganese produced a significant effect in this system. In a group of four animals, a preliminary comparison of the activity of this element with that of chromium gave the results of Table IV.

DISCUSSION

Whereas it was previously shown that trivalent chromium is the active ingredient of the glucose tolerance factor, effective in the intact animal in reconstituting low intravenous glucose tolerance in deficient rats to normal, the present studies demonstrate a direct action of the element on glucose utilization by peripheral tissue, elicited in vitro. Although other potential sites for the GTF effect, for instance hepatic glucose deposition and release or superimposed endocrine systems, have not been ruled out, it appears that the effect in the periphery could account for the increase of glucose removal rates produced by the element in GTF-deficient, intact animals. In the latter, 20 μg of chromium per 100 g of body weight will bring a deficient increase of glucose tolerance in vivo (3)) as well as the enhancement of glucose uptake by fat tissue, described by others (7), is related to the fact that it gives rise to TPNH and DPNH, necessary for acetate assimilation, and possibly also that tricarbon intermediates of glucose breakdown are needed as precursors of glycerol during fat synthesis.

**TABLE IV**

*Effect of chromium supplementation in vitro on incorporation of carbon¹⁴ into fat*

<table>
<thead>
<tr>
<th>Substrate and supplements¹</th>
<th>Tissue</th>
<th>Incorporation</th>
<th>Increase due to Cr(III)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With Cr(III)b</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>c.p.m./100 mg</td>
<td></td>
<td>μM C¹⁴/100 mg/hr³</td>
</tr>
<tr>
<td>Glucose-C¹⁴, insulin</td>
<td>618</td>
<td>1,080</td>
<td>0.061 ± 0.004</td>
</tr>
<tr>
<td>Acetate-1-C¹⁴, glucose, insulin</td>
<td>7,860</td>
<td>11,850</td>
<td>0.078 ± 0.02</td>
</tr>
<tr>
<td>Acetate-1-C¹⁴, glucose</td>
<td>6,300</td>
<td>7,820</td>
<td>0.032 ± 0.005</td>
</tr>
<tr>
<td>Acetate-1-C¹⁴, β-hydroxybutyrate</td>
<td>1,890</td>
<td>2,280</td>
<td>0.019 ± 0.004</td>
</tr>
<tr>
<td>Acetate-1-C¹⁴, insulin</td>
<td>3,300</td>
<td>3,500</td>
<td>0.033 ± 0.016</td>
</tr>
</tbody>
</table>

¹ Incubation was carried out in 2 ml of medium, with the following concentrations per flask: uniformly labeled glucose C¹⁴, 11 μM; acetate-1-C¹⁴, 60 μM; β-hydroxybutyrate, 22 μM; insulin, 1 milliunit.

² Cr(III), 0.1 μg, as chrome alum.

³ Mean of 5 experiments ± standard error.
was significantly increased by those two elements at concentrations of around 10 × 10⁻⁴ M. This effect did not depend on the presence of insulin, and the doses per amount of tissue were 260 to 520 times those effective in our system (12). Recently, von Brand identified manganese as the active ingredient of a heat-stable factor from liver which increases incorporation of acetate-C¹⁴ into fat by a soluble enzyme system from pigeon liver (13). These phenomena may be due to the fact that manganese is a cofactor in a number of enzymatic reactions involved in acetate utilization for fat synthesis, or in auxiliary mechanisms.

The effective dose levels of chromium are physiological, i.e. they are within the limits of chromium contents normally found in tissues or nutrients. Many older data in the literature reporting "zero" chromium contents in organs or other materials of biological origin are misleading since they were obtained with methods too insensitive to assay the amounts physiologically present. From the above mentioned dose levels, no conclusion can be drawn as to the minimal amount of chromium effective in this system. The form of trivalent chromium present at the site of action is unknown. Chrome alum, for example, containing the element as the hexaaquo complex, [Cr(H₂O)₆]³⁺⁺⁺, undergoes a number of reactions when dissolved and partially neutralized in water (14). Some of the water molecules of the inner shell are hydrolyzed, which gives rise to hydroxo-aquo complexes; others are substituted for by migration of the SO₄⁻ into the inner shell of the complex. Also, hydroxo groups secondarily form polynuclear complexes byolation, i.e. by formation of hydroxo bridges between several chromium atoms. The three processes, hydrolysis, migration of anions, and olation occur simultaneously with varying speeds. It is impossible to determine which of the resulting compounds are biologically active. Similarly, it is likely that chromium compounds added to the medium form at varying speeds a variety of complexes with components of the latter, which may be of varying biopotency. These relationships remain to be clarified elsewhere. It is obvious, however, that only certain chromium complexes are GTF-active, whereas many are ineffective in vivo and in vitro. This means that only the bioassay, and not a chromium determination, can give information about the GTF activity of compounds as well as natural materials. A similar situation exists with regard to selenium and Factor 3 against necrotic liver degeneration (15).

The effects of chromium reported in this publication have been obtained with GTF-deficient animals. Experiments with rats raised on other rations will be reported separately. The demonstration of the action of chromium is limited to experimental conditions which produce relatively low basal uptake of glucose. The greatest effects are obtained at basal uptakes below 60 μg of glucose per 100 mg of tissue an hour. Even highly potent chromium complexes increase uptake only to a certain, normal level and not beyond. This ceiling of the chromium effect cannot be overcome by higher doses of the element. If uptake is spontaneously high, or if high doses of insulin are applied, addition of chromium is without influence. Whether this is due to an elimination of the need for chromium, or to a more effective utilization of the small traces of chromium which are invariably present, cannot be determined at this time. The present data have been obtained with Krebs-Ringer phosphate medium. The basal uptake was found to be greatly affected by the concentration of CO₂. Experiments with bicarbonate buffer will be reported separately.

The action of chromium in the in vitro system shows a distinct optimum. With levels higher than 0.1 μg of element per flask, as neutralized chromium, the effect diminishes. Similar observations were made measuring the effect of stomach-tubed GTF on intravenous glucose tolerance in intact rats (4). These results may possibly suggest a regulatory function of the element within the physiological range of glucose utilization, but this conclusion must await clarification of the mechanisms through which the organism handles and regulates its trivalent chromium supply. An analogy may exist between substrate inhibition in enzyme systems and the inhibition by higher than optimal chromium levels. In systems showing substrate inhibition, the substrate is bound to the enzyme at two or more sites of the molecule at the moment of enzyme action. Excess substrate diminishes the chance that both binding sides are found free by a substrate molecule. Chromium, at the site of action, also may be bound by more than one of its six coordinate valences.

The available data give no indication as to the site of action of chromium in metabolism, but they show that the chromium effect depends on the presence of insulin. It is possible, vice versa, that insulin depends for its action on the presence of very small amounts of chromium. Chromium is most effective in the presence of glucose as substrate; when acetate is used, the effect is much less pronounced. This may suggest that the active site of chromium is close to that of insulin. Whether an exact stoichiometric relation exists between insulin and chromium for optimal activity or for stimulation of glucose uptake in general cannot be deduced from the data on hand, particularly since it is not possible to define what part of the applied levels of chromium is in a biologically effective form and is entering actively into the system.

SUMMARY

The effect of trivalent chromium, previously identified as the active ingredient of the glucose tolerance factor (GTF), on glucose uptake by rat epididymal fat tissue was investigated.

Supplementation of a GTF-deficient diet with chromium, as hexaaqua chromium(III)trichloride, resulting in a significant increase of intravenous glucose tolerance in the intact animal, produced a 67% higher glucose uptake by fat tissue in vitro as compared to tissue from unsupplemented controls.

Addition in vitro of suitable chromium complexes enhanced glucose uptake by fat tissue from GTF-deficient rats. The effect of chromium was found dependent on the presence of small amounts of insulin. With 1 milliunit of insulin, the response to trivalent chromium, added as a neutralized solution of chrome alum, showed an optimum at 0.01 to 0.1 μg of element per flask, which yielded increases of 88% and 94%, respectively. Of 13 other elements tested, only manganese stimulated glucose uptake of the system, but to a lesser degree.

Similarly, with uniformly labeled glucose, 0.1 μg of chromium enhanced the incorporation of glucose carbon into fat by 76% in the presence of 1 milliunit of insulin. A lesser effect was observed with acetate as substrate.

REFERENCES

Effect of Trivalent Chromium Complexes on Glucose Uptake by Epididymal Fat Tissue of Rats
Walter Mertz, Edward E. Roginski and Klaus Schwarz