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An Improved Assay for Biologically Active Chromium

Richard A. Anderson,* John H. Brantner, and Marilyn M. Polansky

Biologically active forms of chromium were measured by determining their insulin potentiation on glucose oxidation by isolated fat cells. Adipocytes were isolated from the epididymal fat tissue of rats maintained on a Cr deficient diet. All natural and synthetic Cr compounds tested that yield real biological activity in previous assay systems were active in our assay. Biologically active organic Cr complexes potentiated the action of insulin more than 300% in this bioassay but inorganic Cr complexes yielded little insulin potentiating activity. Nicotinic acid, which interferes in the assay with fat tissue (Roginski, 1974) gave only a slight positive response in the fat cell assay. Cells isolated from two rats can be used for approximately 150 assays and large numbers of control samples are not needed because individual animal variation within an assay is virtually eliminated. This assay is more sensitive and more reproducible and can be used routinely for more samples than any of the previous assay techniques for biologically active chromium.

The biological function of chromium, an essential trace element for man and animals, is closely associated with that of insulin. Systems that respond specifically to chromium also respond to insulin and the interaction between chromium and insulin is postulated to occur at the cell membrane of insulin sensitive tissue (Mertz, 1969). Chromium metabolism differs from that of other trace elements in its strict dependence on the chemical form in which the element is present. Natural organic chromium complexes isolated from Brewer's yeast or other natural products differ from simple inorganic chromium compounds in intestinal absorption, tissue distribution, access to special chromium compartments, and in placental transport (Mertz, 1969). Therefore, total chromium is not a meaningful indicator of the chromium nutriture of animals or their diets. In this respect chromium requirements are similar to those of cobalt because total cobalt content means very little but it is the cobalt-containing vitamin B_{12} that is important. Likewise, the biologically active form of Cr is the important indicator of Cr nutriture, and once a rapid, accurate, reproducible assay method for biologically active chromium is developed, nutritional data for Cr may be expressed in units of biologically active Cr rather than total Cr (Anderson and Mertz, 1977).

Present assays for chromium are tedious and require a large number of animals for assay of a small number of samples, and the results cannot be compared among laboratories. We have developed an assay that is sensitive, accurate, and reproducible and can be used to assay routinely a large number of samples using very few animals, e.g., 75 assays using the fat cells from one rat. A preliminary report of this work has been presented (Brantner and Anderson, 1978).

MATERIALS AND METHODS

Animals and Diet. Weanling male Hilltop Sprague Dawley rats (Charles River, Wis.) were raised in plastic cages using glass water bottles and drinking tubes. Drinking water was purified by a Milli-Q Reagent-Grade Water System (Millipore Corp., Bedford, Mass.). The rats were fed ad libitum a Cr-deficient Torula yeast-based diet (WR No. 6, Teklad, Madison, Wis.) containing less than 40 ng of chromium/g of diet.

Preparation of Isolated Adipocytes. Fat cells from rats (160–180 g) raised on the above low-Cr diet were isolated by a modification of the method of Kahn et al. (1977) based on the Rodbell procedure (Rodbell, 1964). Plastic containers were used exclusively for fat cell isolation and assay. Two rats were sacrificed by decapitation and their epididymal fat pads removed. The distal portion of the fat pads (about 2 g) was rinsed with 0.9% NaCl, minced with scissors, and incubated at 37 °C for 40 min in 6 mL of Krebs Ringer Phosphate (KRP) buffer containing 12 mg of collagenase (CLS 47B177P, Worthington, Freehold, Mass.) in a water bath shaker at 200 rpm. The KRP contained 118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, and 16.2 mM Na_2HPO_4–HCl (pH 7.4). The digested tissue was passed through a silk screen (Adcom, Bethesda, Md.) using a 10-mL syringe. The preparation was then washed three times by centrifugation with KRP containing 2% albumin (Fraction V, Lot No. R53808, Armour Pharmaceutical Co., Kankakee, Ill.). Both KRP and KRP–albumin were millipore filtered and gassed with O_2. The material below the floating fat cells and any fat above the adipocytes were removed by aspiration. KRP–albumin (10 mL) was added to disperse the washed fat cells. Fats cells remained viable for at least 4 h.

Glucose Oxidation Studies. Insulin and the indicated amount of sample to be tested were added to tubes that contained 1.9 mL of KRP–albumin, 0.4 μCi [U-^{14}C]glucose (313 Ci/mol), and 67.5 μg of dextrose. Adipocytes (0.06 mL) were added, and caps containing center wells (Kontes) were used to seal the tubes. After incubation for 2 h at 37 °C and shaking at 150 rpm, 0.2 mL of hyamine hydroxide (10×) (Packard) was added to the center well, and 0.3 mL of 2 N H_2SO_4 was injected into the incubation mixture to stop the reaction. The tubes were incubated for 30 min to allow the hyamine to trap $^{14}CO_2$. The center wells were removed, carefully wiped, and added to 10 mL of Aquasol II (New England Nuclear, Mass.) and counted in a Beckman Liquid Scintillation Counter.

Biologically Active Cr Complexes. Synthetic Cr complexes were synthesized from Cr, nicotinic acid, and glutathione or Cr, nicotinic acid, and glycine and were partially purified by Dowex and high-pressure liquid chromatography (Toepfer et al., 1977; Anderson et al., 1978a). Natural chromium complexes were from an ammonia extract of Brewer's yeast (Anderson et al., 1978b). Sulfhydryl content of the chromium-containing extracts was measured by the method of Tietze (1969). Chromium content of low-temperature ashed natural and synthetic chromium complexes was determined using a continuum source, eschelle, wavelength modulated atomic absorption spectrometer equipped with a Perkin Elmer HGA 2100

U.S. Department of Agriculture, Science and Education Administration, Nutrition Institute, Agricultural Research Center, Beltsville, Maryland 20705.

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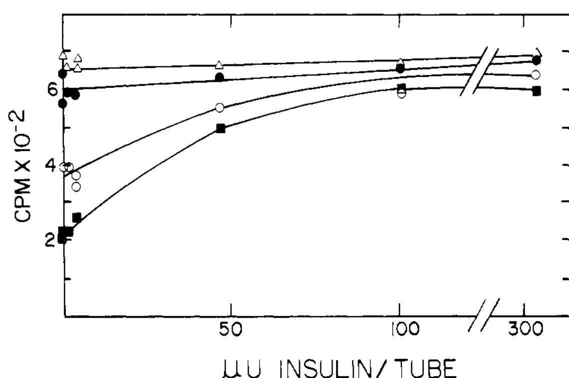

Figure 1. Effect of yeast extract on insulin stimulation of glucose oxidation by fat cells. Cells were suspended in KRP–albumin, pH 7.4, 37 °C. Closed squares (■) represent insulin dose response of glucose oxidation by fat cells, (○) designates levels of insulin + 0.088 mg of yeast extract containing 0.006 ng of Cr; (●) designates levels of insulin + 0.88 mg of yeast extract containing 0.06 ng Cr; (△) designates levels of insulin + 1.76 mg of yeast extract containing 0.12 ng of Cr. All data points are an average of at least four separate determinations.

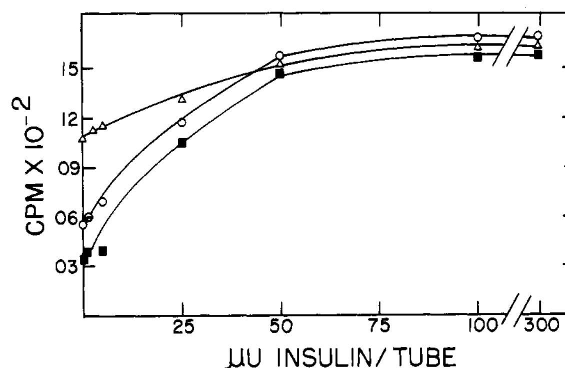

Figure 2. Effect of synthetic Cr complex on insulin stimulation of glucose oxidation by fat cells. Same conditions as in Figure 1. Synthetic Cr complex was prepared from Cr, nicotinic, and glutathione and purified by Dowex and high-pressure liquid chromatography: (■) insulin alone; (○) insulin + 75 μg of complex containing 1 μg of chromium; (△) insulin + 750 μg of complex containing 10 μg of Cr. All data points are an average of at least four separate determinations.

graphite furnace (Zander et al., 1977).

RESULTS

The potentiation of glucose oxidation by isolated fat cells from chromium deficient rats in response to a yeast extract is shown in Figure 1. Increasing amounts of the yeast extract, which is high in biologically active Cr (Toepfer et al., 1973), increased glucose oxidation by the fat cells. At low levels of added insulin, the Cr-containing extract potentiated insulin action more than three times, but at saturating levels, addition of the chromium extract had minimal effect. Insulin potentiation is calculated by determining the ratio of the radioactive CO_2 released at a fixed insulin level with and without added Cr complexes. For example, in Figure 1 there were approximately 200 cpm of CO_2 released at low levels of insulin alone; when 0.88 mg of yeast extract containing 0.06 ng of Cr was added, 600 cpm of CO_2 was released from radioactive glucose. Therefore, insulin potentiation equals 3. The amount of naturally occurring chromium required to potentiate insulin was very low, and even at 0.006 ng/mL of incubation, there was a significant potentiation of insulin. At higher levels of added yeast extract, containing 0.06 and 0.12 ng of Cr, there appeared to be a maximal CO_2 production with only the endogenous insulin present in the assay components, and no additional glucose oxidation was observed upon addition of exogenous insulin. Some preparations appeared to stimulate the oxidation of glucose, even at saturating levels of insulin, but this response varied and yeast extract usually had little effect at high levels of exogenous insulin. Extracts from liver, muscle, diets, and other products that are postulated to contain biologically active chromium (Toepfer et al., 1973) also gave a positive response (data not shown) in our assay system.

Synthetic organic chromium complexes also potentiate the action of insulin (Toepfer et al., 1977; Anderson et al., 1978a). Therefore, we tested such complexes in our assay system. The insulin potentiating activity of a synthetic mixture derived from Cr, nicotinic acid, and glutathione is illustrated in Figure 2. Counts per minute were higher in Figure 2 than in Figure 1 because the number of cells used was greater. The absolute counts are not important but the ratio between of counts observed in the presence and absence of added complexes reflects insulin potentiation. The overall response with the synthetic complex was similar to that of the yeast extract but much higher levels of chromium were required to elicit a given response with this partially purified sample. The effects of individual components of the biologically active synthetic complex were also tested. Chromium chloride and chromium acetate did not elicit any significant response (Table I, lines 2 and 3) and higher levels tended to inhibit the assay. Nicotinic acid which has a significant positive response in the in vitro fat tissue assay (Roginski, 1974) had a minimal effect in our assay (Table I, line 4). Glutathione slightly stimulated glucose oxidation; therefore, we routinely assay for free sulfhydryls (Tietze, 1969) to rule out nonspecific effects (Table I, line 5). The presence of the individual components of our synthetic complex, e.g., chromium, nicotinic acid, and glutathione assayed collectively, did not yield any greater response than glutathione alone (Table I, lines 5–9). We have also synthesized other organic chromium complexes that do not contain glutathione that potentiate the action of insulin

Table I. Biological Activity of Synthetic Cr Complexes and Their Components

sample[a]	insulin potentiation[b]
1. control (no additions)	1.0
2. control + $CrCl_3$	0.96 ± 0.05
3. control + $Cr(C_2H_3O_2)_3$	0.9 ± 0.1
4. control + nicotinic acid	1.06 ± 0.05
5. control + glutathione	1.2 ± 0.14
6. control + glycine	1.1 ± 0.05
7. control + $CrCl_3$ + nicotinic acid	1.1 ± 0.1
8. control + $CrCl_3$ + glutathione	1.3 ± 0.14
9. control + $CrCl_3$ + glutathione + nicotinic acid	1.28 ± 0.11
10. control + Cr, nicotinic acid, glutathione complex	3.18 ± 0.23*
11. control + Cr, nicotinic acid, glycine complex	2.76 ± 0.15*

[a] One hundred micrograms of all samples were added. For more than one addition, 100 μg of each was added. [b] Potentiation of insulin activity when 2 μ units of insulin were added to the fat cell bioassay. Insulin potentiation was calculated by dividing the radiactive CO_2 released in the presence of insulin plus component added by that released in response to insulin alone, e.g., an insulin potentiation of 1 indicates that the component tested had no measurable effect on insulin action. All numbers are mean ± standard deviation of four or more separate determinations. (*) Significantly greater than lines 1–9 at $P < 0.001$.

on fat cells, e.g., Cr–nicotinic acid–glycine complexes (Table I, line 11). Therefore, the insulin potentiating activity of the compounds tested cannot be attributed to sulfhydryl groups.

Using the fat cell assay, a number of samples can be assayed simultaneously and we routinely assay 75 to 95 samples per run with usually less than 10% deviation between duplicate samples. We have assayed the same yeast extract periodically as an internal standard, and over a 3-month period the activity of this preparation has been 2.93 ± 0.63.

DISCUSSION

The isolation and characterization of biologically active forms of Cr have been hampered by the lack of a rapid, accurate assay. The most widely used assay which employs rat epididymal tissue (Mertz and Roginski, 1971) is reasonably specific for biologically active Cr but requires a large number of animals for assay of a relatively small number of samples. For example, six rats are usually used to assay only two or three samples. That assay is acceptable for a very low volume of samples but is not suited for assay of multiple samples such as column fractions, etc. An in vivo assay involving injection of samples into rats and determining changes in blood sugar is also used (Tuman et al., 1978) but again can be used only for a very small number of samples. The effect of chromium on yeast fermentation has been suggested as a bioassay for biologically active chromium (Burkeholder and Mertz, 1967) but to our knowledge has not been used as a routine assay. A microbiological assay (Gutierrez et al., 1974) has the potential to be used for a number of samples but lacks specificity and has not been tested for routine use.

The biologically active chromium compounds employed in this study were not pure since a pure biologically active chromium complex, whether natural or synthetic, has not been isolated. Therefore, some of the activity of the yeast extract (Figure 1) may be due to natural substances, other than chromium. However, the assay also responds to synthetic organic chromium complexes and thus (Figure 2) must measure, at least in part, a biologically active form(s) of chromium. The natural substances in a yeast extract would not be present in the partially purified synthetic complexes, and also the components used in the synthesis do not potentiate insulin activity significantly in the fat cell bioassay (Table I). The oxidation of glucose by fat cells can be stimulated by a number of factors (Rodbell, 1964; Czeck, 1977); therefore care must be exercised when using this assay to be sure that biologically active Cr is measured.

Our fat cell assay is a modification of the fat tissue bioassay (Mertz and Roginski, 1971). Chromium-deficient animals were used because their insulin response to chromium is enhanced (Mertz et al., 1961). Our assay system has several advantages over the present bioassay methods: (1) Individual animal variation within a given assay is virtually eliminated since all fat cells become part of a homogeneous pool; therefore a control does not need to be run for each animal. (2) A large number of assays can be run simultaneously using very few animals, e.g., other bioassays require one animal per sample but the fat cell bioassay requires one animal per 75 samples. (3) Nicotinic acid does not interfere significantly with the fat cell bioassay (Table I) but may interfere with the fat tissue (Roginski, 1974) and whole animal bioassays (Tuman et al., 1978; Bjortorp, 1966). However, the fat tissue assay system does not respond significantly to sulfhydryl compounds but high levels of glutathione may interfere with the fat cell assay system. (4) The fat cell bioassay system is more sensitive than the previous assay methods, e.g., more than 1600 μ units of insulin are required for maximal insulin response in the fat tissue system (Mertz, 1969) while only 50–100 μ units are necessary to achieve maximal insulin potentiation using individual fat cells (Figure 1). Corresponding increases in response to Cr were also observed.

The sensitivity, reproducibility and speed with which a large number of samples can be assayed indicate that this assay has the potential to be used to characterize large numbers of food samples, tissue extracts, column fractions, etc. and hopefully to be used as a clinical assay to determine the nutritional status of man by measuring the biologically active chromium in addition to the total chromium in urine. Toward this end, studies are currently in progress.

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