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Is glucose tolerance factor an artifact produced by acid hydrolysis of low-molecular-weight chromium-binding substance?

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Abstract—A low-molecular-weight chromium-binding substance (LMWCr) has been isolated and purified from porcine kidney and porcine kidney powder and characterized. The oligopeptide is biologically active, has a molecular weight of *ca* 1500 daltons, and is comprised of cysteine, glycine, glutamate, aspartate, and Cr^{III}. Porcine kidney LMWCr is also susceptible to hydrolysis, especially under the conditions for the acid hydrolysis used in the purification of glucose tolerance factor (GTF). Combined with some similarities in the composition of GTF and LMWCr, these results suggest GTF may be an artifact resulting from hydrolysis of porcine LMWCr. © 1997 Elsevier Science Ltd

Keywords: low-molecular-weight chromium-binding substance; chromium; glucose tolerance factor; phosphatase; porcine; kidney.

For the last four decades, a material named glucose tolerance factor (GTF) has dominated the field of chromium biochemistry [1,2]. In 1957, this material from an acid hydrolyzate of porcine kidney powder was found to reverse glucose intolerance in rats fed a Torula yeast diet and proposed to be a new and novel dietary agent [3]. Subsequently, this diet was found to be Cr-deficient; and GTF was found to possess Cr^{3+}, which proved to be the active component [4]. Porcine GTF in *in vitro* assays using rat epididymal fat pads potentiated the effects of insulin [5]. Chromium has since been established to be essential for proper carbohydrate and lipid metabolism in mammals, with deficiency resulting in symptoms comparable to adult-onset diabetes and coronary artery disease [6–8]. However, a material from Brewer's yeast was also found to be active in the fat pad assays and to possess nearly identical properties to porcine kidney powder GTF [8]. The ability to readily obtain significantly larger quantities of the yeast GTF led to abandonment of studies on the porcine material (at least published ones) with one exception after 1960, and the abbreviation GTF is currently used in reference solely to the material from yeast. The yeast GTF has been found to be a low molecular weight molecule which possesses an ultraviolet absorbance maximum at *ca* 260 nm and is comprised of Cr^{3+}, glycine, glutamate, a sulfur-containing amino acid, and nicotinic acid [9]. The presence of glutathione has been proposed, but the tripeptide has never been obtained from GTF preparations. (The isolation procedure of yeast GTF also involves an acid hydrolysis, which uses refluxing 5N HCl for 18 h [9].) However, the presence of nicotinic acid has been seriously questioned [10]. Recent kinetics analyses have suggested that GTF serves only as a readily absorbable form of Cr, possessing no intrinsic function in potentiation of insulin action [11].

Low-molecular-weight chromium-binding substance (LMWCr) is a naturally-occurring oligopeptide of *ca* 1500 daltons, is comprised solely of cysteine, glutamate and aspartate (and perhaps their amines), and glycine, binds four chromic ions in a multinuclear assembly, and is biologically active [12–14]. (Biological activity refers to the ability to potentiate the action of insulin on the conversion of glucose into carbon dioxide or lipid by isolated rat adipocytes or epididymal fat pads [15,16].) LMWCr is a mammalian material, isolated and purified to date from rabbit liver [12], bovine liver [13], bovine colostrum [14], and partially from dog liver [17]. In contrast to GTF, LMWCr has an intrinsic role in insulin potent-

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iation after the binding of insulin to the external surface of the insulin receptor [11]. LMWCr's biological function has recently been shown to be stimulation of insulin receptor protein tyrosine kinase activity, after insulin receptor has been activated by insulin; LMWCr binds to the activated receptor with a dissociation constant of *ca* 250 picomolar resulting in approximately an eightfold increase in insulin-stimulated receptor kinase activity [18,19]. LMWCr activates a membrane-associated phosphotyrosine phosphatase, perhaps in response to insulin [20].

The similar composition of LMWCr and GTF (i.e., both contain glycine, glutamate, and probably cysteine and Cr^{III}) and similar ultraviolet spectra of both species (LMWCr also possesses a maximum at *ca* 260 nm) [12,13] suggest that the two species might be related. As GTF purification involves an acid hydrolysis and is significantly smaller in size than LMWCr [9], GTF could be an artifact resulting from hydrolysis of LMWCr. To test this possibility, this laboratory has isolated LMWCr from the original source of GTF, porcine kidney powder, and examined the properties of the Cr-containing product from hydrolyzing the oligopeptide.

MATERIALS AND METHODS

Porcine kidney powder was obtained from Sigma Chemical Co,; porcine kidney was obtained from a local proprietor. Doubly deionized water was used throughout; all operations in the purification of LMWCr were performed at *ca* 4°C. The purification followed the procedure for bovine liver LMWCr [13]. Amino acid analyses were performed by Commonwealth Biotechnologies, Inc., Richmond, VA. Phosphotyrosine protein phosphatase (PTP) activation assays were performed as previously described [19,21] using *p*-nitrophenylphosphate (*p*-NPP) as substrate. The assay used 5 mM substrate in 0.05 M Tris, pH 7.5. Phosphate ester hydrolyses were allowed to proceed 1 h at 37°C; the extent of hydrolysis was determined at 404 nm ($\varepsilon = 1.78 \times 10^4\ M^{-1}\ cm^{-1}$). Fat cells from male Sprague Dawley rats were isolated by modifications of the procedure of Rodbell [22]. Three rats (not kept on a Cr-deficient diet) were sacrificed by decapitation and their epididymal fat pads removed. Subsequent operations followed Anderson *et al.* [6] with minor modifications except 2% bovine serum albumin media was changed to 1% bovine serum albumin and solutions were not degassed with dioxygen. Isolated rat adipocytes were washed with 1% serum albumin, 50 mM Hepes, pH 7.4 buffer containing 10 mg/mL leupeptin and 5 mg/ml aprotinin. Cells were homogenized with a manual Teflon homogenizer and frozen and thawed five times. The lipid layer was removed, and the cell homogenate was centrifuged for 1 h at 40,000 g. The supernatant was removed, and the pellet was suspended in Hepes buffer and used as a source of membrane phosphotyrosine phosphatase activity. Membrane protein concentrations were determined using the BCA method (Pierce Chemical Co.) with BSA as standard. Cr concentrations were determined using the diphenylcarbizide method [23] utilizing the method of standard addition to minimize potential matrix effects; oligopeptide, by the fluorescamine method [24], unless otherwise noted; and nicotinic acid, by the method of Vilter *et al.* [25]. Analyses and kinetic measurements were performed in triplicate. A Hewlett-Packard 8451A spectrophotometer was used to obtain ultraviolet-visible spectra. Fluorescence measurements were collected on a Perkin–Elmer 204 Fluorescence Spectrophotometer. For HPLC, Shodex OH PAK B-803 (size exclusion) and B-800P columns (Shoko, Co., Ltd.) were utilized; the mobile phase was 0.1 M NaCl. The absorbance of the effluent was monitored at 207 nm.

RESULTS

Purification of porcine kidney and porcine kidney powder LMWCr

This laboratory has previously shown that sufficient quantities of LMWCr can be isolated from bovine liver to allow the oligopeptide to be characterized by spectroscopic and magnetic techniques [13]. This procedure was extended to porcine kidney and porcine kidney powder to determine if appreciable quantities of LMWCr were contained in these materials. For the porcine kidney LMWCr, 6.5 kg of tissue was diced into *ca* one inch cubes. LMWCr was extracted from 1 kg aliquots suspended in 1 L of water containing 3.4 mmol potassium dichromate and the protease inhibitors benzamide (3 mM) and phenylmethylsulfonyl fluoride (0.5 mM). As LMWCr is stored primarily as the apoprotein (i.e., metal-free form), this results in loading the material with chromium. This is necessary as the biological activity assay does not allow for monitoring of LMWCr during the isolation procedure; the assay is sensitive to a variety of substances in both positive and negative fashions. Thus, LMWCr is followed through the purification procedure by assaying of chromium content. Subsequent steps followed the established protocol [12,13] which involves centrifugation and successive ethanol precipitations followed by chromatography on two DEAE-cellulose columns, a G-25 Sephadex column, and two G-15 Sephadex columns (Table 1).

The resulting chromium-containing fractions were concentrated by ultrafiltration (Amicon 8010 and/or 8400 using YC05 membranes) to give a gray–green solution. For the kidney powder material (Table 2), 300 g of porcine kidney powder was extracted with 2000 mL of water containing 0.510 mmol potassium dichromate. This mixture was centrifuged at 11,000 g; the supernatant was treated through ethanol precipitation and subsequent steps in the same manner as the porcine kidney extract. For both the kidney

Table 1. Purification of LMWCr from 6.5 kg of porcine kidney

Purification Step	mg Cr	mg protein[a]	mg Cr/mg protein
Centrifuged homogenate	10.86	4917	0.00220
DEAE #2	0.762	147.9	0.00520
G-25	0.338	5.59	0.00600
G-15 #2	0.127	2.64	0.481

[a] Determined using BCA assay (Pierce Chemical Co.), which varies in sensitivity as a function of protein size and composition; numbers should be considered qualitative.

Table 2. Purification of LMWCr from 300 g of porcine kidney powder

Purification Step	mg Cr	mg protein[a]	mg Cr/mg protein
Centrifuged homogenate	2.830	1931	0.0015
DEAE #2	0.190	53.46	0.0036
G-25	0.060	19.93	0.0030
G-15 #2	0.012	0.77	0.0156

[a] Determined using BCA assay (Pierce Chemical Co.), which varies in sensitivity as a function of protein size and composition; numbers should be considered qualitative.

and kidney powder LMWCr, chromium and protein comigrate from the second G-15 column indicating a higher degree of purity (Fig. 1). The purity was checked by Shodex OH PAK HPLC; only two features were visible in the chromatogram: LMWCr and a hydrolysis product of LMWCr (*vide infra*). The yield was 2.6 mg and 0.8 mg of porcine kidney LMWCr and porcine kidney powder LMWCr, respectively.

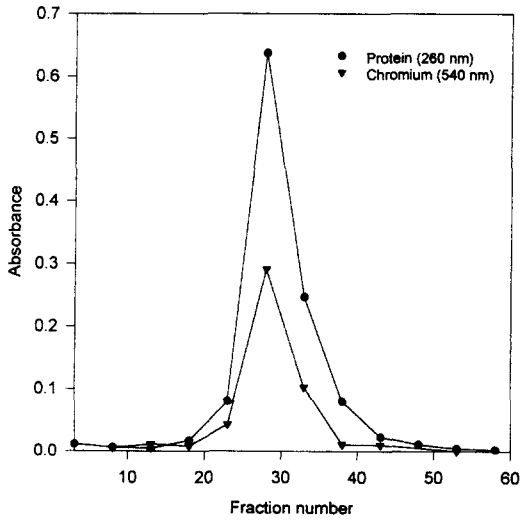

Fig. 1. Chromatography of porcine kidney powder LMWCr: gray–green chromium-containing fractions from first Sephadex G-15 column on second Sephadex G-15 column with absorption at 260 nm (circles) and absorption of chromium assay (triangles).

Characterization of porcine LMWCr

The porcine kidney and kidney powder LMWCr have chromium: oligopeptide ratios of 1.4 and 0.45, respectively. These values are well below the four chromium per oligopeptide found for the bovine liver [13] and rabbit liver [12]. A minimum of four chromium have been shown to be essential for maximal biological activity [18,19]. These data suggest that porcine LMWCr loses Cr during the isolating or the Cr-loading procedure is not completely successful. However, a loss of chromium with time is observed with this material (as with LMWCr isolated from other sources) suggesting the former is probably correct.

On G-15 Sephadex and G-25 Sephadex size exclusion chromatography and Shodex OH-pak HPLC, the porcine materials were found to elute in an identical volume as the bovine material, indicating the materials are almost identical in size and suggesting a molecular weight for the porcine LMWCr's of approximately 1500 daltons. However, for the porcine materials, a smaller molecular weight species also appears in the chromatograms; the species contains organic materials and small amounts of chromium. This species has previously been identified as a hydrolysis product of LMWCr [13] which appears over a period of days to weeks for the bovine liver LMWCr. Unfortunately, the species appears much more rapidly (hours to days) for the porcine materials, suggesting they are much more susceptible to hydrolysis complications than the bovine material. The rapid degradation of porcine LMWCr is probably respon-

sible for the small chromium : protein ratios. This also correlates well with previous reports that the rabbit liver LMWCr loses biological activity with time and that activity is directly related to Cr content [26]. This hydrolysis product turns out to be a potent inhibitor of the activation of rat adipocyte phosphotyrosine phosphatase activity; the bovine LMWCr hydrolysis product also appears to inhibit conversion of glucose into carbon dioxide and lipid by rat adipocytes (C. M. Davis and J. B. Vincent, unpublished work).

Amino acid analysis reveals that porcine LMWCr is comprised of 0.622 cysteine, 2.31 aspartate/asparagine, 4.05 glutamate/glutamine, and 1.45 glycine residues, assuming a molecular weight of 1200 for the organic portion of the isolated oligopeptide. Other amino acids are present only in insignificant amounts. This is quite close to the composition determined for bovine liver LMWCr (approximately 2 cys: 2 asp: 4 glu: 2 gly) [13], especially given that the hydrolysis complications with the porcine material could result in errors in the analysis. Porcine LMWCr's possessed no detectable nicotinic acid.

Concentrated solutions of porcine kidney and kidney powder LMWCr possess a distinct gray–green color. Their visible spectra display three distinct features: a shoulder at *ca* 260 nm and two maxima in the visible region (Fig. 2). These spectra are strikingly similar to that of bovine liver LMWCr, the only LMWCr previously isolated in sufficient quantity for meaningful visible spectra to be acquired. The two bands in the visible region are characteristic of Cr^{III} in a pseudo-octahedral environment. (This is consistent with the assays used to determine the chromium content of the LMWCr's which requires the addition of peroxide, indicating that Cr^{+3} and not a higher valent form of chromium is present [23].) From the visible maximum at 572 nm, an average value for the ligand field splitting, 10 Dq, can be calculated to be 17.5×10^3 cm^{-1}, typical of Cr^{3+} in an octahedral field of six oxygen ligands and similar to that found for the bovine liver LMWCr (17.4×10^3 cm^{-1} [13]).

Biological activity

The ability of LMWCr to activate the phosphotyrosine protein phosphatase activity of rat adipocyte membranes has been shown to be a measure of the biological activity of LMWCr [19], for example, the addition of 12.5 mM bovine liver LMWCr to rat adipocyte membranes results in a 100% increase in PTP activity [20]. Titration of apoLMWCr with chromic ions indicates that this activation is maximal with four chromic ions per oligopeptide; additionally, transition metal ions commonly associated with biological systems are unable to functionally replace chromium

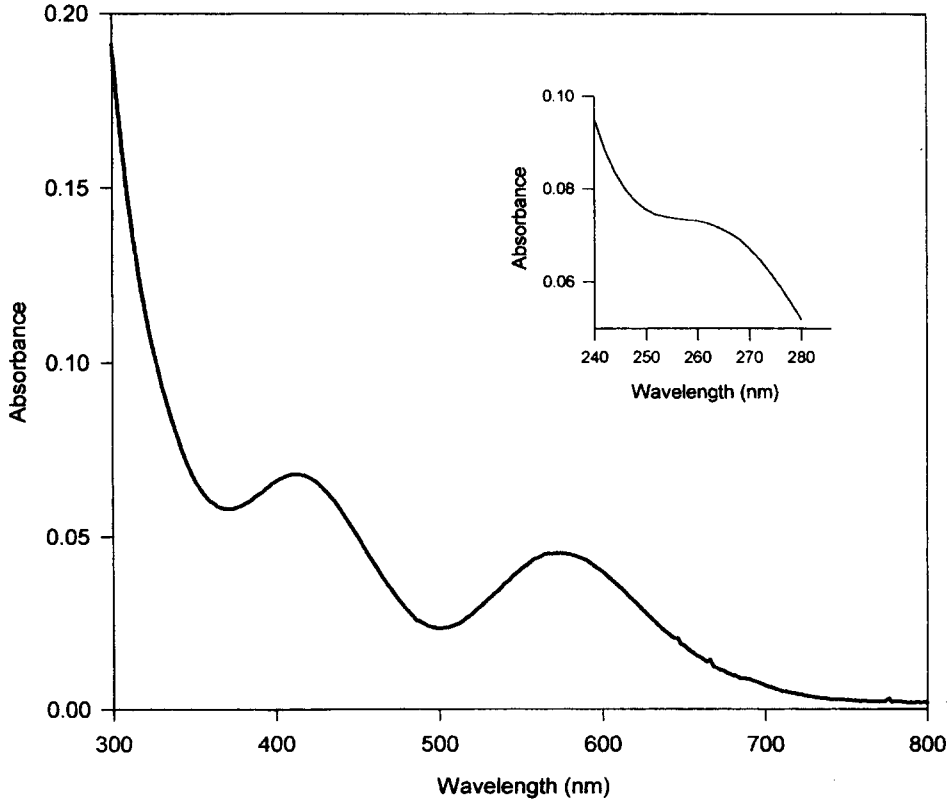

Fig. 2. Visible spectrum of porcine kidney LMWCr in 0.05 M NH_4OAc, pH 6.5. [Cr] = 0.41 mM. Inset: Ultraviolet spectrum of porcine kidney LMWCr in 0.05 M NH_4OAc, pH 6.5. [Cr] = 41 mM.

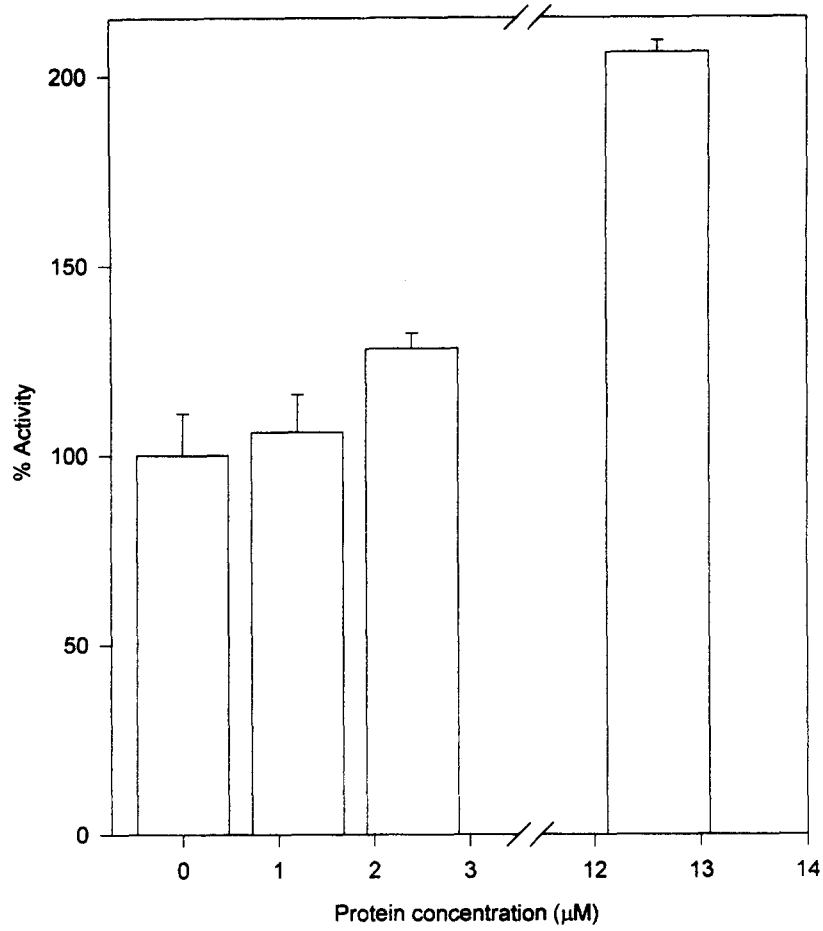

Fig. 3. Activation of rat adipocyte membrane PTP activity by porcine kidney LMWCr.

[20]. Similar behavior is seen in the activation of insulin receptor tyrosine protein kinase activity [18]. The addition of freshly purified porcine LMWCr and 4 equiv. of Cr^{3+} per oligopeptide to rat adipocyte membranes also results in activation of PTP activity, such that 12.5 mM porcine LMWCr results in circa 100% activation of the phosphatase activity (Fig. 3). (Addition of Cr was required as the porcine materials were isolated with less than a full complement of 4 Cr per oligopeptide). Cr^{3+} by itself has no effect on the membrane PTP assay [20]. Thus, the composition, spectroscopic properties, and activity of the porcine materials suggest that they are identical or nearly so to the previously reported liver LMWCr's [12,13].

It is important to note that freshly isolated porcine LMWCr rapidly loses the activation potential with time; use of the isolated hydrolysis product of porcine LMWCr reveals that it is an inhibitor of the membrane PTP activity.

Acid hydrolysis

While the conditions used previously for the acid hydrolysis of porcine kidney powder have not been reported, Brewer's yeast extract (removed from activated charcoal by 1 : 1 mixture of diethyl ether and concentrated ammonia) was refluxed for 18 h in 5 M HCl [9]. Thus, porcine LMWCr was subjected to acid hydrolysis by refluxing in 5 M HCl for 18 h. The resulting product was subjected to G-15 Sephadex size exclusion chromatography; the largest molecular species observed was the hydrolysis product described above. Thus, the conditions used previously in the purification and isolation of GTF result in complete destruction of LMWCr.

DISCUSSION

Relationship of LMWCr to GTF

The literature on the material originally termed 'glucose tolerance factor' is quite sparse. The phrase GTF was given to a material purified form acid hydrolyzate of dried, defatted porcine kidney powder. The material could be absorbed on charcoal and extracted with phenol and isobutanol, was soluble in water, and was not destroyed by wet-washing with HNO_3–H_2SO_4 in terms of its potency to restore glu-

cose tolerance. Concentrates of this GTF material were also shown to prevent the development of low glucose removal levels by rats fed a chromium deficient diet. This work has been reviewed [27]. Unfortunately, no attempts to determine the identity of the material from the kidney or kidney powder prior to acid hydrolysis have been reported. The properties given above for the porcine material are shared by an extract of Brewer's yeast which inherited the name glucose tolerance factor; in fact, the term GTF is currently synonymous with the yeast material. It has thus been assumed the porcine material is essentially identical to the yeast material. Given the lack of further characterization of the porcine material, a similar assumption is presumed in this discussion. Subsequently yeast GTF has been reported to be comprised of nicotinic acid, glycine, glutamic acid, and a sulfur-containing amino acid in addition to chromium and to display an ultraviolet absorption maxima at 262 nm [9]. However, the identification of nicotinic acid has been seriously questioned, and the presence of a tryptophan derivative has been proposed [10]. The entire organic composition has even been questioned [28]. Yet, synthetic mixtures of cysteine, glutamate, glycine, nicotinic acid and chromium seem to have very similar to nearly identical spectroscopic properties and biological activity to the isolated species from acid hydrolyzed Brewer's yeast extracts [9]. Curiously, the amino acid components of GTF are suggestive of the tripeptide glutathione, comprised of cysteine, glutamate, and glycine; consequently, the presence of glutathione has been suggested [2] but has never been detected in GTF preparations (nor has the relative amounts of the amino acids been reported). Recent analysis of the kinetic studies on the biological activity of GTF indicate that this yeast material serves only as a readily absorbable form of Cr and has no intrinsic function in mammals (in fact, it may inhibit insulin action in non-Cr-deficient cells [11]). The synthetic material, as it behaves in a very similar fashion to GTF in the assays, also appears to only serve as a readily absorbable form of Cr, with the same complications in non-Cr deficient cells.

LMWCr in many ways bears some striking similarity to the isolated yeast material (e.g. ultraviolet maximum at *ca* 260 nm, amino acid content—glycine, cysteine, and glutamate (but also aspartate), and biological activity). However, analysis of kinetic studies on the biological activity of LMWCr indicates that it has an intrinsic role in insulin-responsive cells [11]. These data suggest that LMWCr and GTF might be related, i.e. if LMWCr is naturally-occurring chromium-containing species in kidney prior to acid hydrolysis, then the lower molecular-weight species GTF with a similar composition to LMWCr could potentially be an artifact generated from LMWCr by this and other procedures in its isolation. The simplest manner to test this hypothesis seemed to be to attempt to isolate LMWCr from porcine kidney powder (or ultimately porcine kidney) to show that this species did exist and in reasonable quantities and then to examine if the material was sensitive to acid hydrolysis. The presence of LMWCr in porcine kidney seemed quite likely given the levels of a low-molecular-weight, chromium-containing species reported to occur in mouse kidney [29], work from this laboratory had shown that the LMWCr oligopeptide from bovine liver was susceptible to hydrolysis over periods of days or weeks, even in the absence of heat or acid [13]. It is also noted in the early 1960s that rat liver extracts (which have not been subjected to acid hydrolysis) have been shown to possess an agent that is not GTF but can increase glucose tolerance in Cr-deficient rats (i.e. possesses GTF-like activity); this material was water soluble and stable in a boiling water both for 2–3 min [30]. Given the high concentration of LMWCr in liver [29], that it is water soluble, and that it is stable to brief reflux [26], it would appear probable that LMWCr was the agent responsible for restoring glucose tolerance activity in chromium-deficient rats. In the only report on isolating Cr-containing complexes from porcine kidney powder after 1960 [26], one cationic Cr-containing species was separated and concentrated by ionic exchange chromatography (in the absence of a hydrolysis step). The material was extremely active in biological activity assays which used the rate of fermentation of chromium-deficient yeast; this assay reportedly yields results which parallel rat fat pad assays when using GTF as a Cr source [26]. However, since the latter assays can only indicate the ability of Cr to be absorbed and used and not any inherent biological activity unless thorough kinetics experiments are performed [11], the yeast assays can only indicate that porcine kidney powder possessed a form of chromium readily absorbed by yeast. Also the kidney powder was treated by stirring for 18 h in water, standing 2 days in water, and (after homogenization) standing for another 18 h before centrifugation to prepare the extract for further analysis. Given the instability of LMWCr from the porcine kidney powder, most of the LMWCr in the material would have been hydrolyzed before being analyzed by chromatographic techniques.

This work demonstrates that porcine kidney does indeed contain appreciable amounts of LMWCr, that porcine kidney LMWCr is very similar to LMWCr isolated from other sources, and that LMWCr is concentrated in the process to prepare porcine kidney powder (0.8 mg isolated from 300 g powder versus 2.6 mg from 6.5 g kidney). Also porcine LMWCr was found to be especially susceptible to hydrolysis; treatment of the purified chromium–oligopeptide with heat and acid similar to the treatment of Brewer's yeast extracts results in complete conversion of LMWCr into a lower molecular weight, chromium-containing species (which are inhibitory in the PTP activation assays). While the hydrolysis product is not GTF, it is quite conceivable that if the hydrolysis was performed on crude extracts rather than purified materials and the other steps in the isolation of GTF

were followed the product could be GTF or a GTF-like species. However, it is not the intention of this laboratory to track down the hydrolysis products of either yeast or porcine kidney extracts, as this field has a long history of contradictory and irreconcilable results [9,10,28,31–34] and the potential product, GTF, is not the intrinsic biologically active from of chromium. It should also be noted that the pioneering work in the late 1950s which identified GTF was designed and directed towards identifying a heat-stable, acid-stable vitamin or cofactor [27], these studies utilizing acid hydrolyses did indeed recognize that Cr was a necessary cofactor, while unfortunately probably by their very nature destroying any proteinaceous material associated with the Cr.

In conclusion, it appears very likely that for four decades research on chromium biochemistry has focused on an artifact o f acid hydrolysis, GTF, while LMWCr may be the naturally-occurring, biologically-active, chromium-containing species in mammals.

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