Marshall, W. H., Dutson, T. R., Carpenter, Z. L., Smith, G. C., *J. Food Sci.* **40**, 896 (1975).
McWatters, K. H., Cherry, J. P., Holmes, M. R., *J. Agric. Food Chem.* **24**, 517 (1976).
Prendergast, K., *Food Manuf.* **48**(4), 37–39, 57, April (1973).
Preston, F. B., private communication, 1976.
Puski, G., *Cereal Chem.* **52**, 654 (1975).
Sekul, A. A., Ory, R. L., *J. Am. Oil Chem. Soc.* **54**, 32 (1977).
Shiraishi, H., Okuda, K., Sato, Y., Yamaoka, M., Tuzimura, K., *Agric. Biol. Chem.* **37**, 2427 (1973).
Smith, K. J., *J. Am. Oil Chem. Soc.* **48**, 625 (1971).
Sosulki, F. W., McCleary, C. W., *J. Food Sci.* **37**, 253 (1972).
Wilding, M. D., *J. Am. Oil Chem.* **47**, 398 (1970).

Received for review August 15, 1977. Accepted March 7, 1978. Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

Factors Affecting the Retention and Extraction of Yeast Chromium

Richard A. Anderson,* Marilyn M. Polansky, Edward E. Roginski, and Walter Mertz

Retention of radioactive chromium chloride by Brewer's yeast, *Saccharomyces carlsbergensis*, was stimulated by glucose and phosphate. Glucose levels to 25%, increased the amount of chromium retained per gram of cells but high glucose also inhibited growth. Addition of phosphate enhanced Cr retention more than 2.5-fold over the unsupplemented controls. The rate and maximal incorporation of radioactively labeled inorganic chromium salts, which yield little biological activity, was similar to that of organic chromium complexes that display in vitro insulin potentiating activity. The release of the labeled Cr from the yeast cells was pH dependent and more than 85% of the labeled Cr could be extracted with dilute ammonium hydroxide. Disruption of cells with teichozyme-Y released a similar amount of labeled chromium but much lower amounts were extracted with ethanol. Insulin potentiating activity was greatest in the ammonia extract and was lower in ethanol and teichozyme extracts. Essentially all of the radioactive chromium incorporated into the yeast was present in the soluble portion of the cell. These data define conditions for the growth of Brewer's yeast for optimal incorporation of labeled Cr and the conditions for extraction of a biologically active γ-labeled product.

Brewer's yeast, the richest known source of an organic form of chromium, was used in the treatment of diabetes more than a century ago (Herepath, 1854). Several decades later, Glaser and Halpern (1929) described an insulin potentiating effect of yeast by demonstrating that incubation of insulin with a yeast extract potentiated the hypoglycemic action of insulin. McCay (1952) recommended a daily dietary supplement of Brewer's yeast for older people and suggested that yeast would lessen the daily insulin requirement. However, it was not until 1959 that the primary active component of yeast, effective in the treatment of diabetes, was postulated to be an organic form of chromium (Mertz and Schwarz, 1959).

Doisy et al. (1976) studied the effect of Brewer's yeast extract, high in insulin potentiating activity, on 14 subjects over the age of 65 with impaired glucose tolerance. After 1–2 months, glucose tolerance tests were normal for approximately half of the subjects. Liu et al. (1977) also reported that supplementation of the diet of 15 hyperglycemic women with a yeast extract resulted in an improved tolerance to glucose. In both studies release of endogenous insulin and fasting levels of serum cholesterol and triglycerides were reduced.

Little is known of the form or forms of Cr utilized by yeast or higher animals and man. We investigated the optimal conditions for the synthesis of a biologically active form of Cr by yeast and methods to extract this active product. Retention of inorganic Cr salts and biologically active synthetic organic Cr complexes by yeast was also studied.

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

MATERIALS AND METHODS

Brewer's yeast, *Saccharomyces carlsbergensis* (ATC No. 9080) was grown in 4-L Erlenmeyer flasks containing 2 L of purified synthetic medium (Toepfer and Polansky, 1970) or in medium containing 1% peptone, 0.03% phosphate, 10% glucose, pH 4.5. Chromium chloride, 500 μCi/L, was added prior to sterilization in an autoclave at 15 psi for 15 min. Cells were grown routinely at 26 °C, shaking at 125 rpm, in a New Brunswick Controlled Environment Incubator Shaker. Ten days after innoculation, cells were harvested by centrifuging at 10000g for 15 min and washed with 10 volumes of metal-free deionized water until the counts in the cells were constant.

Cells were extracted with ammonium hydroxide by adding 20 mL of 0.1 N NH_4OH to 14 g of wet cells, followed by shaking for 60 min at 175 rpm at 30 °C. Cells were then centrifuged at 15000g for 15 min and washed with water until the counts remaining in the cells were constant. The extract was then concentrated in vacuo and an aliquot was removed and assayed for insulin potentiating activity as described (Mertz and Roginski, 1971). Cells extracted with ethanol were treated similarly except 50% ethanol was added instead of dilute base and the cells were steamed in an autoclave for 5 min. Yeast cells were also treated with teichozyme-Y which was a generous gift from Dr. Norm Lin, Worthington Biochemical Corpora-

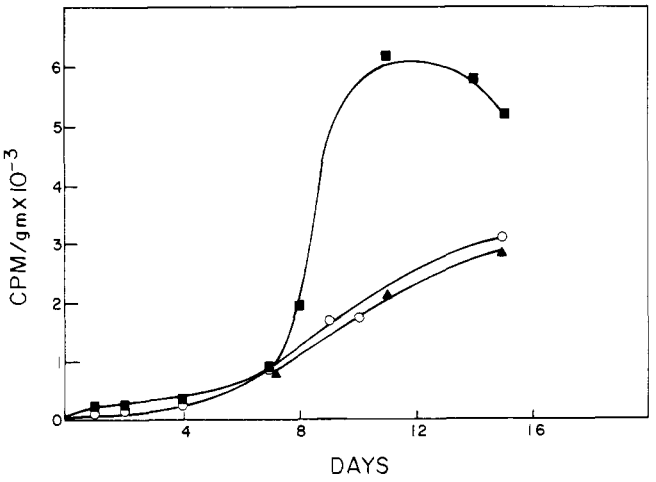

Figure 1. Retention of radioactive chromium by Brewer's yeast grown on purified synthetic medium. All samples contained 5% glucose and 60 μCi $CrCl_3$ in the initial medium: (■) control, no additional glucose added; (o) additional 5% glucose added 5 days after innoculation; (▲) additional 5% glucose added on days 5 and 9.

tion. Teichozyme-Y, 40 mg, was added to 14 g of yeast cells in 20 mL of 0.05 M potassium phosphate buffer, pH 7.15, and incubated with shaking for 12 h at 30 °C. Some batches of cells were then sonicated with a Heat Systems 350 Sonicator for 3 min at half maximal power with a 0.5-in. horn. Cells were then centrifuged and washed as described above.

To determine the cellular distribution of the labeled chromium incorporated into Brewer's yeast, cells were treated with ammonium hydroxide or with teichozyme-Y and centrifuged in a Dupont RC-5 Centrifuge in an SS-34 rotor at 600*g* for 10 min. The supernatant was centrifuged at 12 000*g* for 10 min with the same centrifuge and rotor. After each centrifugation, cells were washed three times with an equal volume of H_2O and the washings were added to the respective supernatants. The supernatant from the 10 000*g* spin was centrifuged at 146 000*g* for 1 h in a Beckman L5-65 Centrifuge equipped with a 50.2 rotor. All cells and supernatants were counted in a Nuclear Chicago small-body gamma counter.

RESULTS

Brewer's yeast, *Saccharomyces carlsbergensis*, was grown under different conditions to determine the optimal conditions for the uptake of labeled Cr and for its incorporation into a biologically active product. When yeast was grown in a purified synthetic medium consisting mainly of acid-hydrolyzed casein and added vitamins and minerals (Toepfer and Polansky, 1970) at pH 4.5 with radioactive $CrCl_3$, the rate of uptake of Cr was low and linear for 8 days, increased sharply for 2 to 3 days, then decreased gradually (Figure 1). Under those conditions, addition of 5% glucose on day 5 or on days 5 and 9 decreased the absorption of labeled chromium. Although the growth of yeast was optimal in the purified synthetic medium (Toepfer and Polansky, 1970) the incorporation of Cr was low and attempts to increase its absorption met with limited success. However, growth of yeast in 1% peptone, 0.03% phosphate, and varying levels of glucose significantly increased Cr absorption (Table I). The endogenous Cr content of the purified medium was 20–50 ppb and that of the peptone medium was 10–30 ppb. Addition of glucose to as high as 25% stimulated the incorporation of labeled Cr (Table I) but the growth of the yeast was inhibited at higher levels of glucose, e.g., increasing glucose from 10 to 25% decreased growth approximately 40%. Addition of glucose after 5 days or 5 and 9 days did not stimulate Cr absorption greater than addition of the total glucose to the medium initially.

Table I. Effects of Glucose and Medium on Cr Uptake by Brewer's Yeast

	Glucose, %	CPM/G[a]
1.	5[b]	6 000
2.	5 + 5[b,c]	2 000
3.	5 + 5 + 5[b,d]	2 000
4.	2	122 000
5.	5	189 200
6.	5 + 5[c]	324 700
7.	5 + 5 + 5[d]	459 000
8.	10	446 000
9.	15	627 616
10.	20	770 000
11.	25	799 000
12.	30	651 000

[a] Dry weight of water washed yeast cells grown 10 days. [b] Yeast cells in lines 1-3 were grown in purified medium (Toepfer and Polansky, 1970) (see Materials and Methods); remaining cells (lines 4-12) were grown in 1% peptone, 0.03% phosphate, and the indicated levels of glucose. Cells were grown in 250-mL Erlemeyer flasks containing 60 μCi $CrCl_3$ in 150 mL of medium at 26° with shaking at 125 rpm in a New Brunswick Controlled Environment Incubator Shaker. CPM/G are an average of three separate determinations. [c] Additional glucose added 5 days after innoculation. [d] Additional glucose added 5 and 9 days after innoculation.

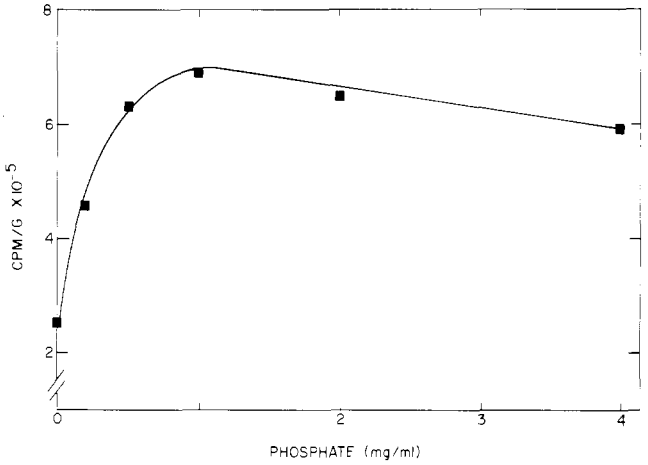

Figure 2. Effect of phosphate on retention of radioactive chromium. Cells grown in 1% peptone, 60 μCi $CrCl_3$, 10% glucose, and varying levels of phosphate, 26 °C, pH 4.5.

In addition to glucose, phosphate also stimulated chromium absorption. Addition of phosphate, 1 mg/mL of medium, stimulated Cr absorption approximately 2.5-fold compared to the unsupplemented controls (Figure 2). Further addition of phosphate decreased slightly the chromium absorbed per gram of yeast.

The absorption of chromium by higher animals and man is influenced strongly by the form of Cr. For example, approximately 0.6% of inorganic Cr salts are absorbed (Doisy et al., 1968; Donaldson and Barreras, 1966) while reports of the absorption of some natural organic Cr products are 10–25% (Mertz and Roginski, 1971). Therefore, the form of chromium absorbed by yeast was investigated. Compounds tested were chromium chloride and sodium chromate which yielded little insulin potentiating activity and biologically active organic Cr compounds synthesized from: chromium, nicotinic acid, glycine, cysteine, and glutamic acid (Toepfer et al., 1977) or from: chromium, nicotinic acid, and glutathione (Anderson and Brantner, 1977; Anderson et al., 1977). For

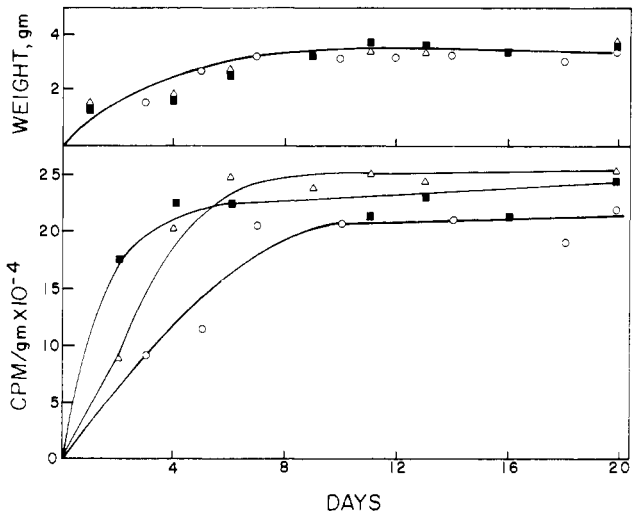

Figure 3. Ligand effect on uptake of radioactive chromium by yeast. Yeast grown in 1% peptone, 0.03% phosphate, 10% glucose, 26 °C, pH 4.5, and radioactive chromium in the form of: (○) $CrCl_3$; (■) Cr, nicotinic acid, glutathione; (△) Cr, nicotinic acid, glycine, cysteine, glutamic acid.

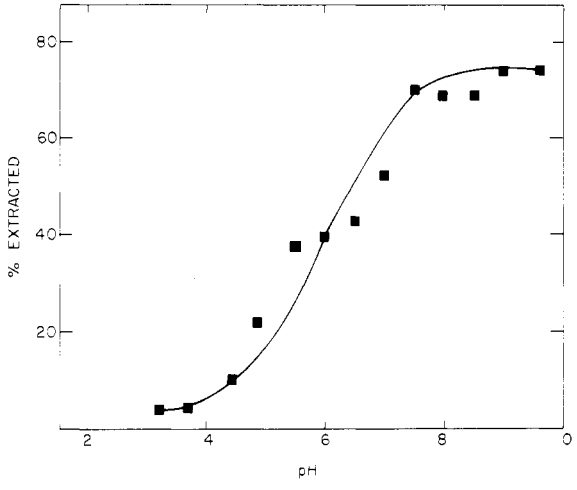

Figure 4. Effect of pH on extraction of labeled chromium from yeast. Acetic acid (0.2 M) and sodium acetate (0.2 M) were mixed to attain the desired pH between pH 3.5 to 5.6, Tris (0.2 M) adjusted with acetic acid (0.2 M) was used at pH values greater than 6.

all forms of chromium tested, rate and maximal level of incorporation were similar (Figure 3). Sodium chromate (data not shown) was also incorporated at a similar rate. However, under these growth additions as opposed to those in Figure 1, there is no lag in the incorporation of radioactive Cr (Figure 3, lower panel) and the incorporation of Cr regardless of form, correlates with the growth of the yeast (Figure 3, upper panel). The biological activity of the extracts was not influenced significantly by any of the forms of chromium added to the growth medium.

After determining the optimal conditions for the incorporation of chromium into Brewer's yeast, we tested methods for extraction of the labeled biologically active Cr. The removal of the labeled chromium was pH dependent and approximately 80% of the radioactive Cr could be removed by shaking the cells for 10 min at a pH greater than 8.0. Little chromium could be extracted at pH values below 4.5, the initial pH of the medium, but there was nearly a linear increase in the amount of labeled Cr extracted between pH 5 and 8 with little increase in the amount of Cr extracted at higher pH values (Figure 4). Since the majority of the Cr could be extracted at pH values greater than pH 8.0, we treated the yeast cells with 0.1 N NH_4OH for 30 min, followed by three water washings and removed more than 85% of the labeled metal (Table II). Cr extracted under these conditions, displayed significant biological activity (Table II, line 1). Ethanol, which has been postulated to remove Cr associated with biological activity (Toepfer et al., 1973), extracted less than 5% of the total Cr but did remove some of the Cr associated with biological activity (Table II, line 2). Treatment of the yeast cells with teichozyme-Y for 12 h ruptured the cells (determined by microscopic examination) and released more than 80% of the labeled Cr, but the bioactivity of the extract was relatively low (Table II, line 3), especially after sonication (Table II, line 4). Apparently, breaking of the cells released factors that inhibited the assay since assaying larger amounts of the extract decreased the relative biological activity but assaying larger amounts of the ammonia extracts yielded progressive increases in bioactivity.

Table II. Extraction of ^{51}Cr from Yeast[a]

	Conditions	Counts extracted	Rel. bioactivity
1.	0.1 N NH_4OH	2 000 000	2.2
2.	50% Ethanol	70 000	1.4
3.	Teichozyme Y	1 900 000	1.6
4.	Teichozyme Y + sonication	1 978 000	1.1

[a] Brewer's yeast, 14 g containing 2.3×10^6 cpm, was extracted under the indicated conditions (see Materials and Methods). The extract was concentrated to 7 mL and 5λ was assayed in the in vitro bioassay. All assays are an average of three separate determinations.

Table III. Distribution of Labeled Cr[a]

Centrifugation conditions	% supernatant	% pellet
600*g*, 10 min	81	19
15 000*g*, 10 min	99[b]	1
146 000*g*, 10 min	98	2

[a] Cells grown in 1% peptone, 0.03% phosphate, and 10% glucose, pH 4.6, were treated with teichozyme-Y (see Materials and Methods). [b] The total counts remaining in the supernatant after the 600*g* spin was considered as 100%.

The radioactivity extracted from yeast grown on labeled chromium in 1% peptone, 0.03% phosphate, and 10% glucose remained in the soluble fraction after centrifugation at 10 000*g* for 10 min and even after centrifuging for 60 min at 146 000*g* (Table III). Approximately 19% of the radioactivity pelleted in the initial centrifugation but some of this radioactivity may be due to incomplete cell breakage and also nonspecific trapping due to the large volume of pellet. Similar cellular distribution was observed for a 0.1 N NH_4OH extract.

DISCUSSION

The recent history of nutrition research on the role of chromium can be traced to the observations that rats fed a torula yeast-based diet, which is low in biologically available Cr, developed an impaired tolerance to glucose while those fed a diet containing Brewer's yeast did not (Mertz and Schwarz, 1955). It was shown that epididymal adipose tissue from chromium supplemented rats and chromium deficient rats had similar rates of glucose uptake, but, in the presence of insulin, the chromium deficient tissue displayed a significantly smaller increase in glucose uptake than that from chromium supplemented

controls. Moreover, addition of less than 0.5 μg of certain Cr complexes to the incubation medium increased glucose uptake of the Cr-deficient tissue to that of the controls. However, not all forms of chromium will reverse the symptoms of Cr deficiency. For example, addition of inorganic chromium to chromium-depleted Brewer's yeast grown in a Warburg flask under a nitrogen atmosphere had little effect on the short-term CO_2 production, but addition of Cr-containing yeast extracts stimulated CO_2 production immediately (Burkeholder and Mertz, 1967). Genetically diabetic mice also do not respond to inorganic Cr compounds but do respond to organic Cr compounds that display in vitro insulin potentiating activity (Doisy et al., 1976). Brewer's yeast is the best known source of biologically available Cr that can be absorbed and utilized by higher animals and man. Therefore, growth conditions for yeast and the forms of Cr that are utilized and synthesized by yeast are of utmost importance to human nutrition.

Yeast grown in a medium, which has been shown to yield optimal growth (Toepfer and Polansky, 1970), retained low levels of radioactive chromium that displayed negligible bioactivity. However, yeast grown in medium containing peptone, phosphate, and glucose retained several times more Cr than yeast grown on purified media and the Cr extracted displayed significant biological activity.

Elimination of exogenous phosphate from the medium decreased Cr retention 2.6-fold. The peptone contained significant endogenous phosphate and elimination of this phosphate would be expected to further diminish Cr retention. Phosphate has been postulated to be involved in the formation of a carrier for the transport of magnesium and manganese ions into yeast and does not appear to be directly involved as a phosphate–metal complex (Jennings et al., 1958); it may play a similar role in the incorporation of chromium.

Glucose, like phosphate, stimulates Cr retention by yeast but this response is critically dependent upon the growth conditions. Our results are not entirely consistent with the previous data (Burkeholder and Mertz, 1967), but due to the variability of the glucose response, interlaboratory comparisons are difficult to evaluate.

Extraction of yeast cells with dilute base released the largest amounts of both radio- and biological activity but we do not know how much of the extracted radioactivity was bound to the surface of the yeast cell. The cells do not appear to be broken after extraction in ammonia or ethanol. A number of metabolites permeate the intact plasma membrane and ethanol plus heat or ammonia would likely alter the permeability of the cell membrane and lead to the release of cellular contents. Release of labeled chromium is similar when cells are disrupted with teichozyme and when cell walls remain visibly intact after treatment with base. The biological activity released, however, is lower after enzyme treatment or enzyme treatment followed by sonication than after treatment with base. This difference may be due to extraction of inhibitory substances, since assaying larger aliquots leads to decreasing responses in the in vitro insulin potentiation assay. Additionally, purification of the teichozyme extract leads to an increase in total biological activity which would also be consistent with the removal of an inhibitory substance.

Essentially all of the counts extracted employing teichozyme-Y or ammonia were in the soluble fraction, indicating that the labeled chromium was not particulate bound. This is consistent with the soluble nature of labeled Cr extracted from leaf tissue from beans grown on labeled Cr (Huffman and Allaway, 1973). However, Mathur and Doisy (1972) found only 12 to 18% of the Cr in the soluble fraction of liver from rats that had been injected intravenously with labeled $CrCl_3$.

These data define conditions for the growth of yeast with radioactively labeled Cr that lead to a biologically active extract containing labeled Cr. However, only a small fraction of the total radioactivity is associated with bioactivity (Anderson and Polansky, 1977). The properties of this biologically active fraction are under study.

LITERATURE CITED

Anderson, R. A., Brantner, J. H., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36**, 1123 (1977).

Anderson, R. A., Polansky, M. M., Brantner, J. H., Roginski, E. E., in "Trace Element Metabolism in Man and Animals", Vol. III, Kirchgessner, M., Ed., presented at the Third International Symposium on Trace Element Metabolism in Man and Animals, Freising-Weihenstephan, West Germany, July 25–29, 1977.

Burkeholder, J. N., Mertz, W., in "Proceedings of the Seventh International Congress of Nutrition", Vol. V, Pergamon Press, New York, N.Y., 1967 p 701.

Doisy, R. J., Streeten, D. H. P., Levine, R. A., Chodos, R. B., in "Trace Substances in Environmental Health", Vol. II, D. D. Hemphill, D. D., Ed., University of Missouri, Columbia, Mo., 1968, pp 75–82.

Doisy, R. J., Streeten, D. H. P., Freiberg, J. M., Schneider, A. J., in "Trace Elements in Human Health and Disease", Vol. II, Prasad, A. S., Ed., Academic Press, New York, N.Y., 1976, pp 79–104.

Donaldson, R. M., Barreras, R. F., *J. Lab. Clin. Med.* **68**, 484–493 (1966).

Glaser, E., Halpern, G., *Biochem. Z.* **207**, 377–383 (1929).

Herepath, W. B., Journal of the Provincial Medical and Surgical Society, **28**, 374 (1854).

Huffman, E. W. D., Jr., Allaway, W. H., *J. Agric. Food Chem.* **21**, 982–986 (1973).

Jennings, D. H., Hooper, D. C., Rothstein, A., *J. Gen. Physiol.* **41**, 1019–1026 (1958).

Liu, V. J. K., Nordstrom, J., Kohrs, M. B., Lorah, E., Dowdy, D., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36**, 1123 (1977).

Mathur, R. K., Doisy, R. J., *Proc. Soc. Exp. Biol. Med.* **139**, 836–838 (1972).

McCay, C. M., in "Cowdy's Problems of Aging", 3rd ed, Lansing, A. J., Ed., Williams and Wilkins, Baltimore, Md., 1952, Chapter 6.

Mertz, W., Roginski, E. E., in "Newer Trace Elements in Nutrition", Mertz, W., Cornatzer, W. E., Ed., Marcel Dekker, New York, N.Y., 1971, Chapter 7.

Mertz, W., Schwarz, K., *Arch. Biochem. Biophys.* **58**, 504 (1955).

Mertz, W., Schwarz, K., *Am. J. Physiol.* **196**, 614–618 (1959).

Polansky, M. M., Anderson, R. A., U.S. Department of Agriculture, Beltsville, Md., unpublished observation, 1977.

Toepfer, E. W., Mertz, W., Roginski, E. E., Polansky, M. M., *J. Agric. Food Chem.* **21**, 69–73 (1973).

Toepfer, E. W., Polansky, M. M., *J. Assoc. Off. Anal. Chem.* **53**, 546–550 (1970).

Toepfer, E. W., Mertz, W., Polansky, M. M., Roginski, E. E., Wolf, W. R., *J. Agric. Food Chem.* **25**, 162–166 (1977).

Received for review January 13, 1978. Accepted March 17, 1978.