Use of Teflon Digestion Bombs for Tissue Analysis: Measurements of the Effect of Estradiol-17β Upon Hepatic Copper in Rats*

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ABSTRACT

Teflon digestion bombs have been used for pressure decomposition of rat liver samples preliminary to copper analyses by atomic absorption spectrometry. The analytical procedure is convenient and is consistently free from copper contamination. The recovery of copper added to liver samples (three μg Cu added per g wet wt) averaged 102 (S.D. ± 3) percent. The accuracy of the copper analyses was verified by use of National Bureau of Standards reference bovine liver. The mean concentration of copper in perfused livers of 16 untreated male rats averaged 16.1 (S.D. ± 2.3) μg per g dry wt. The mean concentration of copper in perfused livers of 8 male rats which received estradiol-17β in dosage of 50 μg per day, s.c., for 21 consecutive days was 21.7 (S.D. ± 4.7) μg per g dry wt (p vs controls = <0.01). This study demonstrates that estrogen administration can cause a significant increase in the concentration of hepatic copper.

Introduction

Atomic absorption spectrometry of trace metals in tissues and other solid biological samples (e.g., hair, feces) has been hampered by cumbersome preliminary techniques for ashing or digestion of the samples in order to destroy the organic constituents. Wet-digestion techniques customarily employ a mixture of nitric and sulfuric acids in conjunction with an oxidizing agent such as perchloric acid or hydrogen peroxide. To achieve adequate wet-digestion, it is usually necessary to add several aliquots of the acidic digestion mixture. Wet-digestion techniques are time consuming and they require the constant attention of an analyst in order to prevent loss of samples by “bumping.” Since even “ultra-pure” acids contain appreciable concentrations of trace metals, such as As, Cr, Ni and Pb, any variations in the quantity of acid which is used for the wet-digestion can result in variable metal contamination.

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Hence, the accuracy and precision of trace metal analyses by wet-digestion techniques are effectively limited by variations in the volumes of acidic digestion mixture which are required for treatment of the blank, standard and unknown samples. Dry-ashing techniques using a muffle furnace have the disadvantages of (1) variations in thermal volatization of metals owing to matrix effects; (2) the hazard of cross-contamination of samples within the muffle furnace; and (3) variations in dissolution of the ashed samples in acid prior to atomic absorption spectrometry. Anderson has recently summarized the relative advantages and disadvantages of conventional methods of wet-digestion and dry-ashing of tissues for trace metal analyses by atomic absorption spectrometry.

Several investigators have attempted to develop improved methods of sample destruction, in order to circumvent the various problems which are associated with customary wet-digestion or dry-ashing techniques. Thompson and Blanchflower have described a system for acid digestion of samples in a specially designed heating block. Lewis and Coughlin have devised a wet-ashing system which uses an all-glass digestion-distillation apparatus. Murphy et al have employed alkaline digestion with aqueous tetramethylammonium hydroxide. Several instrument manufacturers have introduced low temperature dry-ashing apparatuses, which oxidize biological samples in atomic oxygen in a radio-frequency reactor. Each of these methods for
digested of tissues, hair and feces has been evaluated in our laboratory and none of these methods has proven to afford any practical advantage over conventional wet-digestion with nitric, sulfuric and perchloric acids using a Kjeldahl digestion apparatus. On the other hand, the teflon digestion bomb which is illustrated in figures 1 and 2 has proven, in our laboratory, to provide a convenient method for the digestion of biological samples.

Bernas,² who worked for the National Aeronautics and Space Administration, originally developed the teflon digestion bomb in 1968 in order to accomplish rapid decomposition of lunar rock samples in hydrofluoric acid, prior to analyses of metals by atomic absorption spectrometry. The teflon digestion bomb was subsequently used by Hartstein et al⁴ for trace metal analyses of coal; by Mansell and Hiller⁷ for lead analyses of gasoline; and by Holak et al⁵ for analyses of mercury in fish. A larger teflon digestion bomb was described by Paus¹⁰ and has been used for analyses of Hg, Cd, Zn, Cu, Fe and Pb in seaweed and fish.¹¹ A novel digestion apparatus which contains six teflon vessels for the simultaneous decomposition of multiple samples has recently been illustrated in a review article by Mitchell.⁸

The specific biological application of the teflon digestion bomb which will be described in this paper is the determination of copper concentrations in rat livers. This method has been employed in our laboratory for analyses of copper in livers of untreated rats and in livers of rats which received repeated parenteral injections of estradiol-17β.

Methods

**PRINCIPLE OF PRESSURE DECOMPOSITION OF TISSUE FOR COPPER ANALYSES**

Rat liver is homogenized with an ultrasonic disintegrator. Aliquots of the liver homogenate are digested with HNO₃ in the teflon digestion bomb; and measurements of copper in the digested samples are performed by atomic absorption spectrometry. Additional aliquots of the homogenate are dried to constant weight so that the copper content of the liver can be expressed on a dry-weight basis.

**SPECIAL APPARATUS**

*Ultrasonic Disintegrator,* 300 watt output with titanium probe.*

*Teflon-lined Acid Digestion Bombs,* 25 ml capacity.** For routine use in analyses of blank, standard and unknown samples, it is desirable to have at least 12 bombs.

*Atomic Absorption Spectrometer,* fitted with high-solids burner, copper hollow cathode lamp and 10" strip-chart recorder.†

*Disposable plastic knives and forks* which have been soaked in 30 percent (v/v) nitric acid.

**REAGENTS**

*Nitric Acid,* concentrated, ultrapure, (Sp. Gr. = 1.40).†

*Copper Reference Standard Solution,* (1 mg Cu per ml).§

*Copper Working Standard Solutions,* (0.5, 1.0 and 1.5 µg Cu per ml). Into three 2-liter volumetric flasks are transferred, respectively, 1, 2, and 3 ml of the copper reference standard solution. The contents of the flasks are diluted to the marks with distilled, demineralized water.

*Sodium chloride solution,* 8.5 g per liter.

*Caprylic alcohol,* reagent grade.

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** Model 4745, Farr Instrument Co., 211 Fifty-third Street, Moline, IL 61265.
† Model 810 dual-monochromator atomic absorption spectrometer, Jarrell-Ash Division, Fisher Scientific Co., Waltham, MA 02154.
§ Catalog No. 441, E. Merck Co., Darmstadt, Germany, purchased from EM Laboratories, Inc., 500 Executive Blvd., Elmsford, NY 10523.

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PROCEDURE

The rat is anesthetized with ether, and the abdomen is opened in the midline with a scalpel. A short, lengthwise incision is made in the portal vein to permit the insertion of a polyethylene cannula. The cannula is connected by a Luer-lock fitting to a 20 ml syringe, which is filled with isotonic sodium chloride solution. The vena cava is sectioned with scissors, to permit efflux of the perfusion fluid. The liver is very slowly perfused with isotonic sodium chloride solution, until the liver becomes uniformly pale and bloodless. The liver is then excised, blotted with filter paper and weighed.

Approximately two g of liver is minced into one mm cubes by use of the disposable plastic knife and fork. Exactly 1.500 g of liver is weighed into a tared beaker. Five ml of distilled water are added, and the minced liver is completely homogenized by insertion of the titanium probe of the ultrasonic disintegrator, and turning on the ultrasonic signal for approximately 30 sec. The probe is rinsed with one to two ml of water. Three drops of caprylic alcohol are added to the homogenate in order to eliminate bubbles, and the homogenate is transferred quantitatively to a 10 ml volumetric flask, together with water washings. The contents of the flask are diluted to the mark with water.

Two-ml aliquots of the liver homogenate are transferred to teflon digestion bombs. Into additional digestion bombs are transferred two ml of water (“blank” samples), and two ml of each of the copper working standard solutions. Ultrapure nitric acid, three ml, is added to each of the teflon combustion bombs. The bombs are tightly sealed and are placed overnight in an oven at 120°. The oven temperature must be controlled by a reliable thermostat to avoid any possibility that the bomb might be heated above its 150° maximum rating (see cautionary footnote on explosion hazard). It is convenient to perform the wet-digestions overnight, but heating for an interval as short as six hours is equally satisfactory.

Two-ml aliquots of the liver homogenate are transferred to tared weighing beakers, and the wet weights of the samples are measured with an analytical balance. The beakers are then placed in an oven at 100° for 24 hours, so that the samples become dried to constant weight. The beakers are brought to room temperature in a desiccator, and the dry weights of the samples are measured with an analytical balance.

The teflon digestion bombs are removed from the oven and are allowed to cool slowly to room temperature for at least two hours. The steel caps are loosened gently, and the teflon sample cups are carefully removed. The digested samples should be colorless and water-clear.

* The teflon combustion bombs should never be used with samples which are rich in lipids, such as brain, adipose tissue, lipemic serum or milk, owing to the possibility of hydrolysis of lipids to glycerol and fatty acids and subsequent formation of nitroglycerin and other nitrated organic products. Tyler has reported an accidental explosion which occurred when one ml of nitric acid, one ml of sulfuric acid and 0.5 gm (wet wt) of adipose tissue were placed in two teflon digestion bombs, and the tops were screwed on. No heat was applied. After 10 minutes both of the bombs exploded. The laboratory bench was shattered and the analyst suffered lacerations and abrasions. Tyler calculated that the contents of the bombs could have reached temperatures up to 4000°K and internal pressures as high as 15,000 psig. The teflon combustion bombs are only rated to withstand temperatures up to 150° and internal pressures up to 1,200 psig. The manufacturer has specified that the oven in which the bombs are placed must be controlled by a reliable thermostat to avoid any possibility that the bomb might be heated above 150°. For nitric acid digestions of organic compounds, the sample must not contain more than 0.1 g (dry wt) of organic matter, and the amount of concentrated nitric acid added must not be less than 2.5 ml and must not exceed 3.0 ml. Sulfuric and perchloric acids should not be used. Precautions must always be observed to guard against unexplained explosions, which may sometimes occur when treating organic substances with a strong oxidizing acid.
The atomic absorption spectrometer is adjusted to the following parameters: (1) wavelength = 324.7 nm; (2) slit width = 1.0 nm; (3) copper hollow cathode lamp current = 10 ma; (4) acetylene: air flame, 1:5 gas mixture (v/v).

The samples, including blanks, standards and unknowns, are aspirated directly from the teflon cups into the burner-nebulizer of the atomic absorption spectrometer, and the absorption at 324.7 nm is recorded.

The concentration of liver copper is calculated and expressed as µg Cu per gm (dry weight).

ANIMAL INVESTIGATIONS

The experimental animals were 24 male rats of the Fischer strain (200 to 300 g body wt) which were fed Purina rat chow. The control group comprised 16 untreated rats. The treatment group comprised 8 rats which received daily sc injections of estradiol-17β in dosage of 50 µg per day for 21 consecutive days. The protocol for administration of estradiol-17β, and the effects of estradiol-17β upon serum copper and ceruloplasmin concentrations have been reported previously by Sunderman et al.12

RESULTS

EVALUATION OF THE DIGESTION PROCEDURE

In figure 3 is shown an illustrative recorder tracing of atomic absorption spectrometry of copper in reagent blank, standard and liver samples. Based upon analytical runs on 20 consecutive working days, there was no significant contamination with copper during the digestion procedure. The reagent blanks which were carried through the digestion procedure were consistently free of any copper which could be detected by flame atomic absorption spectrometry. Measurements of the recovery of copper added to six rat liver homogenates (three µg Cu added per g wet wt), yielded a mean recovery of 102 ± 3 percent (range = 97 to 105). The accuracy of measurements of hepatic copper by the teflon digestion bomb technique was verified by analyses of National Bureau of Standards Reference Material #1577 (Lyophilized Bovine Liver). Four measurements of 25 mg samples of NBS Bovine Liver yielded a mean copper concentration of 189 ± 4 µg per g (dry wt), range = 185 to 195 µg per g. The certified concentration of copper in the NBS Bovine Liver is 193 ± 10 µg per g (dry wt). To test for the completeness of hydrolysis of organic constituents, aliquots (50 µl) of digested liver homogenates were spotted on cellulose-coated thin layer chromatography plates. Ascending chromatography of amino acids was performed in isopropanol:H2O (70:30, v:v). Inspection of the chromatography plates under long and short wavelength ultraviolet light did not reveal any ultraviolet-absorbing or any fluorescing spots. The thin-layer chromatography plates were sprayed with ninhydrin reagent and were heated at 90° for 30 min. No ninhydrin-reactive compounds were detected.

CONCENTRATIONS OF COPPER IN LIVERS OF RATS

Livers from the control group of 16 healthy, untreated rats were analyzed by

Figure 3. Typical recorder graph for atomic absorption analyses of copper in blank, standard and liver samples which have been digested by use of the teflon digestion bomb. Sample #1 = reagent blank; Samples #2 to 4 = copper standards containing 0.5, 1.0 and 1.5 µg Cu per ml, respectively; Samples #5 and 6 = homogenates of livers from untreated rats.
the procedure which has been described, and the following results were obtained:

1. Dry wt = 31.6 ± 2.7 percent of wet wt (range = 27.5 – 38.5);
2. Copper concentration = 5.1 ± 0.6 µg per g (wet wt) (range = 4.4 to 6.1);
3. Copper concentration = 16.1 ± 2.3 µg per g (dry wt) (range = 13.9 to 19.6).

Analyses of livers from 8 rats which had received daily sc injections of estradiol-17β in dosage of 50 µg per day for 21 consecutive days yielded the following results:

1. Dry wt = 29.0 ± 1.2 percent of wet wt (range = 26.6 to 30.4) (p < 0.05 vs controls by Student's t test);
2. Copper concentration = 6.3 ± 1.6 µg per g (wet wt) (range = 4.1 to 9.0);
3. Copper concentration = 21.7 ± 4.7 µg per g (dry wt) (range = 15.0 to 30.2) (p < 0.01 vs controls by Student's t test).

**Discussion**

The present study has demonstrated that administration of estradiol-17β to male rats in dosage of 50 µg per day for 21 consecutive days results in a mean increase of 35 percent in the concentration of hepatic copper. Sunderman et al. have previously reported that administration of estradiol-17β to rats according to the same dosage schedule stimulates a mean increase of 340 percent in the concentrations of serum copper and serum ceruloplasmin (CPN), without producing pathological alterations of the hepatocytes. Studies by Evans et al. have suggested that estrogen acts as an inducer for hepatic synthesis of CPN messenger-RNA, leading to increased hepatic synthesis of CPN-protein. Insofar as the authors can ascertain, chemical analyses of copper concentrations in liver have not previously been reported in estrogen-treated animals, or in patients who have been treated with estrogens. The increased mean concentration of hepatic copper which was observed in the present study may possibly reflect: (1) an increase in hepatic CPN concentration, resulting from the increased rate of CPN synthesis; (2) a secondary increase in hepatic CPN catabolism, with binding of released copper to lysosomal metallothionein, or (3) a combination of both of these factors. Studies of the subcellular distribution of copper in hepatocytes of estrogen-treated rats are in progress in our laboratory.

From a methodological viewpoint, the use of teflon digestion bombs for pressure acidic decomposition of tissues for trace metal analyses has the advantages of: (1) relative freedom from trace metal contamination, (2) quantitative recovery of the digested sample and (3) convenience and saving of time for the analyst. Disadvantages of the technique include: (1) possible explosion hazard; (2) limited sample size and (3) relatively high cost, since the teflon bombs which are currently available are not very durable. In the authors’ experience, a teflon sample cup can be employed for only 15 to 20 tissue digestions. Thereafter, the sample cup no longer maintains a vapor-tight seal and hence it must be discarded. Leakage of nitric acid from the teflon cup into the steel bomb casing causes (1) loss of sample and (2) possible contamination of the sample with Fe, Ni or Cr dissolved from the steel. To detect such errors, it is necessary to analyze each sample in duplicate, and to repeat the analyses with new teflon cups whenever the results of the duplicate analyses are not in close agreement. Leakage of nitric acid can also result in corrosion of the steel bomb casing, which makes it impossible to maintain a vapor-tight seal even with use of a new teflon sample cup. When a bomb casing has become corroded, it must be discarded. Hopefully, these drawbacks will be overcome by improvements in the design of teflon digestion bombs. Increasing the thickness of the wall of the teflon cup, and providing a screw-thread closure for the inner teflon cover would undoubtedly improve the performance of the digestion
bombs. Fabrication of bomb casings of titanium instead of steel might also be helpful, although this would obviously increase the cost of the apparatus. In the authors’ opinion, when the design of teflon digestion bombs has been improved so that malfunctions rarely occur and so that the explosion hazard has been practically obviated, such digestion bombs should find wide clinical, forensic and research applications for analyses of metals in tissues and other biological materials.

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References