Preparation of the Inorganic Matrix of Bone*

J. B. Williams and J. W. Irvine, Jr.

Radioactivity Center and *Department of Chemistry, Laboratory for Nuclear Science, Massachusetts Institute of Technology, Cambridge*

For many purposes in biology and medicine, it is desirable to obtain intact the inorganic salt fraction of bone separated from its organic environment. The methods in common use at present are glycol-ashing at temperatures greater than 200°C (*1–3*), dry-ashing in a muffle furnace at temperatures in excess of 500°C, and high-temperature autoclaving (*4*).

Glycol-ashing, or boiling in a KOH-glycol solution, alters the cation and anion makeup and can leave collagen in bone (*5*). Treatment at elevated temperatures can cause serious alteration in the crystal structure as well as in the gross structural pattern (*6*).

Among the requirements of an extraction solvent are that it must attack vigorously the organic matrix of bone at as low temperature as possible and volatilize easily, so that it can be readily distilled from possible nonvolatile contaminants. Extraction must be achieved with a minimum of erosion and dislocation of gross inorganic particulates.

These conditions have been met by the use of a constant-boiling (118°C) aqueous (80 percent) mixture of ethylene diamine in a soxhlet extractor. A filter paper thimble is used for the extraction of small or fragmented samples. The process is carried out either by placing the bone in the liquid distillate or by suspending it in the vapor. Whole bones are split to expose their marrow cavities to the hot extractant during the 20 to 30 distillation cycles required.

Soxhlet extractors of any size and geometry may be used, with the qualification that large extraction chambers may require insulation or external heating to maintain the solvent at near-boiling temperatures. Large flat sections of bone have been extracted in a specially constructed large-area soxhlet. To minimize erosion and agitation, the tip of the water-cooled condenser has been bent to deliver the extractant along the side of the chamber. All glass equipment with ground glass joints (used without lubricants) makes the most satisfactory system. Cork, rubber, stopcock grease, and some metals are attacked by the solvent.

One hundred cubic centimeters of ethylene diamine is usually sufficient to extract a 5- to 10-g fresh bone sample. However, a replacement of the diamine may be required when the amount of organic material removed is large, since this dilution can cause a much larger decrease in the vapor concentration of the ethlylene diamine. Because of the extreme solubility of ethylene diamine in water, several extraction cycles with distilled water are sufficient to remove essentially all the solvent. Because of the slight toxicity of ethylene diamine, the process is usually carried out in a hood.

* Supported in part by the joint program of the Office of Naval Research and the Atomic Energy Commission.

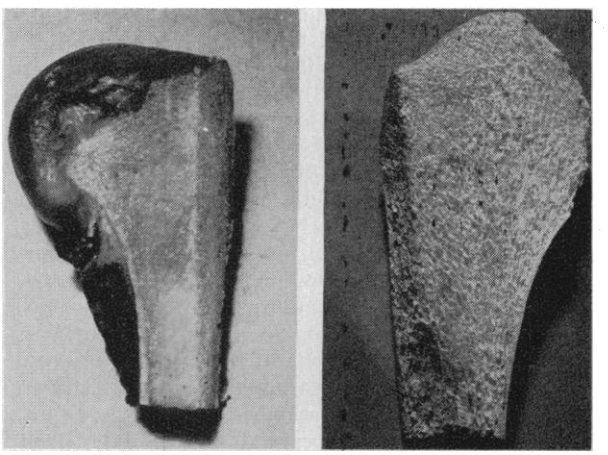

Fig. 1. (Left) Adjacent longitudinal sections of the distal epiphysis of a human femur, fresh. (Right) The same after extraction with ethylene diamine.

Comparative chemical analyses (*7*) of adjacent bones, one prepared by extraction, the other by ashing at 550°C, are tabulated as follows:

	Extracted (%)	*Dry-ashed* (%)
Calcium (Ca)	38.1	39.4
Phosphate (PO_4)	49.8	51.6
Carbonate (CO_3)	5.6	3.1

Preliminary differential thermal analyses (*8*) of extracted bone show a large exothermic reaction at

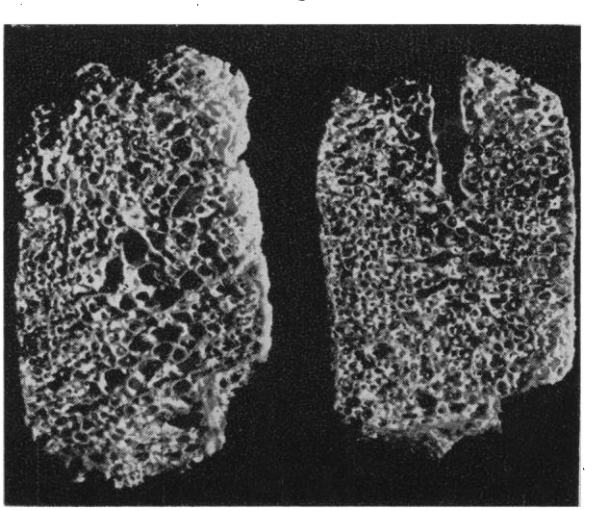

Fig. 2. Cross sections of two of a child's vertebrae after extraction.

580°C, indicating a possible recrystallization. For this reason, the dry-ashing procedure was carried out at 550°C. However, an apparent partial decomposition of the carbonate may still be noted from the tabulated results.

A preliminary x-ray diffraction powder pattern of this low-temperature extracted bone gives qualitatively the same spacing as bone prepared by other methods. (*9*) Electron-microscope studies of ground extracted bone show no trace of collagen confirming a nitrogen analysis (Kjeldahl) finding of less than 0.1 percent

Bone samples have been prepared by this technique (Figs. 1 and 2) for autoradiography, for demonstration of trabecular stress patterns, for radium analysis of normal bone, and for the separate analyses of tracers in the two fractions of bone. Extracted bone and bone powder have been implanted in dogs and are disposed of in a manner metabolically similar to that of fresh bone and other crystalline material without apparent organic reaction.

References and Notes

1. S. Gabriel, *Z. physiol. Chem.* **18**, 257 (1894).
2. S. Margulis, *J. Biol. Chem.* **93**, 455 (1931).
3. C. D. Crowell, H. C. Hodge, and W. R. Line, *J. Dental Research* **14**, 251 (1934).
4. R. A. Robinson and F. W. Bishop, *Science* **3**, 657 (1950).
5. S. Orell, *J. Bone and Joint Surgery* **35**, 873 (1937).
6. W. F. Neuman and B. J. Mulryan, "Studies on the Surface Chemistry of Bone," AECU-132 (Univ. of Rochester, 1949).
7. Analyses were made by Skinner and Sherman Co., Boston, Mass.
8. Analyses were made by the Soil Mechanics Laboratory, Department of Civil Engineering, M.I.T.
9. R. A. Robinson, *J. Bone and Joint Surgery* **34A**, 389 (1952).

Received March 12, 1954.

Trace Element Content of Cancerous and Noncancerous Human Liver Tissue*

Kenneth B. Olson, George Heggen, Carl F. Edwards, and L. Whittington Gorham

Department of Medicine, Subdivision of Oncology, Albany Medical College and Saratoga Springs Commission Research Laboratory, Saratoga Springs, New York

In a previous publication, a method (*1*) has been described for the quantitative study of 14 trace elements in biological material by spectrochemical means. In experimental rat hepatomas induced by p-dimethylaminoazobenzene (DAB), it has been shown (*2*) that zinc decreased in concentration during the period of liver damage and then increased with regeneration and appeared to be at its peak before gross neoplasia was evident. It seemed of interest to study trace elements in human tissue and the following is a preliminary report on the study of 12 trace elements in the livers of six patients without tumor, two patients dead of carcinoma of the esophagus and with hepatic cirrhosis but without tumor involvement of the liver, four cases with metastasis to the liver from cancer of the gastrointestinal tract, and one case of acute lymphatic leukemia (*3*). Table 1 lists the cases studied. In the six cases without cancer, histological examination of the livers revealed varying degrees of congestion.

* These studies are supported in part by U.S. Public Health Service grant No. C-1121 to the Albany Medical College.

Table 1. Cause of death.

Case No.	Diagnosis	Age
1	Generalized arteriosclerosis; bronchopneumonia	82
2	Myocardial infarction	68
3	Esophagitis and hemorrhage from esophageal varices	76
4	Chronic pyelonephritis	81
8	Cerebral artery thrombosis	80
10	Intestinal obstruction	54
23	Carcinoma of esophagus; portal cirrhosis	59
13	Carcinoma of esophagus; portal cirrhosis	62
17	Carcinoma of rectum*	69
18	Adenocarcinoma of colon*	67
14	Adenocarcinoma of colon*	41
6	Adenocarcinoma of stomach*	39
7	Acute lymphatic leukemia†	9

* Tumor metastasis to liver.
† Hepatic involvement.

Table 2 lists the analyses for zinc and indicates that this element is significantly elevated in the uninvolved portions of livers with metastatic carcinoma. Zinc is increased by 112 percent in the noncancerous portion of the livers with tumor as compared with nontumorous livers. In two patients with carcinoma of the esophagus and with cirrhosis of the liver without metastasis, the zinc concentration was essentially the same as in the noncancerous livers. In these last two cases, the copper concentration was strikingly elevated (165 percent). Zinc has been determined by the method used with an accuracy of ±3.27 percent and copper with an accuracy of ±9.02 percent; thus, it would seem that these findings are of significance. In one specimen, it was difficult to separate all the tumor tissue from liver tissue, and this sample had the lowest concentration of zinc (53.0 ppm), suggesting that considerable tumor tissue was analyzed.

Molybdenum was increased by 37 percent in the liver tissue of patients dying with metastasis to the liver, which is of questionable significance. Manganese, chromium, and tin were present in measurable amounts, but no significant difference was noted in cancerous and noncancerous livers. Nickel, aluminum, silver, lead, and cobalt were present in a few samples, but in most they were below the level of detection for the method of analysis used. Perhaps if larger samples are analyzed or with increased sensitivity of the method, further studies may reveal significant changes in these elements.

In all the tumor tissue studied, the trace elements were markedly decreased as compared with either the liver tissue to which metastasis had occurred or with