100 percent phosphoric acid at 50°C in an "in-line" vacuum system connected to a mass spectrometer (V. G. Micromass model 602C). After measuring the isotopic composition of the released CO_2 against that of a known CO_2 reference gas, we calculated the oxygen and carbon isotopic values for the samples with respect to Pee Dee belemnite (per mil) by the usual procedure [H. Craig, *Geochim. Cosmochim. Acta* **12**, 133 (1957)]. The reference gas was calibrated against standard National Bureau of Standards sample NBS-20 limestone treated in the same way as the samples. The analytical precision expressed as $1 - \sigma$ (where σ is the standard deviation) for NBS-20 standard carbonate was 0.06 per mil, and the average difference between 60 duplicate foraminiferal samples was calculated at 0.12 per mil for $\delta^{18}O$ and 0.11 per mil for $\delta^{13}C$.

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18. An extremely high $\delta^{18}O$ value of +0.73 per mil was found at 33 cm in Bx 92 for *Pulleniatina obliquiloculata*. We were unable to duplicate this spike in other species or in a subsequent sample and have therefore omitted it from consideration.
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21. Core ERDC Bx 92 has been dated by the ^{14}C method in great detail by T. H. Peng in the laboratory of W. S. Broecker, Lamont-Doherty Geological Observatory. Dr. Broecker supplied the dates shown in Fig. 1 [see (*14*)].
22. A low salinity layer covering the ocean as a consequence of glacial melting and a consequent buildup of CO_2 and increase in carbonate dissolution was proposed by L. V. Worthington [*Meteorol. Monogr.* **8**, 63 (1968)].
23. We have disregarded the effects of mixing in this discussion, although we are aware of possible complications. For example, the ages of the particles that were analyzed for stable isotopes need not exactly coincide with the ^{14}C ages of the surrounding bulk sediments [see H. E. Suess, *Science* **123**, 355 (1956)].
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31. Research was supported by the National Science Foundation (Oceanography) under grant OCE 75-04335 and by the Office of Naval Research under contract USN N 00014-69-A-0200-6049. This is the first contribution of the Stable Isotope Sediment Laboratory at Scripps Institution of Oceanography. The laboratory was set up with NSF support (DES 75-04497, to W. H. Berger and M. Kastner) and with support from the Scripps Industrial Associates and from Chevron Oil, California. We are indebted to M. Hall (Cambridge University) for advice and to M. Sommer (Brown University) for his direct help in setting up operations. We thank E. Vincent for supervising sample preparation and for helpful discussions. We are grateful to F. B. Phleger, J. Gieskes, and M. Kastner for constructive criticism.

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Biochemical Identification of Homogentisic Acid Pigment in an Ochronotic Egyptian Mummy

Abstract. *Roentgenograms of an Egyptian mummy, dating from 1500 B.C., showed extensive calcification of the intervertebral discs and articular narrowing in both hip and knee joints. Biopsy cores from the right hip showed parallel black zones in the region of the articular surfaces, leading to a clinical diagnosis of ochrinosis. The black pigment was extracted, analyzed, and compared to an air-oxidized homogentisic acid polymer. The two substances apparently were identical. The chemical evidence thus confirms the clinical finding of ochronosis, an autosomal recessive disorder. This is, so far as known, the earliest verified case of this disorder.*

Ochronosis is a hereditary disorder in which arthritic symptoms appear in affected individuals because of the accumulation of a black pigment in cartilagenous and fibrous tissues (*1*). The formation of the black pigment has been linked to the metabolism of phenylalanine and tyrosine. The enzyme homogentisic acid oxidase is absent, and the normal degradation product of tyrosine accumulates. The homogentisic acid in turn undergoes oxidative conversion to quinone derivatives, and these polymerize to form the pigment. This disease has been suspected in Egyptian mummies because of characteristic roentgenographic findings (*2*). Although the black pigment has also been ascribed to the materials used in the process of mummification, the mummies were preserved by dehydration, usually with inorganic salts. Hence, this origin for the pigment appeared to be unlikely. A mummy with apparent arthritic symptoms and black pigment accumulation was available to us, and we have therefore carried out chemical studies to ascertain whether the pigment is related to homogentisic acid. This report is believed by us to be the first documentation by modern biochemical methods of a genetic disease in early civilized man.

The mummy Harwa served as a custodian of a granary in Egypt at approximately 1500 B.C. His cause of death is unknown. Roentgenograms suggest that he was in his early thirties. As is usual with many bodies in ancient Egypt, the internal organs were removed prior to mummification. For the past 60 years the mummy has been exhibited in the Field Museum of Natural History in Chicago. It was lent for study to Northwestern University Medical School.

The body was in a good state of preservation with only the face exposed. Roentgenograms of the entire body revealed extensive calcification in all of the intervertebral discs without secondary arthritic features (Fig. 1). Articular narrowing was present in both hip joints and both knee joints. With the use of fluoroscopic control with a Steinman pin, a percutaneous Craig needle biopsy of the right hip was performed.

The tissue obtained was two com-

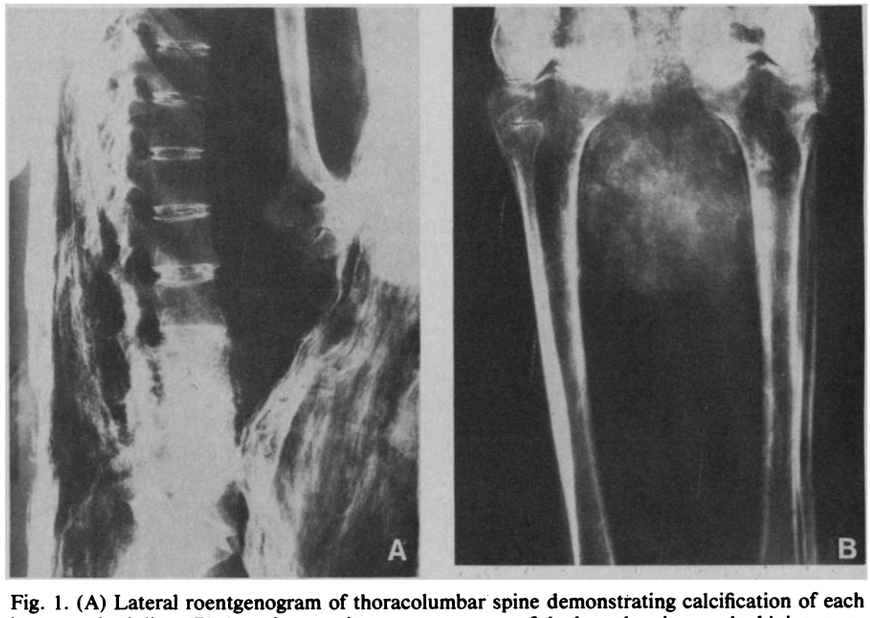
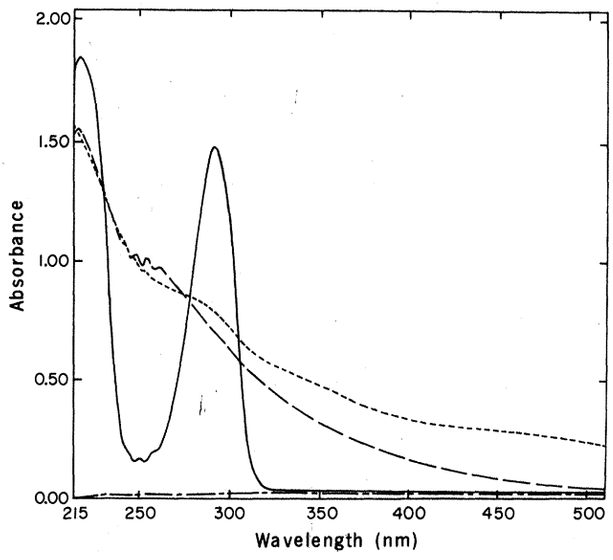


Fig. 1. (A) Lateral roentgenogram of thoracolumbar spine demonstrating calcification of each intervertebral disc. (B) Anterioposterior roentgenogram of the legs showing marked joint space narrowing at the knees bilaterally.



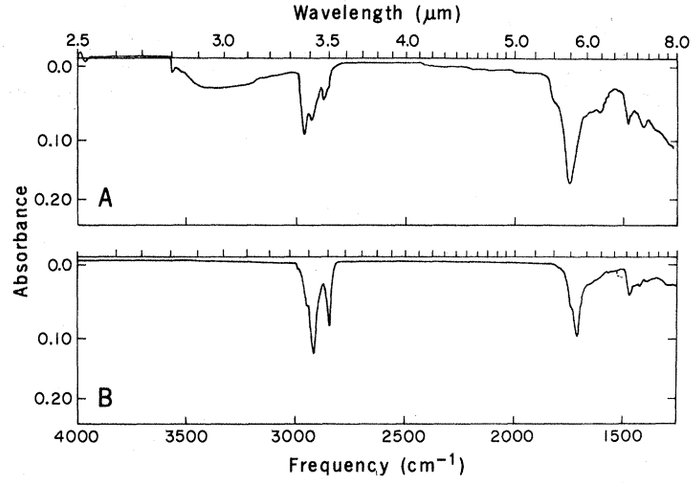


Fig. 2 (left). The ultraviolet spectra of untreated homogentisic acid (—); oxidized homogentisic acid (----); pigment extracted from affected areas of Harwa (— — —); and extract of nonpigmented cartilage (— - — -). Spectra were obtained in *n*-butanol with solute concentration at 0.1 mg/ml. Spectra were blanked with *n*-butanol. Fig. 3 (right). Infrared spectra of oxidized homogentisic acid (A) and pigment extracted from Harwa (B). Spectra were obtained on thin films spread from ether solutions directly on the sodium chloride cell windows. The films were air-dried.

pacted cores of dry bone. It was observed that in the portion of the biopsy that represented the articular surfaces of the acetabulum and femoral head, there were two black zones of a millimeter in width. These parallel zones were present in each of the two biopsy cores. These findings would lead, in the modern patient, to the diagnosis of ochronosis.

A core of Harwa's pelvic bone, weighing 489 mg and containing the black pigment and articular surface, was dissected with a scalpel to remove 11 mg of the most heavily pigmented region. An unpigmented section of the same biopsy core and the articular region of another mummified bone of unknown origin, but free of any evidence of disease or black pigment, served as controls. The bone shavings were added to 2 ml of 0.1*N* NaOH to dissolve any homogentisic acid or pigment. The pigmented bone suspension became a deep amber, whereas the two control sample suspensions remained pale yellow or clear. The suspensions were centrifuged to remove the insoluble material, and the supernatants were acidified to *p*H 1 with 1*N* H_2SO_4. The amber solution became cloudy as a precipitate developed. However, the dark pigment remained in solution.

The acidified supernatant was extracted with an equal volume of *n*-butanol, and most of the amber color was extracted into the organic phase. The phases were separated after 30 minutes. Ultraviolet and visible range spectra were determined directly on the butanol solutions. The *n*-butanol solutions were evaporated to dryness, and the dried pigment served as the starting material for other studies.

Homogentisic acid was polymerized by the method of Milch *et al.* (*3*). Homogentisic acid (Sigma, H-0751, grade II; molecular weight of the free acid was 168.1) was dissolved in 0.1*N* NaOH (1 mg/ml), and oxygen was bubbled through the solution. The solution became dark amber in color. When color development appeared to cease, the solution was either acidified to *p*H 1 with 6*N* HCl or treated with an equal volume of glacial acetic acid. The dark-brown pigment remained in solution both at room temperature and at 0°C. The pigment could be readily extracted from the acidic aqueous solution into an equal volume of *n*-butanol, but not into methylene chloride, benzene, or diethyl ether. The *n*-butanol extract was taken to dryness at 40°C on a Buchler Evapo-Mix. The dried residue, a synthetic "ochronotic" pigment, was used for comparison with the pigment obtained from Harwa's affected cartilage.

Gel filtration experiments were carried out with Bio-Gel P-2 (Bio-Rad, exclusion limit ~1800) and the pigment was dissolved in 0.025*M* triethylammonium–bicarbonate buffer, *p*H 8.5. Columns were monitored at 270 nm.

Infrared spectra were recorded with a Perkin-Elmer 237 B grating infrared spectrophotometer. The pigment, natural or synthetic, was dissolved in diethyl ether and then cast as a thin film on an NaCl window by evaporation of the ether solution.

The synthetic pigment was soluble in aqueous triethylammonium–bicarbonate buffer at *p*H 8.5, and in this solvent was excluded on Bio-Gel P-2. Homogentisic acid has a molecular weight of 168, and the column exclusion limit is ~1800; thus, it is clear that the oxidized homogentisic acid had undergone extensive polymerization. Many phenols are oxidized to dimeric and polymeric products by abstraction of a hydrogen atom from the phenol and coupling via carbon-carbon bonds, exclusively at positions ortho- and para- to the hydroxyl group (*4*). Hydroquinones, including homogentisic acid, are readily oxidized to semiquinones, which can then add nucleophiles—such as hydroxyl anions, amines, or phenolates—to yield hydroquinones, aminoquinones, or polymeric "humic-acid" products. This type of reaction apparently occurred in the alkaline oxygenated homogentisic acid solution to produce the polymeric product. The polymer was readily extracted into *n*-butanol from the aqueous phase after acidification with sulfuric acid.

The dark pigment in the mummy cartilage was also extractable from the tissue with aqueous alkaline solutions and could be extracted from that aqueous solution into *n*-butanol after acidification in a like manner. Bio-Gel P-2 chromatography of the mummy pigment, in triethylammonium–bicarbonate solution (*p*H 8.5), also yielded the extracted pigment in the column void volume.

The ultraviolet spectrum of the synthetic pigment and that of the extracted pigment are compared in Fig. 2. These spectra are virtually identical, and each has an absorbance maximum at 270 nm. Under the same conditions unpolymerized homogentisic acid has a very different spectrum.

The infrared spectra of the synthetic and extracted pigments are also similar,

if not identical (Fig. 3). The spectra show absorbances at 2800 to 3000 cm^{-1} and at 1465 cm^{-1} characteristic of methylene groups ($-CH_2-$), and at ~1720 cm^{-1}, characteristic of six- and seven-membered ring ketones, as is expected for an oxidized homogentisic acid polymer.

The synthetic and extracted pigments were stable to acid hydrolysis. Hydrolysis of the extracted pigment, in 6*N* HCl at 110°C for 20 hours, did not yield any nitrogenous ninhydrin-reactive components. Amino acid analysis of the extracted bone, on the other hand, showed that the bone matrix was typically collagenous. The infrared spectrum of the extracted pigments did not contain any absorption bands that could be attributed to amino acid or peptide components. It has been suggested that ochronotic pigment is covalently associated with protein or proteoglycan components of the connective tissue (*5*). The pigment isolated in the articular cartilage of Harwa, however, was readily extractable in dilute alkali and, as indicated above, was free of protein or amino acid components.

Normal human skin and cartilage contain metalloenzymes, homogentisic acid polyphenol oxidases, which appear to be responsible for the oxidation of accumulated homogentisic acid not normally metabolized. However, neither the chemical nature of the extract of the ochronotic pigment nor the mechanism of its biosynthesis is known. We have not, therefore, attempted a more complete characterization of either the synthetic or extracted pigments.

However, the above data show that the solubility and properties of the spectra of the extracted pigment from Harwa's cartilage are nearly identical with those of the synthetic pigment produced from the oxidative polymerization of homogentisic acid. We can thus conclude that the black pigment found in Harwa's joints and bones was derived from homogentisic acid and that Harwa, living at about 1500 B.C., suffered from the heritable disorder of ochronosis. The common observation of black pigments in Egyptian mummy cartilages suggests that this autosomal recessive disorder was of frequent occurrence in ancient Egypt, probably a result of frequent intrafamilial marriage.

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Mechanically Induced Wall Appositions of Plant Cells Can Prevent Penetration by a Parasitic Fungus

Abstract. *Localized, paramural wall appositions resembling appositions commonly induced by fungal attack, were induced in kohlrabi (*Brassica oleracea *L. gongyloides) by mechanical wounding (bending) of the root hairs before the hairs were inoculated with zoospores of a compatible parasitic fungus. The appositions were effective in preventing fungal penetration at the wound sites, which shows that wall appositions can prevent fungal ingress into plant cells.*

All living tissues respond characteristically to wounding. Plant tissues may respond morphologically by producing new cells and modifying existing cells at the periphery of the injured region, thereby containing the damage. The accompanying metabolic changes include increased respiration and altered phenolic and protein metabolism. Because these and other wound-healing responses closely resemble plant disease-resistance reactions, it is widely held that wound healing is a major disease-resistance mechanism (*1*).

Individual plant cells rapidly respond to both wounding and fungal attack by accumulating a mass of seething cytoplasm (cytoplasmic aggregate) at the site and depositing localized, amorphous, heterogeneous masses of materials paramurally—between their plasmalemmas and cell walls (*2*). The paramural depositions were termed wall appositions (*3*) and were postulated to operate as a defense mechanism against fungal ingress, although the evidence is not conclusive (*2*). By mechanically inducing wall appositions before inoculation and determining whether or not a fungus can penetrate them, I have obtained evidence that this widespread plant cell reaction can prevent fungal penetration.

The parasitic fungus *Olpidium brassicae* (Wor.) Dang. was maintained in roots of *Brassica oleracea* L. *gongyloides*, kohlrabi, as described previously (*4*). Zoospores of this fungus encyst on root hairs and initiate penetration 2.25 hours later by means of penetration tubes which grow through the cell walls into the host cells. The parasite protoplasts are then injected through the tubes into the host cytoplasm. Each cyst requires about 15 minutes to penetrate, and a synchronized population of cysts will normally complete penetration between 2.5 and 3.0 hours after inoculation. Host cytoplasmic aggregates and wall appositions are always associated with penetrations, and most appositions are initiated after the penetration tubes are fully extended (*4*).

Wall apposition formation was induced mechanically in hydroponically cultured kohlrabi seedlings (*4*). The seedlings were first taken from their growth-supporting salt solution and suspended in a moist chamber. Fluids adhering to the root axes bent the hairs near their bases and held them flat against the roots. Localized wall appositions (Fig. 1A) were deposited by cytoplasmic aggregates (*4*) along creases (wounds) in the walls where the hairs were bent. Thirty minutes later the roots were returned to the solution for an additional 30 minutes; deposition continued even though the root hairs straightened out in the solution. This procedure was then repeated with the roots of each seedling receiving a final incubation of 60 minutes in the solution, to ensure both a high frequency of well-formed appositions and complete termination of cytoplasmic aggregates before the next step. Some roots were then inoculated immediately with a suspension of *O. brassicae* zoospores (*4*), while others were heat-shocked at 41°C for 20 minutes (*5*) and then inoculated. Heat-shocking was done to inhibit new apposition formation (*5*) in response to pene-