

Biochemical Discovery of Homogentisic Acid Pigment in an Ochronotic Egyptian Mummy

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Introduction

In this report an infrared absorption technique was used to identify homogentisic acid pigment eluted from the hip cartilage of a 1500-year-old Egyptian mummy whose intervertebral discs showed the calcification characteristic of ochronosis. Ochronosis is a hereditary disorder of phenylalanine and tyrosine metabolism caused by the absence of homogentisic acid oxidase. Arthritis occurs in response to the accumulation of black homogentisic acid pigment in cartilaginous and fibrous tissues.

Alkaptonuria and Ochronosis

Historical comments

In the seventeenth and later in the nineteenth centuries, Scribonius,¹ Schenck,² Lusitanus,³ and Marcet⁴ reported the presence of dark urine in some patients. In 1859 Boedeker⁵ noted that such urine reduced Fehling's solution but not Nylander's solution (bismuth hydroxide) and that the addition of alkali brought out the dark coloration. For this reason, he named the condition "alkaptonuria" because of the avidity for oxygen in an alkaline solution. Ochronosis was so named by Virchow⁶ in 1866 after he discovered a grey to blue-black surface on the hip and knee joints of a 67-year-old man, in which the microscopic appearance of the cartilage suggested brown ochre. Five years later, in 1891, Wolkow and Baumann⁷ identified a specific chemical abnormality in the urine as homogentisic acid (2-5 dihydroxy phenyl acetic acid), which is related to gentisic acid (2-5 hydroxy benzoic acid). It remained for Albrecht⁸ in 1902 to show that ochronosis and alkaptonuria represented different components of the same disease. At the same time, Sir

Archibald Garrod⁹ pointed to alkaptonuria as a metabolic freak related to a specific enzyme defect. Then, two years later, Osler¹⁰ described alkaptonuria in two brothers. In 1941 Beadle and Tatum¹¹ elaborated a concept showing that in this disease state a defective gene results in a metabolic block of a single enzyme reaction. Reisch and Zander¹² in 1955 brought attention to the distinctive appearance of the intervertebral discs. More recently, intervertebral calcification suggesting ochronosis has been reported in Egyptian mummies by Simon and Zorab,¹³ Wells,¹⁴ and Gray.¹⁵

Incidence

The disease is due to a single recessive autosomal gene and occurs in consanguineous marriages. O'Brien, *et al*¹⁶ collected 604 cases in a world review of the literature from 1584 to 1962. Dent¹⁷ reported its incidence as 1 in 100,000 and Sacks¹⁸ as 1 in 10 million.

Biochemistry

It is now known that in man the amino acids tyrosine and phenylalanine are normally reduced to homogentisic acid, which in turn is reduced by homogentisic acid oxidase to maleyl acetoacetic acid; this is further reduced to fumaric and acetoacetic acid. In the absence of the specific enzyme homogentisic acid oxidase, which is normally produced by the kidney and liver, benzoquinone acetic acid is produced. This agent polymerizes and becomes bound to connective tissue macromolecules, thereby resulting in tissue changes associated with ochronosis.

Symptoms

Although ochronosis is inherited, manifestations in youth are rare. However, in the asymptomatic phase the first clue to the diagnosis could be the darkening of the urine to brown or black after it stands for some time.

In patients over 30 years of age who have alkaptonuria, pigment is deposited in the cornea, conjunctiva concha, antihelix, tragus of the ear, the tympanic membrane, underlying tendons beneath the skin of the hands, in the costal, laryngeal and tracheal cartilages, in fibrocartilaginous tend-

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ons and ligaments, especially the articular cartilage of the shoulder, hip and knee joints, in the endocardium and intima of the large vessels, and in the sweat and skin about the axillary and genital regions. The urine becomes black on standing or when it is alkalinized.

The spine is most frequently involved with a narrowing of the intervertebral discs (Figure 1) related to pigment deposition, deterioration and possible disc herniation, loss of height, loss of flexibility of the spine, and often calcification of numerous disc spaces. Clinically, the sacroiliac joints are not involved and osteophytosis is not a feature, though some stiffness and rigidity of the spine may occur.

Deposition of pigment in the articular cartilage occurs at most peripheral joints, but the knee, hips and shoulders are the major sites of degenerative changes and symptoms of osteoarthritis.

Prostatic calculi are common in men and are also pigmented.

Case Report

Our subject was the mummy Harwa, who served as a custodian of a granary in Egypt about 3500 years ago. According to roentgenograms, he was in his early thirties when he died from unknown causes. Like many bodies in ancient Egypt, the internal organs were removed before mummification. For the past sixty years, this mummy has been exhibited in the Field Museum of Natural History in Chicago, but it was loaned for study to physicians at the Northwestern University Medical School.

The body was well preserved and only the face was exposed. Roentgenograms of the entire body revealed extensive calcification in all intervertebral discs without secondary arthritic features (Figures 2 and 3). Articular narrowing was present in the hip and in both knee joints.

Using fluoroscopic control with a Steinman pin, a percutaneous Craig needle biopsy of the right hip was performed and two compacted cores of dry bone were removed. In the portion of the biopsy which represented the articular surfaces of the acetabulum and femoral head, two black zones, each a millimeter wide, were observed. These parallel zones were present in each of the two biopsy cores.¹⁹ In the modern patient, these findings would lead to a diagnosis of ochronosis. Biochemical analysis²⁰ was undertaken to substantiate this diagnosis in Harwa's case.

Materials and Methods

Mummified specimen (Extracted pigment)

A sample of pelvic bone, weighing 489 mg, was received and stored at room temperature. A clean scalpel was used to

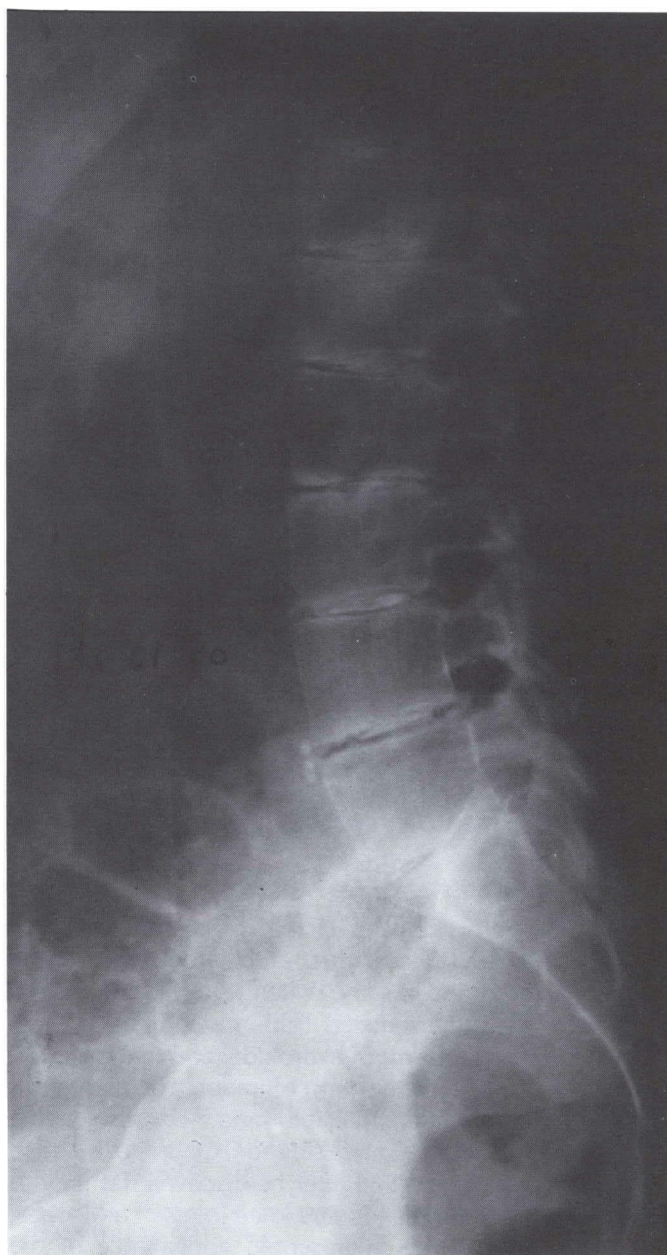


Fig. 1
Wafer-like deposits of calcium in intervertebral lumbar cartilages in ochronotic patient of Howard Duncan, MD, Division of Rheumatology, Henry Ford Hospital.

remove 11 mg of the most heavily pigmented area. Another portion of the biopsy core served as a control. Two ml of 0.1 N NaOH were placed in a glass centrifuge tube and as the bone shavings were added, the solution became amber. After ten minutes, the suspension was centrifuged to remove the insoluble material. The supernatant was acidified to a pH of 1 with 1 N H₂SO₄. The amber solution became cloudy. An equal volume of n-butanol was added and most of the

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Fig. 2 Harwa. Lumbar spine

Wafer-like plaques of calcium in intervertebral spaces. From Department of Radiology, Lee F. Rogers, MD, Northwestern University Medical School.

amber color was extracted into the organic phase. After thirty minutes, the phases were separated and the ultraviolet and visible spectra of the n-butanol extract were recorded. For infrared studies, the organic phase was evaporated to dryness. The dried residue, designated as extracted pigment, was dissolved in distilled diethyl ether and thin films were prepared by casting on NaCl windows. The polymeric nature of the extracted pigment was examined by gel filtration. A portion of the extracted pigment was hydrolyzed.

Synthetic pigment

A synthetic "ochronotic-like" pigment was prepared. Homogentisic acid was polymerized by the method of Milch, et

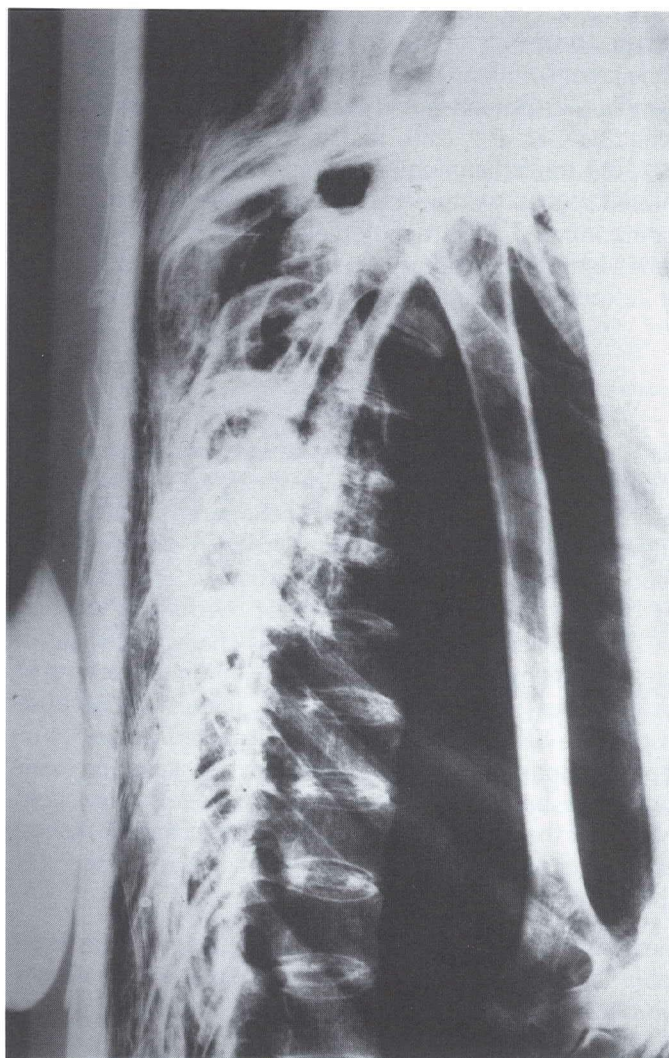


Fig. 3 Harwa. Lumbar spine, oblique view

Characteristic chondrocalcinosis of ochronosis. From Department of Radiology, Lee F. Rogers, MD, Northwestern University Medical School.

a^{21,22} as follows. Homogentisic acid (Sigma, H-0751, grade II, free acid molecular weight of 168.1) was dissolved in 0.1 N NaOH (1 mg/ml) and oxygen was bubbled through the solution. Acidification to pH 1 with 6N HCl or the addition of an equal volume of glacial acetic acid failed to precipitate the dark brown pigment, either at room temperature or at 0° C. Following acidification to pH 1 with 1 N H₂SO₄, however, the pigment was readily extracted into an equal volume of n-butanol, but it was insoluble in either methylene chloride, benzene, or diethyl ether. The ultraviolet and visible spectra of the n-butanol solution of this synthetic pigment were recorded in 1 cm-path quartz cells using a Beckman DB-GT recording spectrophotometer. For infrared studies, the n-butanol was evaporated to dryness at 40°C on a Buchler Evapo-Mix. The dried residue was dissolved in distilled

diethyl ether. A thin film of the synthetic pigment was prepared on the surface of the NaCl window by evaporation of the ether solution. The polymeric nature of the synthetic pigment was demonstrated by exclusion on gel filtration using Bio-Gel, P-2 (Bio. Rad, exclusion limit 1,800), in 0.025 M triethylammonium bicarbonate buffer, pH 8.5. Infrared spectra were recorded using a Perkin-Elmer 237B grating infrared spectrophotometer. A portion of the synthetic pigment was hydrolyzed in 6 N HCl *in vacuo* at 108°C for 22 hours and the acid was removed by evaporation.

Results

The ultraviolet spectra of the synthetic pigment and the extracted pigment are virtually identical, each showing an absorbance maximum at 270 nm. The infrared spectra of the synthetic and extracted pigments are also similar, if not identical. These spectra show absorbances at 2800-3000 cm^{-1} and 1465 cm^{-1} indicative of methylene groups ($-\text{CH}_2-$), and at $\sim 1720\text{cm}^{-1}$ which is characteristic of six and seven-membered ring ketones. The infrared spectrum of the extracted pigment is particularly interesting because absorbance bands which would be attributed to amides or peptide bonds are absent. Thus, the extracted pigment appears to be free of protein components. Amino acid analysis of the extracted bone, on the other hand, showed that the matrix from which the pigment was extracted was typically collagenous.

The synthetic pigment and extracted pigment were both stable to acid hydrolysis under conditions which would hydrolyze proteins and polysaccharides.

Discussion

The ochronotic pigment observed in connective tissues of affected alkaptonuric individuals is assumed to be a polymer derived from the substituted hydroquinone, homogentisic acid. The exact chemical nature of the pigment and the mechanism of biosynthesis in ochronosis are unknown. Zannoni, *et al*^{23,24} have demonstrated that normal mammalian skin and cartilage contain metalloenzymes, called homogentisic acid polyphenol oxidases, which catalyze the oxidation of homogentisic acid to an ochronotic pigment. These authors have proposed one possible pathway for the *in vivo* oxidation of homogentisic acid via benzoquinone acetic acid to an ochronotic pigment in the connective tissues of affected individuals. In an attempt to elucidate a possible mechanism for the *in vivo* formation of ochronotic pigment, several investigators have examined the *in vitro* oxidation of homogentisic acid solutions alone and in the presence of chondroitin sulfate or biological amines.

The homogentisic acid, treated with oxygen gas in the presence of 0.1 N NaOH according to the procedure of Milch, *et al*,^{21,22} produced a dark brown pigment. This synthetic pigment was soluble in aqueous triethyl ammonium bicarbonate buffer at pH 8.5, and in this solvent was excluded on Bio-Gel P-2 indicating that polymerization had occurred. Homogentisic acid has a molecular weight of 168; the column exclusion limit is ~ 1800 . 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. 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 occurred in the alkaline oxygenated homogentisic acid solution to produce a polymeric product. The polymer was extracted into n-butanol from the aqueous phase after acidification with sulfuric acid.

The dark pigment in the mummy tissue was also extractable with an aqueous solution and could be extracted from that aqueous solution into n-butanol after acidification. Bio-Gel P-2 chromatography of the n-butanol solution showed the extracted pigment to appear in the column void volume.

The solubility characteristics and spectral properties of the extracted pigment are similar to those of the synthetic pigment produced from the oxidative polymerization of homogentisic acid. In the mummified remains available to us, there was no evidence that the ochronotic pigment was covalently associated with protein components of the connective tissue.

Summary

The demonstration of homogentisic acid pigment in the cartilage of Harwa, the Egyptian mummy, convinces us that his basic disease was ochronosis. It is clear that infrared absorption studies will need to be done as a specific biochemical test on the intervertebral cartilages of other Egyptian mummies reputed to have ochronosis.

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