

# MICROSCOPIC DETERMINATION OF BONE PHOSPHORUS BY QUANTITATIVE AUTORADIOGRAPHY OF NEUTRON-ACTIVATED SECTIONS

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## ABSTRACT

Longitudinal sections of human cortical bone were submitted to thermal neutrons.  $\gamma$ -ray spectra were recorded repeatedly during 15 days following irradiation. They showed that  $\text{Na}^{24}$  is predominant as early as 3 hours after activation and that all the  $\gamma$ -emitters have decayed on the 15th day. When the  $\gamma$ -rays have disappeared,  $\beta$ -rays are still produced by the sections. It was proved by the absorption curve in aluminium that all these  $\beta$ -rays are issued from the  $\text{P}^{32}$  induced in the sections by activation of  $\text{P}^{31}$ . Therefore autoradiograms registered 15 days after activation reveal the distribution of  $\text{P}^{32}$  in the sections.  $\gamma$ -ray spectra and  $\beta$ -ray absorption curves of neutron activated sections of ivory demonstrated a mineral composition similar to that of bone. Autoradiograms of ivory sections activated for various times were used to establish the relation between the optical density of the autoradiograms and the radioactivity in  $\text{P}^{32}$ . When the bone autoradiograms are compared with the ivory standards of known radioactivity, the optical densities of single osteons (Haversian systems), can be related to their phosphorus contents. Autoradiograms and microradiograms of the same sections were examined side by side. The least calcified osteons, that contain 80 per cent of the calcium of the fully calcified osteons, also contain about 80 per cent of the phosphorus of the fully mineralized osteons. It is concluded that the Ca:P ratio remains constant while mineralization of bone tissue is being completed.

## INTRODUCTION

Microradiography has demonstrated considerable variations in quantitative distribution of the mineral salts in compact bone. The relative calcium contents in different Haversian systems (or osteons) have been measured by densitometry of microradiograms (1, 4-8).

The present study aims to provide similar information about bone phosphorus. The use of monochromatic x-rays has yielded some quantita-

tive determinations of phosphorus in single osteons (4).

If it were possible to transform selectively stable phosphorus present in bone sections into radioactive phosphorus, the autoradiographic record of the  $\beta$ -rays would provide histological maps of the phosphorus contents in the sections. Actually this transformation may be performed by bombarding the stable isotopes of a compound specimen with nuclear particles. The elements present

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become radioactive isotopes, and this process is called activation. In practice, the activation is done with the aid of thermal neutrons in a nuclear reactor. The isotopes induced in the reactor emit radiations with characteristic energies and half-life, and their amounts are dependent upon the concentration of the original elements. The composition of a neutron-activated specimen may be determined by the qualitative and quantitative analysis of the activated elements.

Neutron activation of bone specimens was applied at first to sodium analysis, since the spectrum of  $\text{Na}^{24}$  prevails a few hours after irradiation (3). Autoradiograms of cortical bone obtained 3 hours after removal from the reactor show that the least calcified osteons are less radioactive, and therefore contain less sodium than the fully calcified osteons (11). The quantitative estimation of these differences in sodium contents cannot be relied upon, because about a quarter of the darkening is actually due to other radio-elements, even 3 hours after activation (12).

On the other hand, when  $\text{Na}^{24}$  (half-life, 15 hours) induced in the sections has decayed,  $\text{P}^{32}$  (half-life, 14.3 days) is still present and may be localized on photographic emulsions, as exemplified by autoradiograms of whole rats exposed to thermal neutrons (2).

It is suggested that neutron activated sections of compact bone, when autoradiographed, together with standards of known radioactivity in  $\text{P}^{32}$ , can be a useful material for relative measurements of bone phosphorus at the histological level.

#### MATERIALS AND METHODS

Human cortical bone from ribs or tibiae, removed by surgery or at autopsy, was fixed in 96 per cent ethanol. Longitudinal sections were cut at about  $40 \mu$  with a thin-sectioning machine (Gillings-Hamco). The thicknesses of the sections were not equal or uniform and each was carefully measured with a micrometer gauge.

The sections were washed twice in distilled water and then dried.

Microradiograms were registered on Spectroscopic films 649-GH (Eastman Kodak Co., Rochester, New York) by an exposure of 12 minutes to x-rays generated by a Philips apparatus set at 5 kv and 1.8 ma.

The sections were submitted for 4 hours to a flux of  $3.3 \times 10^{11}$  thermal neutrons/centimeter<sup>2</sup>/second in Trico (Triga Reactor, General Atomic).

The  $\gamma$ -rays emitted by the bone samples were studied in a 400 channel analyzer<sup>1</sup> equipped with a lead-shielded crystal of 3 x 3 inches. Spectra were recorded at 15 minutes, at 3, 16, and 50 hours, and at 6 and 15 days after the end of activation ( $t_0$ ).

Since the  $\gamma$ -activity had completely decayed 15 days after activation, the remaining  $\beta$ -activity was later analyzed easily by the absorption curve in aluminium. The measurements were made through absorbers (model C-101, Nuclear-Chicago Corp., Chicago) by a lead-shielded Tracerlab G. M. counter with a 2.5 mg/cm<sup>2</sup> window and by a Baird atomic scaler (model 123-A).

On the 15th day after activation, the bone sections were sandwiched between two Scientia 19 D 50 Films (Gevaert). When exposed for 48 hours, the films were developed in D19b, and mounted in Canada balsam under a coverslip.

After unsuccessful attempts to include sufficient amounts of phosphorus in hard compounds suitable for cutting, ivory was chosen as reference for the autoradiographic studies.<sup>2</sup> Actually bone and ivory are very similar in their hardness and their crude mineral composition, but we observed by means of microradiography that the mineral salts are distributed more uniformly in ivory than in compact bone. At the magnifications used for our microradiographic analysis of bone sections, ivory does not reveal any significant variations of mineral density.

Ivory pieces of unknown origin were cut and washed the same as the bone samples.

Ivory sections were put into the reactor at intervals of 15 minutes. One ivory section was put into the reactor at the same time as the bone sections. In this way, when all the ivory sections were removed from the reactor, the durations of irradiation ranged from 2 to 6 hours (4 hours for the bone sections), and  $t_0$  was identical for all the sections.

$\gamma$ -ray spectrography and determination of  $\beta$ -ray absorption were performed on ivory using the methods previously described for bone specimens.

The decay of the radioactivity in ivory sections was followed by measurements of the total activity of the sections at successive intervals after activation; the equipment utilized was the same as for

<sup>1</sup> We are grateful to Professor R. Loos who kindly helped us with these studies and reviewed our manuscript.

<sup>2</sup> Following the suggestion of L. Coutelier.

the determination of the  $\beta$ -ray absorption curves but with 1.9 mg/cm<sup>2</sup> window in the G. M. counter. The  $\gamma$ -ray coefficient was determined by similar measurements through a plexiglas absorber (800 mg/cm<sup>2</sup>). The  $\beta$ -activity was derived from the differences between both measurements.

For autoradiography, the ivory sections were placed among the bone sections on the same film in order to secure identical conditions of exposure, pressure, and photographic processing.

Densitometric analysis of autoradiograms was performed with a Lison's histophotometer (Electrophysique). The background of the film outside the imprints of bone and ivory gave the zero value of the optical density. The zero was repeatedly calibrated while reading the densities of the radioactivity imprints.

The darkening produced by the ivory sections appeared to be remarkably constant in areas 1 mm in diameter. Nevertheless it happened that variations were noted between more distant regions of the same imprint. These discrepancies were found to result from variations of the thickness of sections ranging from 35 to 80  $\mu$ .

Random areas were selected in the ivory sections and placed under a 1 mm wide opening of a plexiglas absorber (800 mg/cm<sup>2</sup>). The radioactivity of each area was measured with the equipment used for the analysis of the  $\beta$ -rays. The results were also dependent on the thickness of the measured areas.

Thus it was necessary to correct all the readings in order to refer them to a standard thickness; *i.e.*, 40  $\mu$ . The figures provided by the histophotometer and by the counter were multiplied by 40 and then divided by the thickness expressed in microns of the measured area. After these corrections, the relation between the activity expressed in counts per minute and the duration of the irradiation appeared to be linear.

It can be assumed that auto-absorption, although present, does not influence the interpretation of our results. Indeed, when ivory sections were activated for the same duration, their radioactivity was found to be proportional to their thickness, within the limits of experimental error.

For the photometry of the bone autoradiograms, the optical density was read 5 times in very restricted areas chosen around precise landmarks and corresponding to given areas of the micro-radiogram.

Furthermore, measurements were made step-

by-step along lines crossing the field of the autoradiogram between two landmarks. The stage of the microscope was moved by a motor at the approximate speed of 10  $\mu$  per second, and the motor was stopped every 4 seconds in order to read the optical density.

## RESULTS AND DISCUSSION

It is expected that many radioisotopes are produced in bone and ivory sections by neutron activation. Soon after activation, the  $\gamma$ -ray spectra show numerous peaks that we did not identify. Since most of these radio-isotopes have a short period, the spectra are interpreted more easily a few hours after irradiation.

Fig. 1 reproduces the spectra of bone and ivory recorded 50 hours after activation. It will be noted that bone and ivory give identical energy peaks, confirming the close similarity of the mineral composition of both tissues. Actually the  $\gamma$ -ray spectra of bone and ivory do not differ very much whenever they are registered.

The peaks of Na<sup>24</sup> (1.37 and 2.76 Mev) are clearly recognized in Fig. 1. As early as 3 hours after activation, Na<sup>24</sup> represents more than 90 per cent of the total  $\gamma$ -activity.

Autoradiograms obtained at this time demonstrate chiefly the distribution of Na<sup>24</sup> (11). But at the same time as recording the  $\beta$ -rays of Na<sup>24</sup>, the autoradiograms record also the  $\beta$ -rays of P<sup>32</sup>, which isotope escapes to  $\gamma$ -ray spectrography (12).

The mean half-life of the  $\gamma$ -emitters induced in ivory sections was found to be 14 hours, a value that recalls the half-life of Na<sup>24</sup> (15 hours).

It must be emphasized that the 15-day spectra do not reveal any  $\gamma$ -emitter in either bone or ivory.

The  $\beta$ -rays issued from bone and ivory were studied on the 18th day following activation. The absorption curves in aluminium coincide exactly for bone and ivory. Calculations of the  $\beta$ -ray energy, derived from these curves, give a value of 1.7 Mev ( $\beta$ -energy of P<sup>32</sup> is 1.71 Mev). Furthermore, a sample of phosphorus has been neutron activated under the same conditions as the biological samples. All three  $\beta$ -absorption curves are parallel, as illustrated by Fig. 2.

The half-life evaluated for the  $\beta$ -emission in ivory is very close to that of P<sup>32</sup> (14 days instead of 14.3 days).

It can be concluded from these convergent physical data that P<sup>32</sup> is the only radio-isotope that accounts for the autoradiographic image recorded

two weeks after neutron activation of bone and ivory.

Fig. 3 shows that the optical densities of autoradiograms of ivory sections can be related to their  $\beta$ -activity. The values were corrected for the thickness of the sections. For our purpose, the relation between the optical density and the radio-

Fig. 4 *a* presents the microradiogram of a longitudinal section of the tibial cortex from a 25-year-old man. This x-ray picture shows the distribution of the calcium salts. Densitometric studies of such bone microradiograms have been reported from different laboratories (1, 4-8). They assess for the least calcified osteons a percentage of about 80

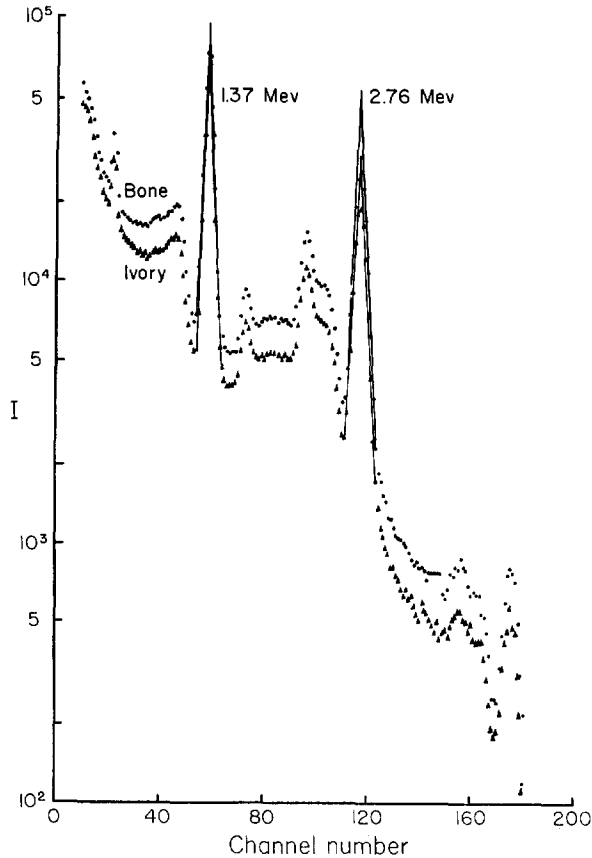


FIGURE 1  $\gamma$ -ray spectra of bone and ivory sections, as recorded 50 hours after neutron activation. The peaks of  $\text{Na}^{24}$  are predominant in both bone and ivory.  $t_0 + 50$  h. Na I 3"; H.V. 1105 V; C.G. 4 A.G. 1/32.

activity may be considered as linear. The straight line of Fig. 3 was calculated according to the method of least squares. It must be pointed out that this Fig. 3 cannot be extrapolated for lesser or higher values of optical density. At least in the range of the values that we found in our autoradiograms, the error due to a slight deviation of this curve would be obviously negligible for the significance of our results. This key diagram will permit us to translate the relative densities read in the bone autoradiograms into terms of activities.

per cent of the calcium amount found in the fully calcified osteons.

The autoradiogram of Fig. 4 *b* must be referred to the microradiogram of the same section. Even with the naked eye, this combined illustration demonstrates that the darkening in 4 *b* is weaker in the areas of poor calcification in 4 *a*. Since some osteons are cut tangentially outside their Haversian canals, the reduced darkening cannot be attributed to haziness of the wall of an empty cavity. In transverse sections, it is often hazardous to distinguish on the autoradiograms the Haversian canal itself from the surrounding calcified tissue.

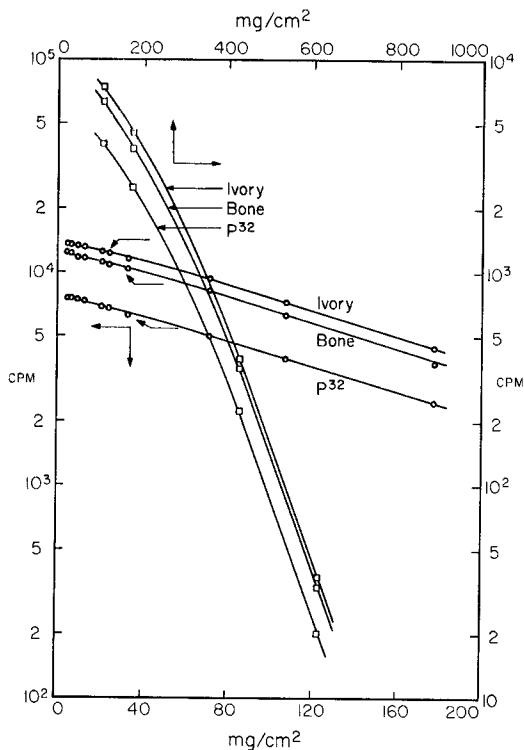


FIGURE 2 Absorption curves in aluminium of the  $\beta$ -rays emitted by bone and ivory sections and by a phosphorus sample on the 18th day after neutron activation. All three curves are parallel, indicating that the  $\beta$ -rays from bone and ivory are due to  $P^{32}$ .

The densitometric evaluation of the darkening in various parts of the autoradiograms has provided maximal differences of 20 per cent between the least and the fully calcified osteons. This percentage is derived from mean figures each obtained by 5 successive readings in minute areas of bone tissue. Each reading covers a circle of about  $20 \mu$  in diameter ( $900 \mu$  at the magnification of Fig. 4). The extreme mean values found by this means in the bone autoradiograms are reported on Fig. 3.

Other densitometric results, just as they were observed along a line crossing the field of Fig. 4 noted under the autoradiogram and expressed in terms of percentages of the full load of phos-

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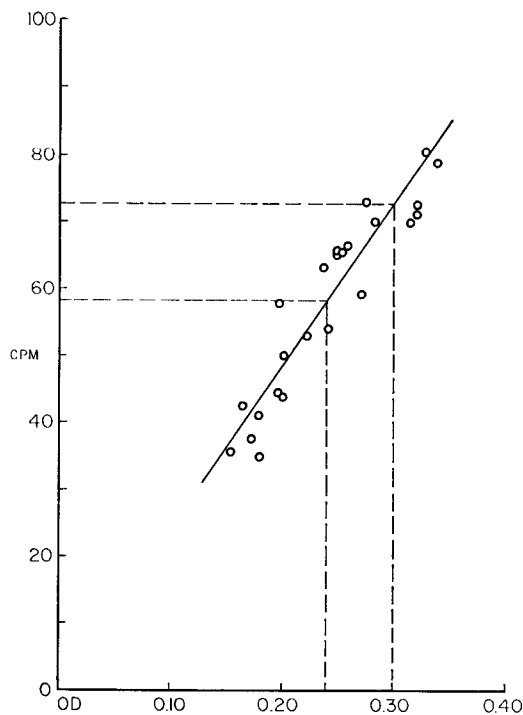


FIGURE 3 Diagram illustrating the relation between the optical densities of ivory autoradiograms and the radioactivity in  $P^{32}$ . The extreme values corresponding to different osteons in bone autoradiograms are reported on the diagram.

phorus. They confirm the visual impression and the previous measurements.

Since the radioactivity is evidently proportional to the amount of phosphorus existing in each unit of bone volume, our data indicate that the load of phosphorus keeps pace with the load of calcium in a given osteon. The Ca:P ratio remains constant while the mineralization of bone tissue is being completed.

Our conclusions are in good agreement with the microchemical studies carried out by Strandh (9, 10) on pools of microdissected osteons selected according to their microradiographic densities.

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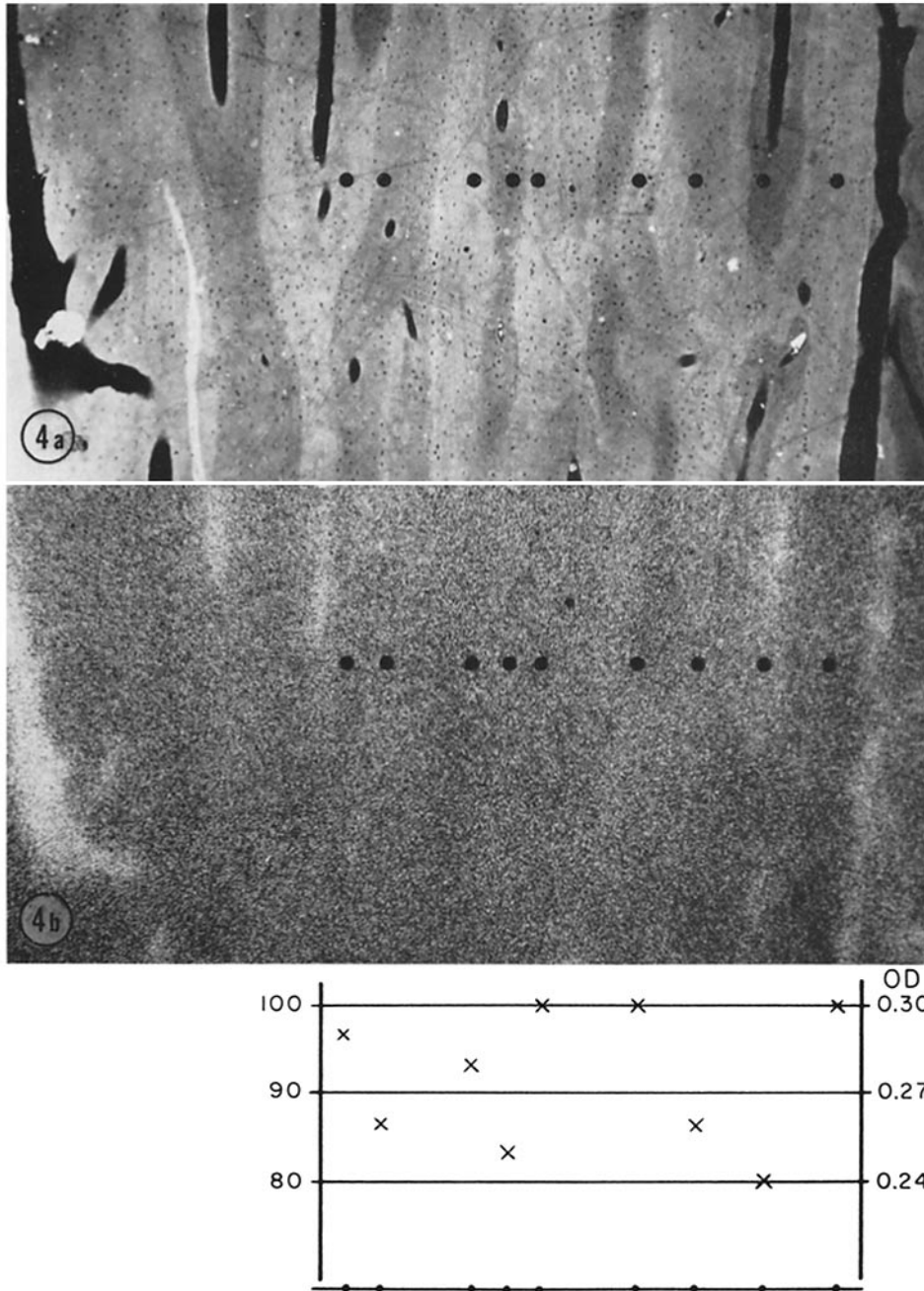


FIGURE 4 Longitudinal section of the tibial cortex from a 25-year-old man. The microradiogram (4a) must be compared with the autoradiogram registered 15 days after neutron activation (4b).  $\times 45$ .

As examples, some densitometric measurements (OD) made in different areas of the autoradiogram and expressed in percentages of the full load of phosphorus are to be related to points marked along a line crossing the field of both pictures and referred on the base line of the diagram.

This illustration demonstrates that the amount of phosphorus parallels the amount of calcium in a given osteon.

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