HISTORADIOGRAPHIC IDENTIFICATION OF ALKALINE PHOSPHATASE

ARTHUR J. HALE. From the Division of Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, England

The technique of historadiography (Engström, 1946) has been used extensively for investigations of the distribution of dry mass and of naturally occurring elements of high atomic number (> 14) in tissues (Engström and Lindström, 1958). Remarkably little use, however, has been made of its possibilities as a technique for the localization and measurement of suitable elements deposited in tissues during staining reactions.

In the classical Gomori-Takamatsu reaction for

alkaline phosphatase the sequence of events is that the enzyme is allowed to hydrolyse a phosphate ester in the presence of calcium. Calcium phosphate is thus deposited at the site of activity but as it is colourless it is converted first to colourless cobalt phosphate and then to black cobalt sulphide.

It is possible to visualize the calcium phosphate at the site of deposition by means of the interference microscope. If sufficient calcium phosphate is deposited, then the resulting change in optical path is seen as a change in light intensity in monochromatic light or in colour in white light (Barter, Danielli, and Davies, 1956; Davies, Barter, and Danielli, 1954). The morphological result is not striking and the preparation is not permanent.

With historadiographic methods it proved possible to localize chromium deposited in the tissues during the chromaffin reaction (Hale, 1958), and thus I decided to investigate the possibility of studying the deposition of enzymically produced calcium phosphate by the same method (Hale, 1961).

MATERIALS AND METHODS

Kidneys from rats, killed by a blow on the neck, were fixed in buffered 10 per cent neutral formalin at 0–4°C for 24 hours. Pieces of tissue were then transferred to, and stored in, a 0.88 M sucrose and 1 per cent gum acacia mixture at 2°C to which some thymol had been added. Frozen sections approximately 4 μ in thickness were cut and incubated in the following mixture for varying times at room temperature.

20 ml 2 per cent diethylbarbiturate
20 ml 3 per cent sodium β-glycerophosphate
40 ml 2 per cent calcium chloride
10 ml distilled water
2 ml 5 per cent magnesium sulphate

After incubation the sections were then

(*a*) Mounted on x-ray plate as described below, or
(*b*) Placed in a 2 per cent solution of cobalt nitrate for 4 minutes and then mounted on x-ray plate, or
(*c*) Carried through (*b*), then immersed in a weak solution of ammonium sulphide for 2 minutes and then mounted on x-ray plate.

Control sections were incubated after heating at 60°C for 1 hour or by using a substrate from which the calcium chloride had been omitted.

Sections were floated, in the darkroom, directly from the last solution used, on to small pieces of Kodak maximum resolution plate which had previously been coated with nitrocellulose. The preparations were hung in the dark over phosphorus pentoxide for 24 hours to dry.

The apparatus for the production of ultra-soft x-rays in the 8 A wavelength region is essentially that of Engström and Lundberg (1957). Specimens were exposed to x-rays produced from a copper target at 1.5 kv and 1.5 mA and filtered through an aluminium window approximately 1000 A in thickness. Exposure times of approximately 90 minutes were used.

After exposure the sections and the protective nitrocellulose coating were removed by washing in an alcohol-ether mixture. The plate was then washed thoroughly in water and developed in Prodox (May and Baker) for 1.5 to 2 minutes. The plate was then washed, fixed in Amfix (May and Baker), washed again, dried in air and mounted with DePeX and a coverslip as with a histological section. It can then be examined under the microscope. Absorption, which is related to the amount and type of elements in the tissue, is seen as bright areas in the preparation.

RESULTS

The deposition of calcium phosphate produced by incubation in the initial substrate mixture alone is readily observed in the brush border of the convoluted tubules (Figs. 1 and 2). It is clearly localized at that site and even after prolonged incubations (up to 3 hours) shows no sign of diffusion. Conversion of the calcium phosphate to cobalt phosphate by exposure of the section to cobalt chloride after incubation in the substrate gives the picture shown in Fig. 3. The distribution is similar to that of calcium phosphate (close examination indicates that some structures other than the brush border, but adjacent to it, may have taken up cobalt phosphate). Conversion of cobalt phosphate to cobalt sulphide produces a picture in which the brush border is still heavily "stained," but considerable absorption is shown by surrounding structures (Fig. 4).

DISCUSSION

It has been shown by Barter *et al.* (1956) that calcium phosphate does not diffuse away from the site of activity of alkaline phosphatase. The present investigation confirms this finding. The similarity of the picture obtained with calcium phosphate and cobalt phosphate means either that the conversion of the former to the latter is good and occurs without diffusion happening to any degree or that very little conversion has occurred at all. The diffusion which occurs on conversion to cobalt sulphide is similar to that seen when exam-

ining the black cobalt sulphide under the light microscope. It is, in general, more difficult to get a good picture of the deposition of cobalt sulphide than of calcium or cobalt phosphate. This may be due either to poor conversion of cobalt phosphate or to diffusion of the cobalt sulphide from the site of conversion. Sometimes the sections used to obtain the image of cobalt sulphide were heavily stained when they were mounted on the x-ray plate, yet the x-ray absorption picture was poor. Thus it appears that black staining by cobalt sulphide can be obtained with an amount of that material which is not readily identifiable by the x-ray method.

The specific extinction coefficients of P, S, Ca, and Co are such (Henke, White, and Lundberg, 1957) that one would expect heavy absorption of x-rays of the wavelength which I have used. The absorptions of each element would be in the ratio 0.22:0.28:0.58:1.0, respectively, at a wavelength of 8.34 A. Changes in degree of absorption on conversion of calcium phosphate to cobalt phosphate to cobalt sulphide will depend on the efficiency of the conversion under the conditions of the technique. With the present equipment it is not possible to study these changes. Quantitative studies with monochromatic x-ray at the absorption peaks of P, S, Ca, and Co would, however, resolve these problems.

In addition to providing a direct demonstration of the deposition of calcium phosphate at the site of enzyme activity, the technique should help quantitative study of the kinetics of alkaline phosphatase. It has been pointed out by Barter and his colleagues that measurement of the amount of calcium phosphate deposited in unit time is not a measure of enzyme activity but that measurement of the amount of phosphate liberated, or deposited, is. They could not calculate precisely the amount of phosphate deposited as they did not know the form in which calcium phosphate was present. Table I shows the formulae of the various forms of calcium phosphate which might be deposited. It also shows the percentage weights of calcium and phosphorus in each of the compounds listed. Using a bent crystal x-ray monochromator (Lindström, 1955), it is possible to detect 0.3 $\times$ 10^{-12} g of calcium or phosphorus in an area of 1 square micron in a histological section 10 μ in thickness by historadiography. Using the interference microscope, an amount of calcium phosphate of the order of 15 $\times$ 10^{-12} g per μ^2 is deposited in sections 10 μ in thickness after they have been incubated in substrate for alkaline

TABLE I

Formulae and Percentage Weight of Phosphorus and Calcium of Different Forms of Calcium Phosphate

Formulae	Atomic weight as percentage of molecular weight		
	P	*Ca*	*ratio P/Ca*
$CaHPO_42H_2O$	18.0	23.3	0.776
$Ca(H_2PO_4)_2H_2O$	24.6	15.9	1.657
$Ca_2P_2O_75H_2O$	18.0	23.3	0.776
$Ca_3(PO_4)_2$	20.0	38.8	0.517
$2Ca_3(PO_4)_2CaO$	16.7	43.8	0.381

FIGURE 1

Historadiograph of rat kidney after deposition of calcium phosphate at the site of alkaline phosphatase activity. $\times$ 75.

FIGURE 2

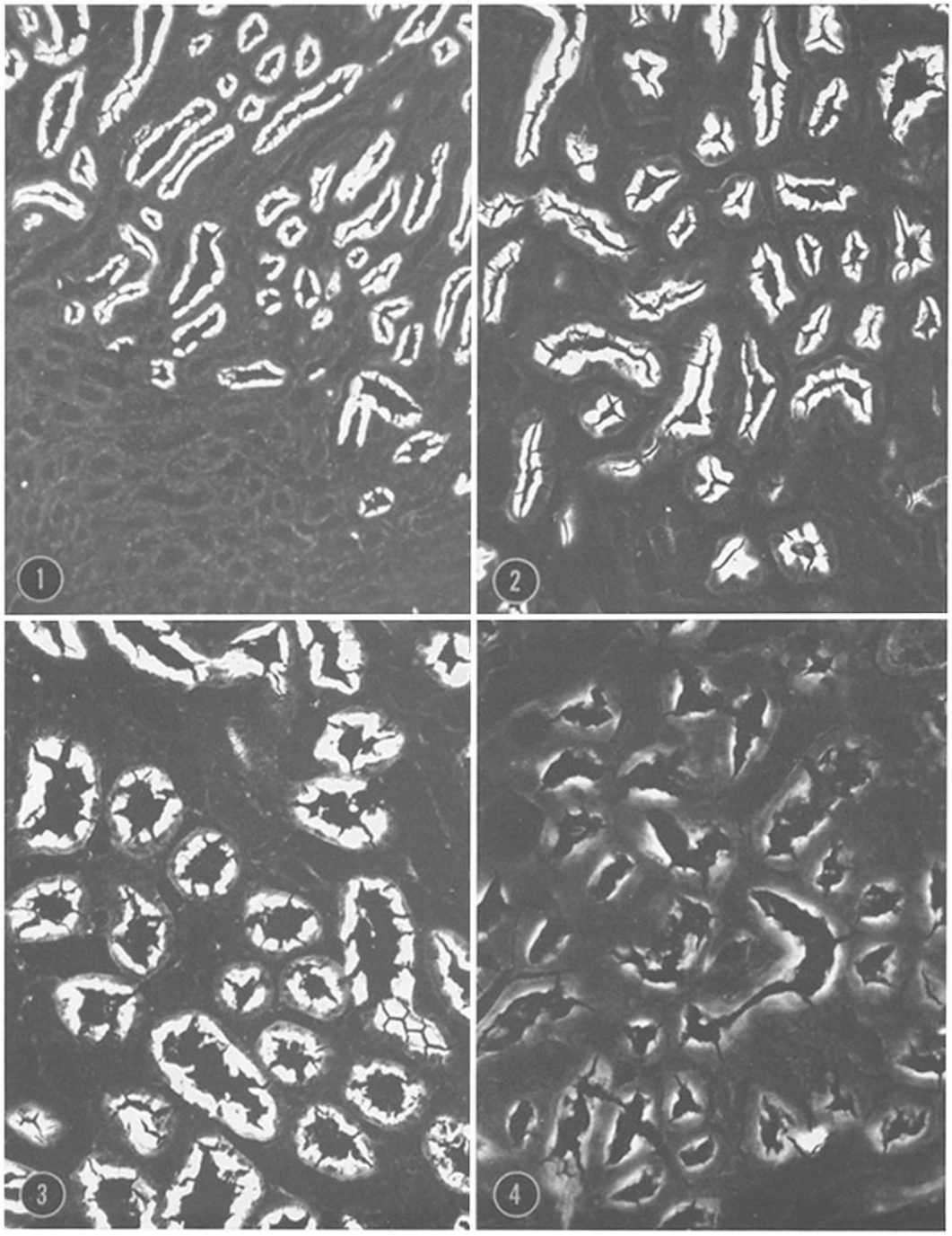
As in Fig. 1 but at a higher magnification. $\times$ 200.

FIGURE 3

Historadiograph of rat kidney after deposition of cobalt phosphate at the site of alkaline phosphatase activity. $\times$ 200.

FIGURE 4

Historadiograph of rat kidney after deposition of cobalt sulphide at the site of alkaline phosphatase activity. $\times$ 200.



phosphatase (calculation based on the data of Barter). Consequently, by combining the two techniques it should be possible to measure the amount of calcium and phosphorus present in a given amount of calcium phosphate and thus determine the form of the calcium phosphate. Having done so, then a true measure of the activity of alkaline phosphatase will be possible.

The investigation is at present being extended to studies of the deposition of elements of suitable absorption characteristics in tissues by enzymic and other reactions.

SUMMARY

The site of deposition of calcium phosphate in the Gomori-Takamatsu method for the identification of alkaline phosphatase has been studied using historadiographic methods. Frozen sections of rat kidney were immersed in the substrate for the enzyme and then mounted on small pieces of x-ray plate. After drying, the specimen and plate were exposed to x-ray wavelengths of 12 A. The calcium phosphate produced from the substrate by the enzyme absorbed the x-rays strongly and a good image of its localization was obtained on the plate. It was also found possible to convert the calcium phosphate to cobalt phosphate and then to cobalt sulphide and to localize these compounds by the x-ray method. The possible value of the method for quantitative studies on products of enzymically controlled reactions is discussed.

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