The Histochemical Demonstration of Carbonic Anhydrase in the Osteoclast

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Carbonic anhydrase (CAH), which has been shown to be a zinc-protein, was discovered by Meldrum and Roughton (1932)\(^1\) in red blood cells. By the action of this enzyme, carbonic acid is split to yield CO\(_2\) and H\(_2\)O, and, in the reverse direction, CO\(_2\) is hydrated to carbonic acid. And its occurrence and characteristics have been fully described by Roughton and Clark (1951)\(^2\).

On the other hand, the hitherto reported histochemical method for demonstration of CAH was only that of Kurata (1953)\(^3\), but many workers were not able to obtain satisfactory results by this method, whether original or modified. And its reaction is not inhibited by Diamox, and therefore its specificity is open to question.

Recently, Häusler (1958)\(^4\) reported a new modification of Kurata’s method for this enzyme. In this presentation, these histochemical methods are compared in a model experiment and the histochemical localization of the enzyme in the osteoclast is studied.

Materials and Methods

CAH used in this experiment is prepared according to Meldrum and Roughton’s method (1934)\(^5\).

Extracting method of CAH

To 10cc. of washed erythrocytes are quickly added 10cc. of 40% ethanol and 5cc. of chloroform, the mixture is stirred in a centrifuging tube until the thick paste becomes a thin sludge and allowed to stand at least 20 minutes centrifuging. The supernatant solution of crude CAH should be clear or pale

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yellow.

Small strips of filter paper are immersed in this enzyme solution, then allowed to dry and fixed in various kinds of fixatives for half an hour. Fixatives tested are absolute acetone, absolute ethanol, absolute methanol and neutral formalin, each being chilled by ice. Histochemical methods used in this experiment are Häusler's (1958), Kurata’s (1953), and Coquoin-Carnot's (1956) methods.

Häusler’s method (1958)

1) Fix section in cold acetone for 1 hour. 2) Transfer directly into incubating medium at 18°-20°, and incubate for 1.5-2 hours. It is important to keep the sections floating on the surface of the medium. (Incub. med.: Sol. A. 0.1M CoSO₄, 1.0cc. 0.05M H₂SO₄, 6.0cc. Sol. B. NaHCO₃ 1.0g. 0.1M Na₂SO₄ 50.0cc.) Before incubation pour solution B into solution A in order to avoid a momentary high concentration of cobalt ions. 3) Wash in distilled water. 4) Transfer to dilute (NH₄)₂S solution. 5) Wash in tap water, and mount in Canada balsam.

Kurata’s Method (1953)

1) Fix slice of fresh tissue (5 × 5 × 2mm.) in cold acetone for 1 hour. 2) Wash in distilled Water for 10-30 minutes. 3) Place in the incubating medium for 30-45 minutes at 37°. (Incub. med.: MnCl₂ 1.0g. 8% NaHCO₃ 100.0cc.) The medium is stirred until the pink color of the manganate has disappeared and then filtered. 4) Wash in distilled water, changing several times. 5) Dehydrate in alcohol, clear in benzene and embed in paraffin. 6) After sectioning and deparaffinization, rinse in absolute alcohol and place in 0.1% aqueous solution of potassium periodate for 48 hours at 37°C. 7) Wash in distilled water, and mount in Canada balsam.

Coquoin-Carnot’s method (1956)

1) Incubate section of fresh tissues in incubating medium for 1 hour at 37°C. (Incub. med.: CoCl₂ 1.0g. 8% NaHCO₃ 100.0cc.) 2) Wash in distilled water. 3) Fix in acetone for 30-60 minutes. 4) Washed in distilled water. 5) Transfer to dilute (NH₄)₂S solution. 6) Wash in tap water, and mount in Canada balsam. As an inhibition test, before incubation filter paper strips are heated at 90°C for 2 minutes, or Diamox (2-acetylamino-1,3,4-thiadiazole-5-sulfomide) at a concentration of 100mg% is added to the incubating medium.

As decalcification is often needed in the case of histological investigation of oral regions, the influences of a 30 minutes’ immersion in the following decalcifying fluids on this histochemical reaction are examined; 1% nitric acid, 1% hydrochloric acid, 5% formic acid, 0.5M ethylenediamine tetracetatic acid (EDTA), Lorch’s citrate-hydrochloric acid buffer (citric acid crystals 14.7g, 0.2N sodium hydroxide 700cc, 0.1N HCl 300cc.), N-acetate buffer (N-acetic acid 520cc., N-sodium acetate 480cc.), N-ammonium citrate-citric acid buffer (N-citric acid 50cc., N-ammonium citrate 950cc.).

The tissue studied is the proximal end of tibia of Wistar rats. Bone, which is difficult to cut by freezing technique, is embedded in paraffin by the
freezing substitution method, and sectioned without decalcification.

These bone sections, undecalcified or after decalcification by Lorch's citrate-HCl buffer, are stained for CAH, Alk. P-ase and Ac. P-ase, and with HE and PAS staining technique. The inhibition test with Diamox is also done.

**Results**

Results in a model experiment are shown in Table 1 and Table 2.

<table>
<thead>
<tr>
<th>Method</th>
<th>Häusler pH 7.5–8.3</th>
<th>Inhibition test (Häusler) pH 7.5–8.3</th>
<th>Coquioin-Carnot pH 7.8–8.4</th>
<th>Kurata pH 7.5–8.1</th>
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<tr>
<td>Fixation</td>
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<tr>
<td>Acetone</td>
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<td>Ethanol</td>
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<td>Methanol</td>
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<td>Neutral formalin</td>
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In bone sections, besides calcified bone spicules, where calcium exchanges cobalt which in turn precipitates there as cobalt salt, osteoclasts are highly positive, but osteoblasts are negative (Fig. 1). Although this positive reaction of osteoclasts may possibly be considered to be due to the bone salt phagocytized, the fact that when decalcified by Lorch's citrate-HCl buffer bone spicules become negative but osteoclasts are still positive (Fig. 2), as well as the fact that this positive reaction of osteoclasts is inhibited by Diamox, indicates that this positive reaction is due to the enzymic activity.

In addition to CAH, Ac. P-ase is also found in osteoclasts, but not in osteoblasts.

In contrast to this, Alk. P-ase is found in osteoblasts, but not in osteoclasts.

**Discussion**

The question of the specificity of the histochemical reaction has been taken up by a number of workers. Recently, Fand et al (1959) after testing histochemically the kidney and pancreas of seven mammalian species and a coujard
Fig. 1 Showing positive reaction of carbonic anhydrase in osteoclasts, but negative in osteoblasts.

Fig. 2 After decalcification of section by Lorch's citrate-HCl buffer of pH 4.4, bone spicules do not stain, but osteoclasts are still positive.

Fig. 1 and 2: Rat, proximal end of tibia (freeze-substituted, acetone fixed, paraffin section). Oc: Osteoclast, Ob: Osteoblast ×400.

slide technique, concluded that the Kurata's method does not demonstrate CAH, and Pearse (1960) also agrees with Fand's conclusion.

In this model experiment also, the paper strips prepared according to the Kurata's method is always very dirty with diffuse amorphous precipitates. Kurata's incubating medium itself during the course of standing at 37°C for several minutes becomes turbid, and is very unstable. When strips are incubated in this solution, it is natural for diffuse precipitates to cover the strips.

In Kurata's method the following reaction formula is postulated.

\[
\text{Co(HCO}_3\text{)}_2 + \text{CAH} \rightarrow \text{CoCO}_3 + \text{H}_2\text{O} + \text{CO}_2
\]

But, Gmelin does not refer to \(\text{Co(HCO}_3\text{)}_2\) in his textbook of inorganic chemistry, therefore this reaction is difficult theoretically to understand. On the other hand, the mechanism of the reaction was explained by Hausler as follows:
The CO₂ produced by (b), enzyme catalysed reaction, escapes and the equilibrium of the first reaction, which is spontaneous, shifts to the right. As the result a large number of CO₃²⁻ ions are produced, these are precipitated by the Co ions presents in the medium. As above mentioned, it seems that Häusler's method is easy theoretically to understand.

By dissolving the bicarbonate in sodium sulfate solution and adding 0.05M H₂SO₄, Häusler's method eliminates the troublesome precipitates associated with the original Kurata's method, and a more distinct localization of CAH can be obtained.

Puchtler et al (1955) and Braun-Falco et al (1955) reported that the specific CAH inhibitor, Diamox, does not inhibit the histochemical reaction. Possible explanations for these observations are the insolubility of Diamox in water, and the fact that the positive reaction by Kurata's method is no more than a nonspecific precipitating reaction. In this model experiment and tissue sections, Diamox (sodium salt) inhibits completely this reaction.

This is proved that the positive reaction of osteoclasts is not due to phagocytized bone salt, but to CAH. By the biochemical method de Bernard et al. (1955) found CAH in the epiphyseal cartilage, and regarded that this enzyme participates in the fixation of calcium by chondroitin sulfuric acid, and Bensch et al. (1944) too, considered that CAH plays an important role in formation of the anion of carbonate of the shell. In this experiment CAH is not found in osteoblasts, so the participation of this enzyme in calcification is not confirmed.

In recent years Siegmund et al. (1959) found the occurrence of the carbonic anhydrase in blood-free epiphyseal cartilages, and considered that this enzyme is concerned in dissolution of bone minerals. Further, from the study on effect of Diamox on calcium metabolism in laying hen, Siegmund et al. (1960) concluded that bone demineralization is caused by the secretion of hydrogen ions by the osteoclast. Cretin (1951) form the study by means of pH indicator solution, concluded that the osteoclast has a regional climate of acidic pH. The localization of CAH in the osteoclast and above mentioned experimental results may indicate that this enzyme is related to resorption of bone.

Further, Burstone (1959) described that the osteoclast shows Ac. P-ase activity, and in this experiment too, Ac. P-ase is found in the osteoclast, but no Alk. P-ase.

These histochemical findings may indicate that this enzyme is involved in regulation of the cytoplasmic hydrogen ion concentration.

Summary

Three histochemical methods for demonstration of CAH, i.e., Häusler's, Kurata's, and Coquoin-Carnot's methods are compared in a model experiment, using a crude preparation of CAH. Häusler's method gives the most constant and clearcut result, but two other methods are not good. For the fixatives,
chilled acetone, ethanol and methanol are all excellent, but neutral formalin is unsatisfactory.

When Diamox 100mg% is added to the incubating medium, or the sample is pretreated at 90°C for 2 minutes, this reaction is completely inhibited, suggesting this reaction is a specific one for CAH.

Of various decalcifying fluids, whose effect on this reaction is tested in the model experiment, Lorch's citrate-HCl buffer after acetone fixation is the most suitable.

In undecalcified bone sections, osteoclasts are positive in contrast with the negative reaction of osteoblasts. This positive reaction of osteoclasts, which is completely inhibited by Diamox, is to be ascribed not to the bone salt phagoytized in them, but to CAH.

Coexistence of CAH with Ac. P-ase in the osteoclast may be considered to indicate this enzyme's intracellular pH regulative function.

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References

Discussion
Dr. Higashi: The decalcifying procedure removes Mg" out of the tissues and the addition of Mg" in the substrate mixture makes it possible to reveal the alkaline phosphatase activity of osteoclasts in the decalcified (by EDTA solution) preparations. Your finding that the osteoclasts did not show the alkaline phosphatase activity is questionable and should be reexamined.

Dr. Yagi: We have observed the phosphatase reactions (azo dye methods) in undecalcified bone tissues by freeze-substitution paraffin sections and have obtained the following results:
osteoclasts: alkaline phosphatase
acid phosphatase
osteoblasts: alkaline phosphatase
acid phosphatase