LOCALIZATION OF SUDANOPHIL MATERIAL AT
THE SITES OF CALCIFICATION IN DENTINE,
AND THE COMPACT BONE AND EPiphySEAL
CARTILAGE PLATE OF TIBIA IN THE RAT
GIVEN BERYLLIUM CARBONATE

Masaru NAGAYAMA, Tohru OKA, *Akira MATSUMOTO,
*Shoji YAMADA, *Keiko SUZUKI, *Tomio MOROHASHI
and *Namika TANAKA
Department of Oral Surgery, Nagoya University School of Medicine,
Nagoya, 466, Japan
*Department of Dental Pharmacology, School of Dentistry,
Showa University, Tokyo, 142, Japan
Accepted August 2, 1982

Abstract......The localization of sudanophil material at dentine, the compact
bone and epiphyseal cartilage plate of tibia of rat given beryllium carbonate
was examined.

Sudanophil material was seen at the boundary parts between dentine and
widened predentine, and between widened preosseous matrix and calcified bone,
but it was not seen at the area corresponding to the zone of provisional
calcification. These facts suggest that the localization of sudanophil material
in hard tissue of rat with Be rickets was similar to that in vitamin D deficient-
-induced rickets.

This sudanophil material was not disappeared by the enzymes such as
papain, pepsin and hyaluronidase as described in vitamin D deficient-induced
rickets (Irving, 1960, 1963). Accordingly, it was suggested that the substance
was not proteins and mucopolysaccharide.
Key words : sudanophilia, dentine, bone, cartilage, beryllium rickets

INTRODUCTION

Generally, if hard tissue is stained by sudan black B without any treatment, the
calcification sites in hard tissue are not stained by that dye. However, Irving (1958,
1959a, 1960) has developed the method to stain the calcification sites in hard tissue by
Masaru NAGAYAMA et al.

placing the hard tissue in benzol or pyridine kept at 60°C for 16 to 24 hours before
decalcification. Using this method, he has reported that the boundary parts between
dentine and widened predentine, and the area between widened preosseous matrix and
calcified bone in vitamin D deficient-induced rickets were stained. But sudanophil
material was not seen in widened epiphyseal cartilage plate (Irving, 1959b) and when
vitamin D was given to the rat with vitamin D deficient-induced rickets, an intense
sudanophil material appeared again at the area corresponding to the zone of provisional
calcification in the widened epiphyseal cartilage plate.

On the other hand, it has been reported that beryllium rickets occurred after the
administration of beryllium carbonate to animal(Jacobson, 1933; Guyatt, et al., 1933;
Goring, 1951).

As beryllium rickets is one of the metal rickets, it is conjectured that the localization
of sudanophilia is similar to that of vitamin D deficient-induced rickets.

The object of present study was to examine whether localization of sudanophil
material in rats with beryllium rickets is the same as that in vitamin D deficient-induced
ricks or not.

**MATERIALS AND METHODS**

*Animals*

Thirty 4-week-old male rats of the Wistar strain, weighing 50 to 60gr. with initial
body weight, were divided into three equal groups of 10 rats. Each group of rats was
fed a normal well balanced diet(control group), a low calcium diet(low Ca group) or a
beryllium diet(Be group) respectively for 4 weeks. During the experimental period, all
rats were given demineralized water *ad libitum*. The composition of diets was shown in
Table 1. The body weight were measured everyday.

*Table 1. Composition of diets.*

<table>
<thead>
<tr>
<th></th>
<th>Control diet(%)</th>
<th>Low Ca diet(%)</th>
<th>Be diet(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn starch</td>
<td>64.547</td>
<td>66.047</td>
<td>63.047</td>
</tr>
<tr>
<td>Casein from milk</td>
<td>20.000</td>
<td>20.000</td>
<td>20.000</td>
</tr>
<tr>
<td>Cotton seed oil</td>
<td>8.000</td>
<td>8.000</td>
<td>8.000</td>
</tr>
<tr>
<td>Vitamin mixture*</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Salts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BeCO₃</td>
<td>0.000</td>
<td>0.000</td>
<td>3.000</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>1.500</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>KCl</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.700</td>
<td>0.700</td>
<td>0.700</td>
</tr>
<tr>
<td>Fe₂(SO₄)₃(NH₄)₂</td>
<td>0.508</td>
<td>0.508</td>
<td>0.508</td>
</tr>
<tr>
<td>SO₄•24H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH₃PO₄</td>
<td>1.700</td>
<td>1.700</td>
<td>1.700</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>MgSO₄•H₂O</td>
<td>0.545</td>
<td>0.545</td>
<td>0.545</td>
</tr>
</tbody>
</table>

*Sally and Bryson*
Sudanophilia in hard tissue to Be rickets

Sudan Black B Staining

All rats were killed under ether anesthesia at the end of the experimental period. Both left and right mandiblae and tibiae were taken out immediately, and then, their adherent tissues were removed. They were fixed in Baker’s formol-calcium solution (Baker, 1946) and then, they were placed in absolute alcohol for 24 hours in benzol at 60°C for 16 hours, and then brought back to absolute alcohol and washed in distilled water by using Irving’s method (1960). Decalcification was carried out in 5 percent nitric formol mixture and then embedded in gelatin. In tibia, longitudinal sections in midsagittal plane, cross sections in the part of one third from upper end of tibia in tibial length and, in mandible, cross sections in the part of one third from apical end of incisor in incisal length were cut in a freezing microtome, setting at 10µm, stained with saturated solution by sudan black B (Merck Co., Ltd., West Germany) dissolved in 70 percent alcohol, mounted in glycelin jelly and used for light microscopy.

Enzymes

The extracted and decalcified sections were treated as follows before staining with sudan black B. Some sections of each group were incubated for 3 hours with a 2.5 percent filtered papain solution of the crude enzyme at 37°C. Other sections of each group were incubated for 4 hours with a 2 percent pepsin solution which was buffered to pH 2.0, or for 2 and 1/2 hours with hyaluronidase dissolved in 0.1M acetate buffer at pH 5.5, whose concentration is 1500I.U. of testicular hyaluronidase per ml at 37°C. The control was placed in buffer solution only.

RESULTS

Growth chart

As shown in Fig. 1, the growth rate of body weight in low Ca group was worse than that of control group. In the case of Be group, it was much worse than that of low Ca group.

Sudanophil material

1) The boundary part between predentine and dentine.

In the control group, the boundary part between predentine and dentine was strongly stained as a narrow, dark blue line with sudan black B (Fig. 2a, an arrow). In low Ca group, the site was similarly stained (Fig. 2b, an arrow). In Be group, the width of predentine greatly increase, compared with those of control and low Ca groups, and sudanophil material was irregularly seen at the boundary part between widened predentine and dentine (Fig. 2c, an arrow).

2) The boundary part between preosseous matrix and calcified bone.

As shown in Fig. 3a, the boundary part between preosseous matrix and calcified bone in control group was stained with sudan black B. In low Ca group, the reaction was similar to that of control group (Fig. 3b). The thickness of cortical bone in low Ca group was thinner than that of control group (Fig. 3b). In Be group, the sudanophil material at the end of preosseous matrix on the periosteal aspect was seen (an arrow)
Masaru NAGAYAMA et al.

and wide sudanophilic reaction was also seen on the periosteal aspect. In addition, wider layer around Haversian canals was stained with sudan black B (Fig. 4, arrows). The cortical bone was osteoporotic, compared with that of control group (Fig. 4).

3) Epiphyseal cartilage plate.

In both control and low Ca groups, the intercellular area at the zone of provisional calcification was stained with sudan black B (Figs. 5a and 5b, arrows). In Be group, sudanophilia was not seen at the area corresponding (Fig. 5c, an arrow). The width of zone of hypertrophic cartilage increased.

Enzymes

Staining of sudan black B was not affected by the treatment of papain, pepsin and hyaluronidase to the extracted and decalcified sections. As this substance was not disappeared by these enzymes, it was suggested that it was not proteins and mucopolysaccharide.

Fig.1: Daily changes in the body weights in groups of rats. ○⋯○ Control, ×⋯× Low Ca, ○⋯○ Be groups. Each point represents the average value in each group. Vertical bars represent the standard deviations.
Fig. 2: Cross section in the part of one third from apical end of incisor in incisal length.

a, Control group: Note an intense sudanophil line at the boundary part between predentine and dentine (an arrow).
D, dentine; PD, predentine; P, pulp; OB, odontoblasts; AB, alveolar bone. Sudan black B stain.

b, Low Ca group: Note a sudanophil line at the boundary part between predentine and dentine (an arrow).
D, dentine; PD, predentine; P, pulp; OB, odontoblasts. Sudan black B stain.

c, Be group: Note that the width of the predentine increased and that the sudanophil reaction is diffusible at the boundary part between widened predentine and dentine (an arrow).
D, dentine; PD, predentine; P, pulp; OB, odontoblasts; AB, alveolar bone. Sudan black B stain. The scale in this figure is applicable to Figs. 2a and 2b.
Fig. 3: Coross section in the part of one third from upper end of tibia in tibial length
a, Control group: Note an intense sudanophil line at the boundary part between preosseous matrix and calcified bone (an arrow). CB, calcified bone; OS, preosseous matrix. Sudan black B stain.
b, Low Ca group: Note the sudanophil line at the boundary part between preosseous matrix and calcified bone (an arrow). CB, calcified bone; OS, preosseous matrix; BM, bone marrow. Sudan black B stain.
c, Be group: Note the sudanophil material at the end of preosseous matrix on the periosteal aspect (an arrow). Wide sudanophilic reaction was also seen on the periosteal aspect. CB, calcified bone; OS, preosseous matrix; BM, bone marrow. Sudan black B stain. The scale in this figure is applicable to Figs. 3a and 3b.
Sudanophilia in hard tissue to Be rickets

Fig. 4: Cross section in the part of one third from upper end of tibia in tibial length in Be group. Note the sudanophilic reaction around haversian cannals (arrows). CB, calcified bone; OS, preosseous matrix; BM, bone marrow; HC, haversian cannal. Sudan black B stain.

Fig. 5: Longitudinal section in midsagittal plane of the proximal epiphyseal cartilage plate and metaphysis.

a, Control group: Note the sudanophilia in the intercellular area at the zone of provisional calcification (an arrow). PC, proliferating cartilage; HC, hypertrophic cartilage; ZPC, zone of provisional calcification; TB, trabecular bone. Sudan black B stain.
b, Low Ca group: Sudanophilic reaction in also seen at the zone of provisional calcification as Fig. 5a. (an arrow). PC, proliferating cartilage, HC, hypertrophic cartilage, ZPC, zone of provisional calcification; TB, trabecular bone. Sudan black B stain.
c, Be group: Note that no sudanophilic reaction was seen in the intercellular area corresponding to the zone of provisional calcification (an arrow). HC, hypertrophic cartilage; TB, trabecular bone. Sudan black B stain. The scale in this figure is applicable to Figs. 5a and 5b.
DISCUSSION

It was examined whether sudanophilia localized at the sites of calcification of hard tissue in the rat given beryllium carbonate like the case in vitamin D deficient-induced rickets or not. In the present study, Be rickets was produced by replacing calcium carbonate in normal well ballanced diet with beryllium carbonate as previously described (Jacobson, 1933; Guyatt, et al., 1933; Goring, 1951). In Be group, sudanophilia was found at the boundary part between widened predentine and dentine and the boundary part between widened pre osseous matrix and calcified bone as those in vitamin D deficient-induced rickets (Irving, 1960, 1963). As this substance was not disappeared by enzymes such as papain, pepsin and hyaluronidase, in the case of vitamin D deficient-induced rickets, it was not proteins, mucopolysaccharide but lipid-like substance (Irving, 1960, 1963). However, in the present study, as other lipid stain such as oil red O, Nile blue and fat blue 4R was not done, it may be rather difficult to insist that this substance is lipid-like substance.

In the teeth and compact bone of rat with Be rickets, sudanophilia was seen. This fact suggests that calcification process does not stop as Irving (1959a) has reported in vitamin D deficient-induced rickets. In the present experiment, mineralized dentine or calcified bone was not stained with sudan black B. This may be due to the fact that the lipid content in the calcification tissues decrease remarkably (Peck and Dirksen, 1966). The histological changes of epiphyseal cartilage plate in Be group was similar to those in vitamin D deficient-induced rickets. Sudanophil material was not observed at the area corresponding to the zone of provisional calcification in the rat with Be rickets, as that in vitamin D deficient-induced rickets (Irving, 1958).

If Be rickets was produced by the failure of conversions of an inactive form of vitamin D to active one (DeLuca, 1971; Corradino, et al., 1971), both beryllium-induced rickets and vitamin D deficient-induced rickets would be caused by the same mechanism. Although there have been many reports that the lipid must play an important role at the sites of calcification in hard tissue (Leach, 1958; Irving, 1959b; Cruess and Clark, 1965; Shapiro, Wuthier and Irving, 1966; Sakai and Cruess, 1967; Irving and Wuthier, 1968; Wuthier, 1968; Havivi and Bernstein, 1969), it seems that the significance of the presence of lipids in dentine, bone and cartilage is not well known (Wolinsky and Guggenheim, 1970).

Further more investigation will be required as to the role of sudanophilia at the sites of calcification in hard tissue.

REFERENCES

Sudanophilia in hard tissue to rickets

WUTHIER, R. E. 1968: Lipids of mineralizing epiphyseal tissues in the bovine fetus. J. Lipid Res. 9, 68-78.