Resumen por el autor, Oscar V. Batson.

La coloración diferencial del hueso.

I. El teñido de ejemplares conservados.

El autor ha probado una serie de derivados de la alizarina y mordientes con el propósito de hallar, si esto fuese posible, un colorante que ofrezca una coloración de contraste con la obtenida por el rojo de rubia ordinario. Para los experimentos ha empleado embriones de pollo y cerdo. El material fué fijado en formol al 10 por ciento. Para eliminar el color de la sangre se empleó una solución débil de peróxido de hidrógeno. La solución tintórea fué una solución saturada del colorante en alcohol de 95°, una parte, y veinte partes de alcohol de 95°. Los ejemplares fueron diferenciados en una solución al medio por ciento de ácido sulfúrico en alcohol de 95°, después de lo cual fueron deshidratados y aclarados según el método de Spalteholz. Los veinte colorantes que pudieron obtenerse fueron los siguientes:

Negro de alizarina ácido, R. Meister, Lucius & Brüning	Pardo de alizarina RB, polvo, Fr. Bayer & Co.
Negro de alizarina SR, polvo, Fr. Bayer & Co.	Negro de alizarol 3G, Nat. Aniline and Chemical Co.
Azul de alizarina Br3g, polvo, Fr. Bayer & Co.	Carmín de índigo, Grüber
Negro de alizarina Bayer NR, Polvo, Fr. Bayer & Co.	Ácido carmínico, Grüber
Negro de alizarina SBB, polvo, Fr. Bayer & Co.	Rojo de Burdeos, Grüber
Pardo de alizarina AR, polvo, Fr. Bayer & Co.	Naranja, Grüber

THE DIFFERENTIAL STAINING OF BONE

I. THE STAINING OF PRESERVED SPECIMENS¹

OSCAR V. BATSON

Anatomical Laboratory, University of Wisconsin, Madison

The study of ossification centers, of healing in fractures, and of bone growth in general has been greatly facilitated by the use of alizarin in its natural and synthetic forms. That madder root when fed to animals colors the bone red has long been known. It seems that Belchier (1736) rediscovered this fact and brought it to popular attention. Almost 150 years earlier, however, Laevinus Lemnius (1581) mentioned this property of madder root.² Considering the numerous editions and translations of Lemnius' "Hidden Miracles of Nature," it seems remarkable that no use was made of this observation.

Biologists and chemists have examined madder root to find the active staining principles. The chief of these have been found to be alizarin and purpurin, mainly the former. Since the pure synthetic alizarin has been available it has been largely used in the staining of preserved specimens, for the demonstration of the skeleton. This staining technic coupled with clearing methods has been presented in detail by Lundvall ('04, '05) and Spalteholz ('14). Alizarin in the form of its soluble salt, sodium alizarin monosulphonate, has also been used to supplant madder root in the vital staining of bone. Gottlieb ('14), in a very thorough paper, describes the use of the above salt for vital staining. Brooks ('17, '20) more recently has reported a series

¹A series of specimens stained with the described dyes was demonstrated at the meetings of the American Association of Anatomists at The Wistar Institute, Philadelphia, March, 1921. This demonstration included a set that had been prepared in August, 1920.

²"... erythrodanum seu rubea, qua ossa pecudum sandicino rubentique colore imbuunt, . . ." De Miraculis Occultis Naturae, p. 390, lines 30 and 31.

of studies in which the same salt was used, and it is quite probable that sodium alizarin monosulphonate is now used in most laboratories for vital staining of bone.

Graebe and Lieberman first produced alizarin synthetically in 1868. Since that time many synthetic alizarin derivatives have been produced by the dye chemists. A glance at the catalogs of the dye firms reveals alizarin reds, browns, blacks, blues, greens, and yellows. Apparently none of these have been used for the successful staining of bone. Ehrlich ('85) used alizarin blue S in studying oxidation in tissues, but with this stain did not report any staining of bone. Rawitz ('96) who used alizarin extensively in microscopic technic did not mention bone staining. Spalteholz ('14) uses small quantities of alizarin cyanatum in staining preserved specimens to improve the color of the alizarin red. Gottlieb ('14) unsuccessfully tried to stain bone *intra vitam* with alizarin blue and alizarin green (brand or formula not mentioned).³ In reporting Bardeen's technic for the study of ossification centers, Hill ('06) describes the former's use of alum carmine, a dye of a wholly different chemical series. Miller ('21) in a recent paper refers to the same work.

In certain types of bone work a stain affording a contrast to the red-brown of the usual alizarin would seem desirable. It might be possible to alternate the red with a contrasting color in *intra vitam* work, or it might be possible to bring out more detail after preservation by the use of a counterstain for that part of the bone not colored during life. With the view of possibly finding such a stain, the following study of alizarin derivatives and mordant dyes was begun.

Among the readily soluble alizarin cloth dyes available those were selected which gave promise of affording a contrast to the alizarin red. The non-alizarin mordant dyes of the histological laboratory were tried out, partially to see if the anthraquinone grouping (present in the alizarins) is an essential element of a bone stain and partially in the hope of finding a more brilliant bone stain.

³ "Ich möchte hier noch kurz erwähnen, dass ich mit einer Reihe verwandter Farbstoffe (Alizarinblau und Alizarin grün) nach dieser Richtung Versuche angestellt habe, sämtlich aber negativ ausgefallen sind" (S. 192).

In investigating the properties of the various stains the following technic was used. The tissue consisted of portions of pig embryos ranging in size from 3.5 cm. to 8 cm. These embryos were part of the laboratory stock obtained from the packing house fixed in 10 per cent liquor formaldehyde. The blood color was removed from part of the material by bleaching in a $\frac{1}{2}$ per cent by volume solution of hydrogen peroxide (the ordinary peroxide of commerce is 3 per cent by volume), followed by removing the gas bubbles with the suction pump.

Stock solution of stain

Strain being investigated.....	250 mg.
Water.....	5 cc.
Shake well and add of 95 per cent alcohol.....	95 cc.

In no case did all of the stain go into solution.

1. Material washed in water.
2. Stained twenty-four to forty-eight hours in

Stock solution.....	1 part
95 per cent alcohol.....	20 parts

3. Differentiated in $\frac{1}{2}$ per cent solution of sulphuric acid in 95 per cent alcohol.

4. Dehydrated and cleared after the method of Spalteholz.

To the present time twelve stains have been found to combine differentially with the bony skeletal elements. A list of the stains, with a brief comment on their behavior and the results obtained, is given below.

Alizarin group

1. Acid alizarin black R. Farbwerke vorm. Meister, Lucius, & Brüning. Hoechst o/M. Stains the skeletal elements a deep reddish purple. The soft tissues and cartilages decolorize rather slowly; however, differentiation is good from the beginning.

2. Alizarin-black SR powder. Farben fabriken vorm. Fr. Bayer & Co., Elberfeld. This stain likewise imparts a purple color to the bones. Satisfactory stains may be obtained from 1 to 250 dilutions. When this is done the bone is not stained so intensely, but decolorization is easier. Must be differentiated in sulphuric acid alcohol.

3. Alizarin-blue Br3G powder. Bayer. Stain is fairly differential from the start. Bone is an azure blue.

4. Alizarin-black-Bayer NR powder. Bayer. This stain gives a good purple to the bones. The other tissues decolorize readily and the end-result is a very sharp stain. See no. 8.

5. Alizarin black SBB powder. Bayer. Bones a sharp indigo. The other tissues take this stain slightly. The differentiation is rapid and sharp. This stain seems to be one of the most satisfactory. Its color is devoid of reddish tinge.

6. Alizarin brown A R powder. Bayer. Imparts a reddish brown to bone.

7. Alizarin brown R B powder. Bayer. Very similar to no. 6.

8. Alizarol black 3 G. National Analine and Chemical Co., New York. A new American stain, supposedly an alizarin. The shade is slightly different from no. 4, but otherwise the dye is quite similar. This is a very satisfactory stain. It is available in quantities.

9. Indigocarmine, Grübler. This stain can be used in a saturated solution or in any dilution. It decolorizes perfectly in water as well as in the acid alcohol mentioned. The resulting stain is a medium indigo. One of the easiest stains of the series to handle and one producing an excellent preparation.

10. Carminic acid, Grübler. This stain like the previous one can be differentiated in water. If the reaction of the mounting fluid is acid the color of the bone in the specimen is an orange-red, if the reaction is alkaline the color approaches a Bordeaux. If acid decolorization is used the specimen should be alkalinized before mounting. Dr. Barden's use of alum carmine was referred to earlier. It is perfectly obvious that the use of carminic acid is but a modification of this technic.

11. Bordeaux red, Grübler. This stain can be used in a saturated solution if desired, but this makes decolorization a little more tedious. The skeletal elements come out a brilliant shade of Bordeaux and if decolorized properly the rest of the structures are colorless. The end-results seems much better than those obtained with the usual alizarin red.

12. Aurantia, Grübler. Solutions near saturation work best. The bones stain a bright orange-yellow. The crystalline stain gives a deeper stain than the amorphous.

In working with the individual stains certain modifications as noted above were found to be desirable, but on the whole the standard technic was entirely satisfactory for the preliminary work. Portions of the above specimens were subjected to treatment with 5 per cent sulphuric acid in 95 per cent alcohol to test the permanence of the stains. Some decolorization (not decalcification) was noted when the time in the 5 per cent acid was extended over forty-eight hours. This permanence to sulphuric acid applies to the skeleton of the chick at hatching and to the

skeletons of pig embryos of the stages mentioned. Sulphuric acid has not been satisfactory when used on the bone of young rats. Lundval ('05) must have had in mind the acids that form soluble calcium salts when he comments, "Da das Alizarin in sauren Lösungen beinahe vollständig entfärbt wird, kann man keine von den schon beschreibenden Knorpelfarbenmethoden verwenden." Calcium sulphate is quite insoluble. It should be noted here that in differentiating specimens stained by sodium alizarin monosulphonate, the acidulation of the alcohol with sulphuric acid causes the red soft tissues to become immediately yellow, while the bone remains red. This yellow color is readily extractable. Spalteholz ('14) made use of these facts in his earlier technic by adding small amounts of acetic acid to the staining solution itself.

SUMMARY

Of these new bone stains indigo-carmin, Bordeaux red, alizarol black 3G (National Analine and Chemical Co.) and alizarine black SBB powder (Bayer), seem particularly suitable for staining preserved specimens.

The number of stains shown to combine differentially with bone indicates that there is a large number of mordant or adjective dyes with this property.

The anthraquinone grouping is not necessary for bone staining.

Weak solutions of sulphuric acid are adequate and satisfactory decolorizing agents for soft tissues in differentiating bone stains.

Staining of bone in preserved specimens is largely a matter of differential decolorization.

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