LXXXII. VITAL STAINING OF BONES WITH MADDER

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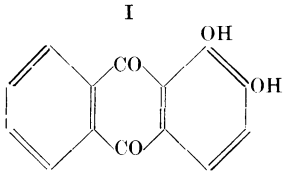
It has been known for many years that the bones of animals are coloured red by feeding with the root of the madder plant *Rubia tinctorum*, which contains a number of colouring matters related to alizarin. The work of Duhamel, Hunter and others showed that the growing parts of the bones of young animals are stained much more strongly than the fully formed bone of old animals: when young growing animals are fed intermittently on madder the bones become stained in concentric rings. Summaries of the literature are given by v. Möllendorff [1926] and Cameron [1930].

The selectivity of the madder stain in differentiating between growing bone and fully formed bone has become of considerable value in studying the growth of bones; but the method suffers from the disadvantages that the amount of stain in madder is variable, and certain animals, such as cats, cannot be made to eat the considerable quantities of madder necessary for effective staining. Serres & Doyerre [1842] found it necessary to tie up the mouths of dogs fed on madder to prevent their vomiting, and a number of their animals died. Madder is also somewhat toxic to rats and to young chicks.

Since the isolation of alizarin from madder a number of investigators have tried replacing madder by alizarin, but Reimers & Boye [1905] were unable to obtain any staining of the bones of dogs with alizarin. The method of vital staining by injecting sodium alizarinsulphonate has been used successfully by Gottlieb [1914], but he found that it did not show the same selectivity as the madder stain. He found that the bones of rats were tinted by feeding with alizarin, but the colour was different from that produced by feeding with madder. Schreiber [1904], who was unable to obtain any staining of the bones of frogs or pigeons with alizarin, concluded that ruberythric acid, a soluble glycoside of alizarin which is also present in madder, might be responsible for the staining; but he did not test this possibility.

A reinvestigation of the colouring matters of madder has shown that in addition to alizarin (I), ruberythric acid and purpurin (II), madder contains considerable quantities of *purpurin-3-carboxylic acid* (III) and its glycoside galiosin, which have been isolated in the pure crystalline condition [Hill & Richter, 1936; Richter, 1936].

I



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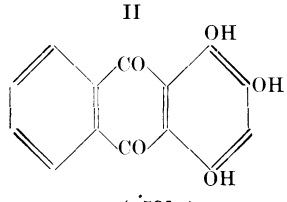
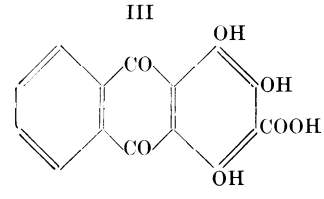


III



When young rats and pigeons, 2 to 3 months old, were fed with pure specimens of the various colouring matters it was found that only purpurincarboxylic acid gave a staining of the bright carmine red colour typical of madder-stained bones. The bones of rats fed on relatively large amounts of alizarin were tinted slightly, but the colour was a pale bluish shade of pink. Feeding with the glycoside galiosin is equivalent to feeding with purpurincarboxylic acid since

	Rats		Pigeons	
Stain	Dose g./kg.	Result	Dose g./kg.	Result
Alizarin	1·4	±	0·16	–
Ruberythric acid	0·23	–	0·17	–
Purpurin	0·51	–	0·16	–
Purpurin-3-carboxylic acid	0·17	+ + +	0·16	+ + +

the glycoside is very unstable and is hydrolysed in the stomach and gut. It can be concluded that purpurincarboxylic acid is the colouring matter mainly responsible for the staining of bones by madder.

This was confirmed by extracting the colouring matter from madder-stained bones. On digesting the finely ground bones (rat femur) with a mixture of dilute hydrochloric acid and toluene the colouring matter passed into the toluene layer and could be identified spectroscopically by the absorption bands at 5650, 5320 and 4950 Å. The toluene solution was extracted with sodium hydroxide which gave a purple solution with a band at 5500 Å., due also to purpurincarboxylic acid, but no trace of the alizarin bands could be observed.

The bone-substance showed a certain specificity in that of these four closely related colouring matters only one acted as an effective stain. At first it appeared likely that their different solubilities might be a determining factor, but all these substances were readily absorbed from the gut and excreted in the urine. The alizarin and purpurin were excreted in the form of derivatives, possibly hexuronates, which were insoluble in toluene but which gave the original hydroxyanthraquinones again on boiling with dilute hydrochloric acid.

With most stains the staining properties depend mainly on the physical properties of the tissues on which the stain is adsorbed or in which it is dissolved; but there is reason to believe that with stains related to alizarin the staining depends rather on their specific chemical affinities for calcium [Gottlieb, 1914]. This was supported by some experiments on staining *in vitro*.

Staining properties of bone in vitro

Fresh bones of young rats (femur) were incubated at 37° with solutions of (*a*) alizarin, (*b*) purpurin and (*c*) purpurincarboxylic acid in buffer solutions containing (*a*) *M*/15 phosphate buffer *p*H 5·7 and (*b*) *M*/15 acetate buffer *p*H 4·6 (it has been estimated that *p*H values in the neighbourhood of *p*H 4–5 obtain in the interstitial fluid of pericardial bone [Schulze, 1925; Schulze & Ornstein, 1928]. It was found that after 3 hours the bones in the alizarin and purpurin solutions were only slightly tinted, while the bones in purpurincarboxylic acid solution were stained a deep red. These results obtained *in vitro* agree with the observations made on vital staining.

Purpurincarboxylic acid resembles alizarin in forming a coloured calcium salt which is highly insoluble in water and is probably of the nature of a lake. Calcium compounds such as the carbonate and the phosphates are readily stained by purpurincarboxylic acid solution, which forms a thin surface layer of the insoluble red calcium salt. When a solution containing equal amounts of

alizarin and purpurincarboxylic acid (0·2 mg.) dissolved in a solution of sodium carbonate (0·05 g.) and sodium bicarbonate (0·05 g.) in 20 ml. water was passed through a Tswett chromatographic adsorption column packed with calcium carbonate, it was found that the more acidic purpurincarboxylic acid was adsorbed preferentially in the first layer while the alizarin was displaced into the second coloured zone. This may explain why purpurincarboxylic acid is more effective than alizarin in vital staining.

The calcium salt of purpurincarboxylic acid which was formed in alkaline solution was purple in colour while the calcium salt formed in neutral or acid solution was red. It is probable that in the former compound the phenolic groups take part in salt formation, while in the latter compound only the carboxyl group is concerned: in both compounds the calcium atoms are likely to be co-ordinated with the oxygen atoms of the neighbouring hydroxyl groups to form chelate rings.

In attempting to understand the selectivity of purpurincarboxylic acid in staining growing bone more strongly than old bone it appeared at first possible that this might be due to the greater vascularity of growing bone. But Gottlieb [1914] found that after an injection of sodium alizarinsulphonate the bones of old animals are also rapidly coloured, so that the vascularity can hardly be the determining factor. Cameron [1930] observed a selective differentiation in the staining of young and old bone implanted subcutaneously into animals injected with sodium alizarinsulphonate. This again shows that the selectivity in staining is a property of the bone substance itself.

When a soluble calcium salt is added to a dilute solution of purpurincarboxylic acid at the *p*H of blood serum the red calcium salt of purpurincarboxylic acid is rapidly formed, but it may remain for some time in colloidal solution before finally separating out. Since serum contains a considerable concentration of calcium ions it is probable that the dilute purpurincarboxylic acid which circulates in the blood under the conditions of vital staining is present as the calcium salt. A colloidal solution of the calcium salt in Ringer solution, stabilized by the addition of 0·25 % gelatin, gave a solution that stained much more selectively than purpurincarboxylic acid itself.

Bone (femur of 2-month rat)	+ + +	Apatite, $Ca_5(PO_4)_3$ (Cl, F)	−
Bone (femur of 12-month rat)	+ + +	Decalcified bone (by HNO_3)	±
Calcite, $CaCO_3$	−	Bone mineral (by NaOH)	+ + +
$CaCO_3$, precipitated	−	Calcium oxalate	−
$Ca_3(PO_4)_2$, precipitated	+ + +	$MgHPO_4$, $3H_2O$	+
$CaHPO_4$, $2H_2O$, precipitated	+ + +		
$CaH_4(PO_4)_2$, H_2O, precipitated	±		

The substances to be stained were incubated for 4 hours at 37° with a solution of the stain prepared as follows: 24 mg. purpurincarboxylic acid were ground with a slight excess of NaOH in 10 ml. water and the solution was neutralized with 5 % acetic acid. To this was added (*a*) a solution of 0·5 g. gelatin in 50 ml. water, (*b*) a solution containing 0·18 g. NaCl, 0·046 g. $CaCl_2$ and 0·05 g. KCl, and 20 ml. *M*/1 buffer solution. The mixture was filtered and diluted to 200 ml. The staining experiments gave similar results at *p*H 4·6 (acetate buffer) and 6·2 (phosphate buffer).

Bone was deeply stained by the solution described, and young bone was generally coloured more deeply than old bone though the difference was not always very marked. The calcium phosphates $Ca_3(PO_4)_2$ and $CaHPO_4$, $2H_2O$ were strongly stained, while crystalline apatite, calcite and calcium carbonate were hardly stained at all. These observations suggest that bone may contain,

in addition to the crystalline mineral of the apatite series, another calcium compound with staining properties similar to those of the more basic calcium phosphates, and this may be present in larger amounts in young than in old bone.

Preparation of purpurincarboxylic acid

A specimen of commercial madder was found to contain 0·7 % of purpurincarboxylic acid, which could be prepared from madder in small amounts. The dried madder root (500 g.) was suspended in water (1000 ml.), the mixture acidified with dilute HCl and shaken with toluene (700 ml.). Kieselguhr (10 g.) was added, the solution decanted and filtered and the toluene layer separated. The purpurincarboxylic acid was obtained as its sodium salt by shaking the toluene layer with a paste of sodium bicarbonate (10 g.) in water (10 ml.). The toluene was then returned to the madder suspension and the extraction repeated several times, until the toluene no longer went pink on shaking with the madder. On adding glacial acetic acid to the sodium salt purpurincarboxylic acid separated: after filtering, washing well with water and drying, it formed a bright red powder. It was recrystallized from chloroform in which it is sparingly soluble. The yield was 2·5 g.; M.P. 118–120° with decomposition.

Synthetic preparation. The synthesis of purpurincarboxylic acid has been described by Hill & Richter [1936]. The following method is suitable for its preparation in large quantities. Purpurin (10 g.) and sodium hydroxide (10 g.) were stirred vigorously with 2000 ml. water, and 38 % formaldehyde (20 ml.) was added. The mixture was warmed to 70°, acidified with HCl and allowed to cool. The clear solution was then removed by decantation, an equal bulk of kieselguhr added to the yellow precipitate and the mixture filtered, washed and dried. The resulting 3-hydroxymethylpurpurin (10 g.) was then dissolved in 200 ml. concentrated sulphuric acid (in a large flask to allow for frothing), and boric acid (15 g.) and sodium nitrite (15 g.) were slowly stirred in. The mixture was heated slowly to 147°, at which temperature it was kept for 10 min. It was then cooled and poured on to ice. The red precipitate of purpurincarboxylic acid mixed with kieselguhr was filtered off, washed thoroughly with water and after drying on a porous plate recrystallized from chloroform. The yield was 8 g., M.P. 118–120°, with decomposition.

Purpurincarboxylic acid easily decomposed on standing in the amorphous state with dilute acids or on boiling with solvents. This would appear to explain the observation of Schreiber [1904] that vital staining cannot be obtained with madder that has been sterilized by heating with superheated steam.

Dosage required for vital staining. Purpurincarboxylic acid appeared to be completely non-toxic to rats. When rats were fed with quantities of the order of 20 mg. (0·17 g./kg.) a marked general coloration of the tissues could be observed, and the ears and paws became pink. With larger amounts, up to 200 mg. (1·7 g./kg.), the staining was not noticeably darker; the excess of purpurincarboxylic acid was excreted unchanged in the faeces. If the feeding with purpurincarboxylic acid was continued for a week or more the colour of the bones became extremely dark. The best results were obtained by adding the stain in small amounts to the food in the course of 2–3 days; but even when only one dose was given, a distinct coloration, sufficient for histological purposes, could afterwards be observed. Since purpurincarboxylic acid can be easily prepared and gives an effective staining in very small doses, some of the difficulties formerly associated with vital staining with madder can be avoided.

SUMMARY

1. Purpurin-3-carboxylic acid is responsible for the vital staining of the bones of animals fed on madder.

2. The staining properties of bone and of certain calcium compounds have been compared.

3. Methods of preparing purpurincarboxylic acid are given.

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