Histochemie 27, 87–95 (1971)

On the Structure of Carminic Acid and Carmine

SUSAN N. MELOAN, LINDA S. VALENTINE and HOLDE PUCHTLER

Department of Pathology, Medical College of Georgia
Eugene Talmadge Memorial Hospital, Augusta, Georgia, U.S.A.

Received May 5, 1971

Summary. The chemical mechanism and histochemical significance of carmine stains are not yet understood. To determine possible effects of dye configuration on staining patterns we built models of dye molecules with the Stuart-Briegleb-type of atomic models. However, steric hindrance prevented construction of carmine according to the formula suggested by Harms. A review of recent chemical literature showed that the widely accepted formula of carminic acid is incorrect; the carboxyl group is not in the 5- but in the 7-position, and the side-chain is not a methylpentose but a hexose. Models based on the revised structural formula could be combined to 2:1:1 carminic acid—Al—Ca complexes. But formation of the central Al—O—Ca—O—Al bridge of the conventional 4:2:1 carminic acid—Al—Ca formula of carmine was still impossible. It is suggested that carmine may be a 2:1:1 compound analogous to the 2:1:1 alizarin—Al—Ca complex established by Kiel and Heertjes. Investigations of carmine were rendered difficult by wide variations in the staining properties of dye samples and the lack of data concerning the composition of various batches of carmine.

Introduction

Carminic acid and carmine were widely used in histology during the second half of the 19th century (P. Mayer, 1892, 1917). Though their importance declined when numerous synthetic dyes became available, a few procedures, e.g. Best's carmine for glycogen, mucicarmine, acetocarmine, are still listed in current textbooks of histochemistry and staining technics. But the chemical mechanism of these procedures, and hence their histochemical significance, are not yet understood. In 1952 Monné and Harde suggested that Best's carmin may not even stain glycogen but a basic substance associated with it (Harms, 1957a, original not available). It was therefore deemed of interest to extend previous studies of hydroxyanthraquinone dyes of the alizarin group (Puchtler *et al.*, 1969) to carminic acid and chelates derived from it. However, interpretation of staining patterns was difficult and it was not possible to devise a hypothesis which would explain all observations. We therefore built models of these dyes to determine possible effects of the configuration on dye binding. This report will be limited to studies of the molecular structure of carminic acid and carmine.

Material. All dye molecules were built with atomic models of the Stuart-Briegleb type (manufactured by Leybold, Köln, Germany; distributed in USA by LaPine Scientific Company, 6001 South Knox Avenue, Chicago, Illinois 60629). The elements are colorcoded: carbon is black, hydrogen is white and the red oxygen atoms appear gray in the black-and-white photographs. Metal wedges denote double bonds; benzene rings have six metal wedges to indicate resonance. Wire hooks and loops designate hydrogen bonds. In chelates of this type aluminum is represented by octahedron with six coordination sites. A model of planar ionic metal atoms is used to denote calcium.

Conventional Structural Formulas of Carminic Acid and Carmine

Fig. 1 shows the formula of carminic acid as given in the Colour Index (1957); hence, most double bonds are omitted. According to the literature available for this study, the structure of carmine has not yet been established. Harms (1957a) suggested a formula (Fig. 2) analogous to the Rütishauser structure for the alizarin-aluminum-calcium compound. The Ca and H_2O groups enclosed by squares could not be demonstrated by chemical analysis and were omitted in a later

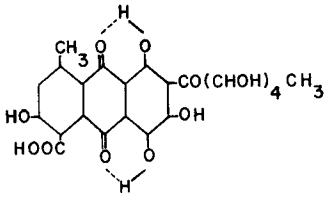

Fig. 1. Conventional structural formula of carminic acid

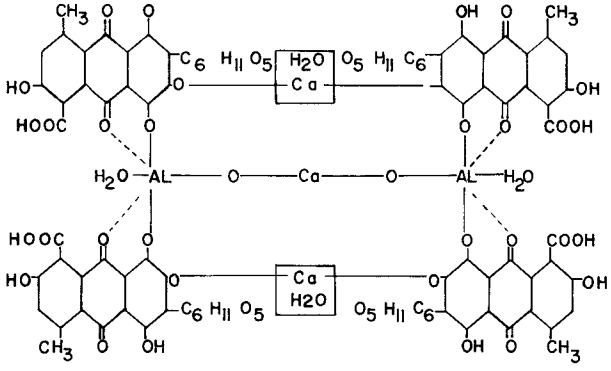

Fig. 2. Structure of carmine suggested by Harms (1957a) on the basis of the Rutishauser formula for the alizarin—Al—Ca complex. The groups enclosed by squares were later omitted (Harms, 1957b)

paper (Harms, 1957b). Fig. 3 shows a model of carminic acid. The 1- and 4-OH groups form hydrogen bonds with the oxygen atoms in the 9- and 10-position respectively. Such hydrogen bonds have been demonstrated by infrared spectroscopy in other anthraquinone dyes containing 1,4-OH groups (Flett, 1948), and it appears probable that such hydrogen bonds exist also in carminic acid. The other substituents have limited freedom of rotation.

However, combination of four molecules of carminic acid as shown in Fig. 2 proved to be impossible (Fig. 4). Steric hindrance by the carboxyl groups in the 5-position prevents the close approach of the aluminum atom necessary for chelate formation with the oxygen atoms in the 4- and 10-positions of the second dye molecule. Complex formation with the 1- and 9-oxygen atoms is equally impossible because of the bulky side-chain and the methyl group in the 8-position; furthermore, chemical studies indicate that chelate formation by hydroxyanthra-

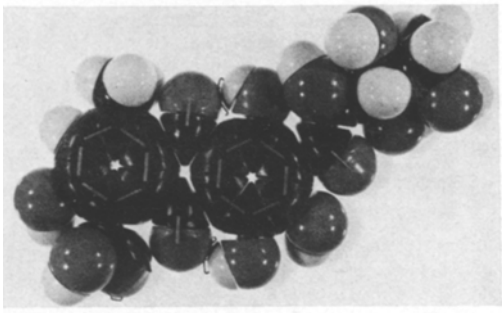

Fig. 3. Model based on conventional formula of carminic acid. (Fig. 1)

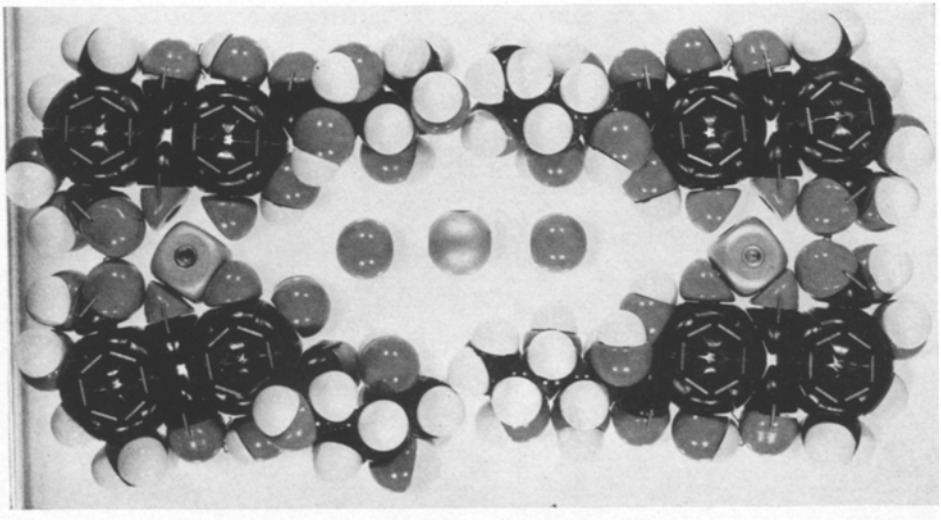

Fig. 4 Model of carmine built according to Fig. 2. Steric hindrance by the bulky carboxyl groups prevents chelation of the Al with a second dye molecule. The large side-chains preclude formation of the Al—O—Ca—O—Al bridge

quinones requires a hydroxyl group in ortho position to the complex forming hydroxyl group (Hüttig, 1914; Attree and Perkin, 1931; Kiel and Heertjes, 1963a, b). The large side-chains obviously preclude formation of the Al-O-Ca-O-Al bonds indicated in the drawing (Fig. 2). As already mentioned, this drawing was based on the Rutishauser formula for alizarin—Al—Ca compounds which has meanwhile been disproved by Kiel and Heertjes (1963a, b, c). However, even formation of a 2:1 (dye:metal) complex of carminic acid is impossible owing to the steric hindrance from the carboxyl groups. Nevertheless, carminic acid—Al—Ca compounds prepared in this laboratory behaved like complexes. Furthermore, Jain (1960) demonstrated 2:1 complexes of carminic acid with Cu^{++}, Pb^{++}, Th^{++} and Zr^{++}. These observations raised the question whether the widely accepted formula for carminic acid suggested by Dimroth and Kämmerer (1920) is correct. We therefore searched the chemical literature for more recent data on the structure of carminic acid.

Review of the Literature

Nature of the Side-chain. Dimroth and Kämmerer gave only the summary formula $—C_6H_{11}O_5$; the exact constitution was still obscure. Miyagawa (1929) studied the effects of ozone on carminic acid and presupposed that the side-chain was a methylpentose or corresponding acid; he therefore suggested the formula $—CO(CHOH)_4CH_3$ (Figs. 1, 3). This sugar was not identical with any known methylpentose and Miyagawa (1929) therefore regarded it as a new sugar, but the amount obtained was too small for detailed analysis. Miyagawa's (1929) formula indicated that though the side-chain is essentially a sugar group, it is not bound to the anthraquinone as a glycoside (F. Mayer, 1943). However, earlier studies by Hlasiwetz and Grabowski (1867, original not available) suggested that carminic acid is a glycoside which can be decomposed into a sugar and a new dye carmine red (Perkin and Everest, 1918), but this concept was attacked by Liebermann (1885) and Liebermann and Liebermann (1914).

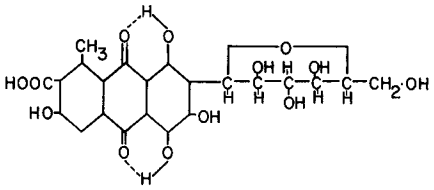

Fig. 5. Revised formula of carminic acid

Miyagawa's (1929) formula was difficult to reconcile with the formation of coccinin when carminic acid is fused with alkali; it seemed more likely that carminic acid is a C-glycosyl compound. Ali and Haynes (1959) therefore reinvestigated the side-chain. Their chemical data indicated that the side-chain in carminic acid is a glucopyranosyl and there can be little doubt that carminic acid is the D-glucopyranoxyl derivative (Fig. 5).

Position of the Carboxyl Group. According to a review of the chemistry of carminic acid by Perkin and Everest (1918) the carboxyl group was assigned to the 5-position of cochenillic and coccinic acid by Liebermann and Voswinkel. Dimroth (1909) accepted this arrangement in his formulation of carminic acid as carminazarin (a naphthoquinone) and also in the later hydroxanthraquinone formula (Dimroth, 1913). However, a reinvestigation of cochenillic acid by Overeem and Van der Kerk (1964) showed that the carboxyl group had been assigned to a wrong position. These studies led to a modification of the widely accepted structural formula of carminic and kermesic acid; the carboxyl group was transferred from the 5- to the 7-position and the hydrogen moved to the 5-position (Fig. 5). Parenthetically, kermesic acid differs from carminic acid only in its side-chain $—CO—CH_3$. The revised structure of carminic acid was confirmed by Bhatia and Venkataraman (1965) in chemical and nuclear magnetic resonance (NMR) studies. In the NMR spectrum of carminic acid in DSMO, a single aromatic proton appeared at 2.33 τ and it must therefore be an α- and not a β-proton in the anthraquinone nucleus. The carboxyl group in the structure for carminic acid must be moved to the β-position adjacent to the α-methyl group (Bhatia

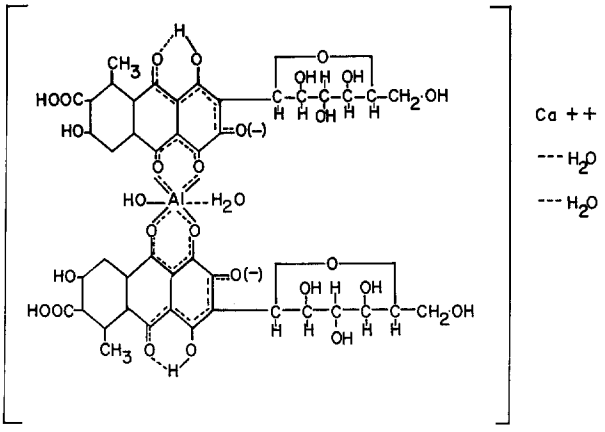

Fig. 6. Formula of a 2:1:1 carminic acid—Al—Ca complex analogous to the 2:1:1 alizarin—Al—Ca complex established by Kiel and Heertjes (1963a, b)

and Venkataraman, 1965) as shown in Fig. 5. The positions of the OH groups and the side-chain remain unaltered. According to F. Mayer (1943) carminic acid in alkaline solutions behaves like 1,3,4,6-hydroxyanthraquinone (hydroxyanthrapurpurin) but differs significantly from 1,2,4,6-hydroxy-anthraquinone (hydroxyflavopurpurin).

Structure of Carmine. Spectroscopic and recent analytical data on the composition and configuration of carmine could not be found in the available literature. It has been suggested that carmine is a carminic acid—Al—Ca lake or complex and the similarity of carmine and alizarin—Al—Ca compounds has been emphasized (Liebermann, 1885; P. Mayer, 1892; Dimroth, 1913; Harms, 1957a, b). In contrast to the alizarin complex carmine contains about 20–25% protein which is in part derived from *Coccus cacti* during preparation of cochenille and in part added during manufacture of carmine (Muspratt, 1860; Napier, 1875; Liebermann, 1885; Harms, 1957a, b). On the basis of chemical and spectroscopic studies Kiel and Heertjes (1963a, b, c) identified the metal complex of alizarin and its 3-derivatives as 2:1:1 dye—Al—Ca compounds. The literature on this subject has already been reviewed (Puchtler *et al.*, 1969). Al was found to form a chelate with two dye molecules; the Ca cation was bound by salt-type linkages to the dissociated β-OH groups. We adapted the Kiel and Heertjes (1963a, b) formula to carminic acid—Al—Ca (Fig. 6). Since nothing is known about bonds between carmine and the associated proteins, the latter have been omitted. This omission is justified by early observations that the dyeing and staining properties of decayed (verfaultem) or moldy carmine were superior to those of fresh carmine (Muspratt, 1860; Mayer, 1892); i.e. the protein moiety is dispensable and can be regarded as a contaminant of commercial dye samples.

Revised Models of Carminic Acid and Carmine

In the revised structure of carminic acid (Fig. 7) the bulky carboxyl group has been moved to the 7-position adjacent to the methyl group. The small hydro-

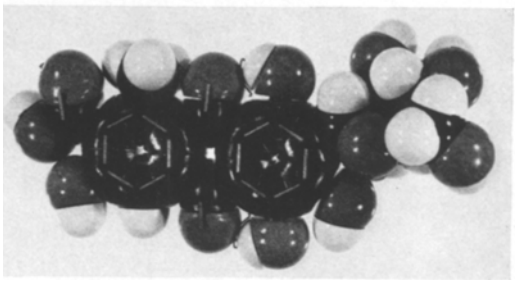

Fig. 7. Model of the revised formula of carminic acid (Fig. 5)

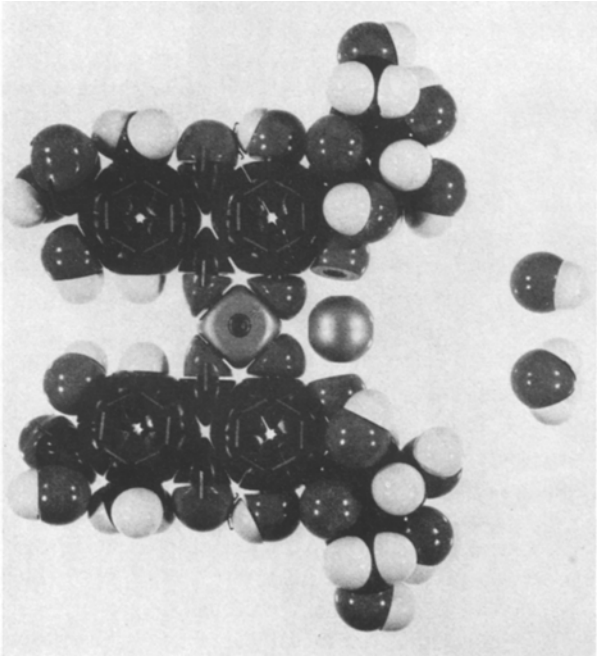

Fig. 8. Model of the 2:1:1 carminic acid—Al—Ca complex (Fig. 6). The revised structure of carminic acid allows chelation of two dye molecules with one Al atom; there is no steric hindrance

gen atom at the 5-position does not pose a steric hindrance to reactions at the 10—C = O and 4-OH groups. However, the revised side-chain is still too large to allow the close lateral approach necessary for formation of the Al—O—Ca—O—Al bonding shown in Fig. 4.

A model of carmine based on the Kiel and Heertjes (1963a, b) formula for alizarin—Al—Ca complexes is shown in Fig. 8. The aluminum atom can form a chelate with the 4- and 10-oxygen atoms of two dye molecules; the resulting 6-membered rings show no strain in the model and should be fairly stable. There is no steric hindrance to the close approach of the two dye molecules. For clarity,

the OH or H_2O ligands have not been attached to the remaining coordination sites of Al but are shown at right. The calcium cation compensates the negative charges of the dissociated β-O^- groups. Salt formation at the β-OH groups should be as important for chelate formation by carminic acid as it is in the alizarin series. In alizarin the pK of the α- and β-OH groups is 11.96 and 8.18 respectively. Chelate formation can occur only after salt formation at the β-OH group; which weakens the hydrogen bond formed by the α-OH group; under the influence of the β-OCa group the dissociation constant of the α-OH group becomes much larger, even larger than the value for the β-OH group (Kiel and Heertjes, 1963d). Physical-chemical data concerning the dissociation constants of OH groups in carminic acid could not be found in the available literature; however, our investigations of alizarin and alizarin red S and carminic acid indicate similar behaviour of the OH groups at various pH levels.

As already mentioned, the Al—O—Ca—O—Al bridge of the Rutishauser-type formula (Fig. 2) appears unlikely owing to steric hindrance from the large side-chains; there would be a large strain in the central bridge. Furthermore, the aluminum atom in this type of complex has a coordination number of 6 and the bonds are directional as shown in the model. In other words, the aluminum atom can combine with two more ligands, each more or less at right angle to the plane of the dye complex; but it seems unlikely that a —O—Ca—O— bridge could connect two coplanar carminic acid Al—Ca molecules at the Al atoms.

However, it must be emphasized that the suggested structure of carmine is hypothetical and based mainly on extensive chemical and spectroscopic studies of hydroxyanthraquinone dyes of the alizarin group. Corresponding studies of carminic acid complexes are lacking; carminic acid and carmine are no longer of interest to the textile and pigment industry. Unfortunately, facilities for spectroscopic studies are not available to us. However, the fact that this structure could be built with the Stuart Briegleb-type of atomic models shows that in contrast to the earlier structural formula (Figs. 2, 4) the proposed structure is at least theoretically possible. The associated proteins may perhaps form salts with the carboxyl groups in the 7-positions.

According to Harms (1957a, b) it appears probable that during preparation of mucicarmine (heating of carmine and $AlCl_3$ in a small amount of water) Al atoms form chelates with the 1-OH and 9-C=O group. This reaction is possible also with the revised formula. In addition, interaction of Al with the 6-OH—7-COOH grouping is theoretically feasible. The ability of o-hydroxy-carboxylic acids to form complexes is well known; for example dyes containing salicylic acid are widely used in the manufacture of premetallized dyes (Venkataraman, 1952). Again, the exact structure of mucicarmine can be established only by spectroscopic studies.

Variations in the Composition of Carmine. Samples of carmine from different manufacturers differed widely, and even samples from the same supplier varied from batch to batch. Such variations were found also in spectroscopic studies (Lima-de-Faria and Bose, 1953). These differences complicate histochemical studies of the chemical mechanism of various carmine stains. Complaints about the wide variations between samples of carmine were already voiced by Grenacher (1879). According to P. Mayer (1917) the manufacturers always marketed good

and bad sorts of carmine; the mode of manufacture of carmine was not disclosed and is still regarded as a trade secret (Harms, 1957a). However, a perusal of early literature yielded a few recipes for the manufacture of carmine. Muspratt (1860) and Napier (1875) gave several procedures which cast doubt on the belief that all samples of commercial carmine are or at least were a carminic acid—Al—Ca compound. Since the composition of carmine should affect its histochemical properties, the modes of preparation will be reviewed briefly.

Muspratt I. Carmine is generally prepared by subjecting cochineal to ebullition with K_2CO_3 or Na_2CO_3, and throwing down the coloring matter with a weak acid or an acid salt; sometimes binoxalate of potassium or a mixture of potassium bitartrate with alum. Occasionally, the precipitation is accelerated by the addition of gelatin or albumen.

Muspratt II. Boil cochineal (carminic acid) in water, add alum, boil briefly, then let stand undisturbed for some time. Decant supernatant and set aside for several days, when carmine is deposited. Additional carmine of rather inferior quality is obtained by permitting the mother-liquor again to repose for a length of time.

Muspratt III. Boil cochineal in water, add potassium bitartrate, boil again, add alum, boil briefly, let cool and decant; carmine is deposited and dried in the shade.

Muspratt IV. Boil cochineal of the best quality in distilled water, add KNO_3, boil briefly, add potassium binoxalate, boil again, remove from heat and let settle for four hours, decant into shallow glass vessels and set aside for 15 weeks. Remove the film of mold formed on the surface, draw the liquid off with a pipette and dry the carmine.

Napier. Boil cochineal in water with soda, then add a little alum, cream of tartar, and the white of eggs or isinglass, which separates the carmine as a flaky precipitate.

Evidently, these formulas do not contain calcium, except what may have been present in the water; furthermore, some products do not even contain aluminum. Such variations in composition would explain the widely differing staining properties of various samples of carmine. Yet, at least since Liebermann's (1885) studies carmine has been regarded as a carminic acid—Al—Ca compound. However, such formulas should obviously be reserved for products of known composition and for compounds prepared in the laboratory. Also, staining patterns obtained with samples of carmine whose composition is guarded as a trade secret should be interpreted with caution and should not be equated with histochemical reactions.

Acknowledgements. This investigation was supported by United States Public Health Service research grant He 12147 from the National Heart and Lung Institute and General Research Support Grant FR-5365.

References

Ali, M.A., Haynes, L.J.: C-Glycosyl compounds. III. Carminic acid. J. chem. Soc. **1959**, 1033–35.

Attree, G.F., Perkin, A.G.: Reduction products of the hydroxyanthraquinones. J. chem. Soc. **134**, 144–173 (1931).

Bhatia, S.B., Venkataraman, K.: The position of the carboxyl group in carminic acid. Indian J. Chem. **3**, 92–93 (1965).
Colour Index, 2nd ed., vol. III. Lowell, Mass.: The American Association of Textile Chemists and Colorists 1957.
Dimroth, O.: Zur Kenntnis der Carminsäure. Ber. dtsch. chem. Ges. **42**, 1611–1627 (1909).
— I. Über die Carminsäure. Ann. Chem. **399**, 1–35, (1913).
— Kämmerer, W.: Über die Carminsäure. Ber. dtsch. chem. Ges. **53**, 471–480 (1920).
Flett, M. St. C.: The application of infra-red spectroscopy to structural problems in the anthraquinone field. J. chem. Soc. **1948**, 1441–1448.
Grenacher, H.: Einige Notizen zur Tinktionstechnik, besonders der Kernfärbung. Arch. mikr. Anat. **16**, 463–471 (1879).
Harms, H.: Handbuch der Farbstoffe für die Mikroskopie, Teil II, 2. Liefg. Kamp-Lintfort: Staufen 1957a.
— Zur Chemie der Karmins und seiner in der Mikroskopie verwendeten Lösungen. Naturwissenschaften **44**, 327 (1957b).
Hüttig, G.F.: Physikalisch-chemische Untersuchungen der Oxy- und Dioxyanthrachinone mit besonderer Berücksichtigung ihres Beizvermögens. Z. physik. Chem. **87**, 129–168 (1914).
Jain, T.C.: Spectrophotometric studies of carminic acid complexes. J. Indian Chem. Soc. **37**, 727–730 (1960).
Kiel, E.G., Heertjes, P.M.: Metal complexes of alizarin. I. The structure of the calcium-aluminum lake of alizarin. J. Soc. Dyers Col. **79**, 21–27 (1963a).
— — Metal complexes of alizarin. II. The structure of some metal complexes of alizarin other than Turkey Red. J. Soc. Dyers Col. **79**, 61–64 (1963b).
— —Metal complexes of alizarin. III. The structure of metal complexes of some 3-derivatives of alizarin. J. Soc. Dyers Col. **79**, 186–188 (1963c).
— — Metal complexes of alizarin. IV. The structure of the potassium and calcium salts of alizarin and of 3-nitroalizarin. J. Soc. Dyers Col. **79**, 363–367 (1963d).
Liebermann, C.: Zur Kenntnis der Cochenille und des Cochenillecarmins. Ber. dtsch. chem. Ges. **18**, 1969–1975 (1885).
— Liebermann, H.: Zur Formulierung der Carminsäure. Ber. dtsch. chem. Ges. **47**, 1213–1219 (1914).
Lima-de-Faria, A., Bose, S.: Comparative analysis of acetocarmine solutions. Caryologia **6**, part II, 652–653 (1954).
Mayer, F.: The chemistry of natural coloring matters. Translated by A.H. Cook. New York: Reinhold Publishing Co. 1943.
Mayer, P.: Über das Färben mit Carmin, Cochenille und Hämatein-Tonerde. Mitt. Zool. Stat. Neapel **10**, 480–504 (1892).
— Über die Reinheit unserer Farbstoffe. Z. wiss. Mikr. **17**, 305–328 (1917).
Miyagawa, I.: Über die Konstitution der Carminsäure. Mem. Coll. Eng. Kyuchu Imp. Univ. **4**, 99–113 (1929).
Muspratt, S.: Chemistry, theoretical, practical and analytical as relating to the arts and manufacture, vol. I. London: William Mackenzie 1860.
Napier, J.: A manual of dyeing and dyeing receipts, composing a system of elementary chemistry as applied to dyeing. London: Charles Griffin & Co. 1875.
Overeem, J.C., Kerk, G.J.M. van der: Mollisin, a naturally occurring chlorine-containing quinone. Part IV. Revised structures for cochenillic acid and for the insect pigments, carminic and kermesic acid. Rec. Trav. chim. Pays-Bas **83**, 1023–1035 (1964).
Perkin, A.G., Everest, A.E.: The natural organic colouring matters. London: Longmans, Green & Co. 1918.
Puchtler, H., Meloan, S.N., Terry, M.S.: On the history and mechanism of alizarin and alizarin red S stains for calcium. J. Histochem. Cytochem. **17**, 110–124 (1969).
Venkataraman, K.: The chemistry of synthetic dyes, vol. 1. New York: Academic Press 1952.

Holde Puchtler, M.D.
Department of Pathology
Medical College of Georgia
Augusta, Ga. 30902, U.S.A.