of it were available in the sequence preceding a -Gly.Leu C-terminal ending for HA, such as -Ala.Ala.Ala.Gly.Leu. From data concerning the specificity of CPase for a number of peptides studied by Neurath, *et al.*,[15] it was suspected that a peptide bond containing alanine as the C-terminal group might be hydrolyzed about ten or fifteen times faster than such a bond containing glycine as the C-terminal group. To substantiate this possibility, carbobenzoxy-L-alanyl-L-alanyl-L-alanyl-glycine was synthesized and digested by CPase. As shown in Fig. 3 and 4, more alanine than glycine was released during any period of time, just as though the C-terminal amino acid of the peptide were alanine, and the ratio of glycine to alanine released from the peptide was about 1:2. These results show that more alanine than glycine can be released from HA if more alanine units follow glycine in the C-terminal sequence. They also show that the bond between the carbobenzoxy group and alanine was not hydrolyzed with CPase.

The digested HA products, which were isolated after digestion of the original HA with CPase for 0.5 hr. in one case and 2 hr. in another, were then further digested with CPase. During any period of time in this digestion, 3.5 moles of alanine, 1 mole of valine and 1.5 moles of leucine were released for 1 mole of glycine. From these results, the C-terminal amino acid sequence of HA is suggested to be -(Leu, Val, Ala_{3-4}) Gly.Leu; however, the exact sequence of the alanines, valine and leucine is not suggested from the present experimental data. As demonstrated by the experiments described herein, if the amino acids of a protein are not arranged according to decreasing rates of enzymic hydrolysis, the amino acids may not be released in order of the C-terminal amino acid sequence. HA is an example of such a protein. Therefore, it should be emphasized that the carboxypeptidase method must be used for the determination of the amino acid sequence, along with another method.

The author also confirmed independently of the report of Biserte[8] that DNP-cysteic acid could not be detected in a hydrolysate of DNP-oxidized HA or oxidized DNP-HA. Therefore, it is supposed that HA has one peptide chain, with aspartic acid as the N-terminal amino acid and leucine as the C-terminal acid as:

Asp.Ala.His-------(Leu, Val, Ala_{3-4}) Gly.Leu.

This was supported by investigation of the physicochemical properties of oxidized and reduced carboxymethylated HA.[16,17]

Acknowledgments.—The author is indebted to Dr. Bruno Jirgensons and Dr. Darrell N. Ward for their helpful discussion concerning this study and also to Dr. A. Clark Griffin, Head of the Department of Biochemistry, for his interest in this work.

(15) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(16) M. J. Hunter and F. C. McDuffie, THIS JOURNAL, **81**, 1400 (1959).

(17) B. Jirgensons and T. Ikenaka, *Makromol. Chem.*, **31**, 112 (1959).

[CONTRIBUTION FROM THE DIVISION OF PURE CHEMISTRY, NATIONAL RESEARCH COUNCIL OF CANADA]

Cytodeuteroporphyrin[1,2]

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The syntheses of the four desmethyl derivatives of deuteroporphyrin 9 have been completed by the syntheses of the 1-, 5- and 8-desmethyl derivatives. The cytodeuteroporphyrin from cytohemin (hemin-a) was identical with the last of these.

Cytohemin (hemin-a) is the prosthetic group of the *sauerstoffübertragende Ferment* (cytochrome-a_3) and of cytochrome-a. Crystalline cytohemin[4] had been degraded to cytodeuteroporphyrin[5] by fusing with resorcinol to split off the labile side-chains and then removing the iron. Cytodeuteroporphyrin contained two carboxyl groups which had survived resorcinol fusion suggesting propionic acid rather than acetic acid or ring carboxyl groups. Its oxidation had led to methylmaleimide and hematinic acid in the same yields as from deuteroporphyrin 9. This and the analysis of cytodeuteroporphyrin had suggested that it might be one of the fifteen deuteroporphyrins[6]; however, it differed from deuteroporphyrin 9 and from the synthetic deuteroporphyrins 1, 2, 3, 5 and 6. Cytodeuteroporphyrin had then been shown to form a tribromo derivative rather than the dibromo derivative expected of a deuteroporphyrin. It evidently had three free positions and one possible structure had been excluded by comparison with synthetic 3-desmethyl-deuteroporphyrin 9(II).[7]

As to the substituents in cytodeuteroporphyrin were not completely defined analytically and particularly as those of cytohemin were evidently not all of normal chain length,[4] the possible structures for cytodeuteroporphyrin could not be definitely limited. According to another approach,[8] two free positions were to be expected on opposite rings in cytodeuteroporphyrin because the spectrum ("oxo-rhodo" type) of the porphyrin corresponding to cytohemin had suggested labile side chains ("rhodifying" groups) in these

(1) This was the subject of a preliminary communication, THIS JOURNAL, **81**, 250 (1959).

(2) Issued as N.R.C. No. 5643.

(3) National Research Council of Canada Postdoctorate Fellow.

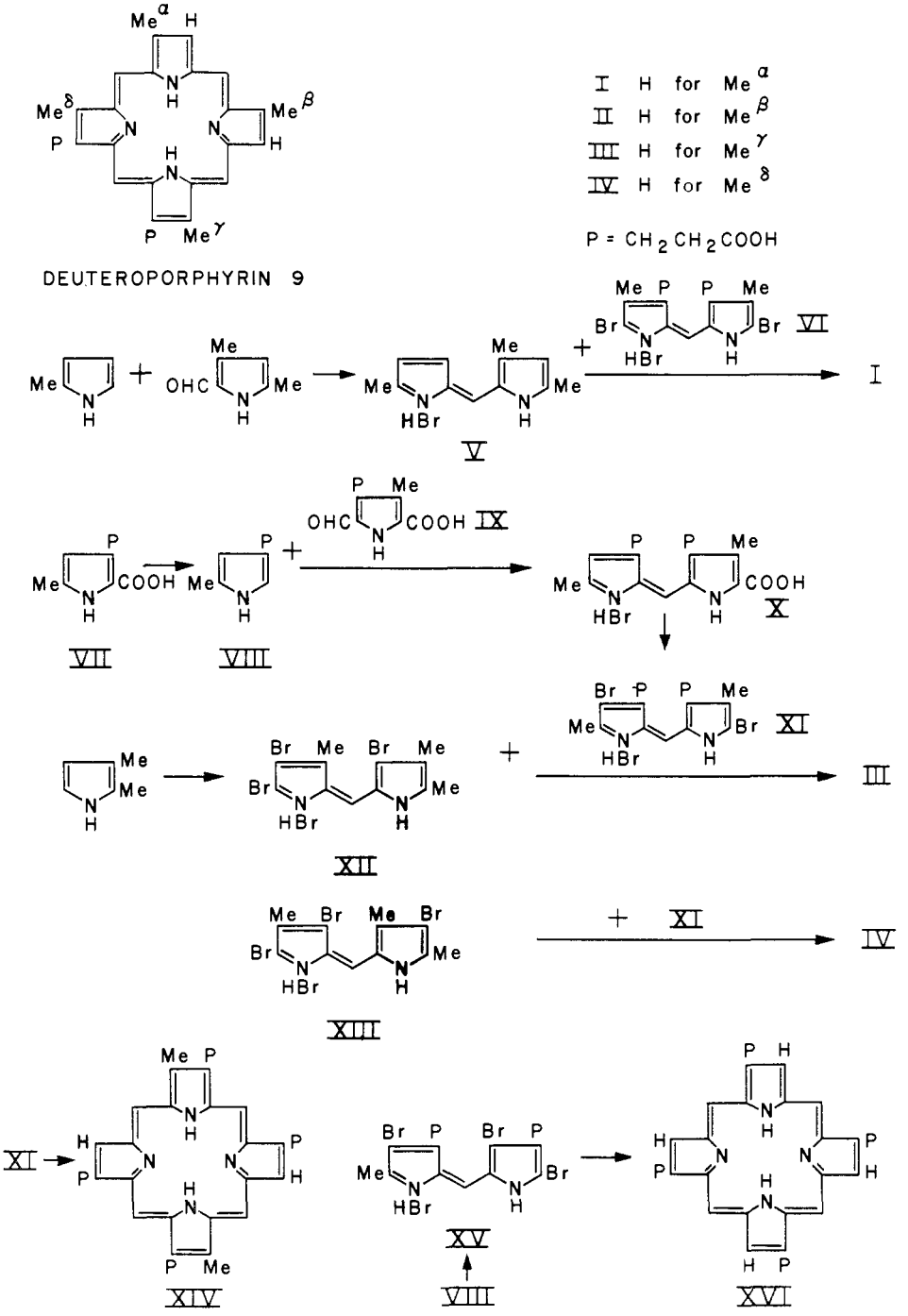
(4) O. Warburg and H. S. Gewitz, *Z. physiol. Chem.*, **288**, 1 (1951).

(5) *Idem, ibid.*, **292**, 174 (1953).

(6) For the nomenclature of the deuteroporphyrin isomers see H. Fischer and H. Orth, "Chemie des Pyrrols," Vol. II/I, Leipzig, 1937, p. 409.

(7) O. Warburg, H. S. Gewitz and W. Völker, *Z. Naturforsch.*, **10b**, 541 (1955). We have continued this work at the suggestion of Professor Warburg, who kindly provided the specimen of cytodeuteroporphyrin methyl ester from crystalline cytohemin *ex* horse heart.

(8) R. Lemberg, *Nature*, **172**, 619 (1953).



positions. Among other uncertainties in this last argument, the lack of model compounds with two "rhodifying" groups in one ring has been pointed out.[8]

We explored two routes to the structure of cytodeuteroporphyrin. The first, a prerequisite for rational synthetic work, was a re-investigation of the maleimides resulting from its oxidation. However, we have not yet separated hematinic acid from its desmethyl derivative by paper chromatography or gas chromatography.[9] The second was synthesis guided by rather arbitrary assumptions which rationalized the analytical evidence and suggested only two structures. There was no reason to question the homogeneity of the methyl maleinimide from cytodeuteroporphyrin. This imide had analyzed well and the possible contaminants were known: maleimide and its dimethyl-, ethyl- and methyl-ethyl derivatives. However, it was conceivable that the hematinic acid from cytodeuteroporphyrin contained the unknown desmethyl-hematinic acid,[10] particularly as the carbon analysis of the hematinic acid was low.[7] If cyto-

(9) Compare H. V. Morley, F. P. Cooper and A. S. Holt, *Chemistry & Industry*, 1018 (1959).

(10) This assumption remains unproved.

hemin were derived from protoporphyrin 9, cytodeuteroporphyrin might then be formulated as 5- or 8-desmethyldeuteroporphyrin 9 (III or IV), structures also consistent with the spectroscopic evidence. The 3-desmethyl derivative of deuteroporphyrin 9 (II) was known.[7] To complete the series and as a control on the criteria of identity we synthesized the 1-desmethyl derivative I as well as the 5- and 8-desmethyl derivatives III and IV.

2-Methylpyrrole and 2,4-dimethylpyrrole-5-aldehyde were condensed to the dipyrromethene V as a hydrobromide and also as a hydrochloride. Bromination of V to its tribromo-derivative confirmed the rational course of this condensation. When the dipyrrylmethenes V and VI were fused together 1-desmethyl-deuteroporphyrin 9 (I) resulted. In this synthesis it was not necessary to use V in the form of its tribromo derivative.[11]

The dicarboxylic acid VII, obtained from its diethyl ester, was decarboxylated to 2-methylpyrrole-4-propionic acid (VIII) and this condensed with IX to the dipyrrylmethene X. Bromine converted the latter to XI which contained perbromide, the halogen content being too high before treatment with acetone and afterward too low. 2,3-Dimethylpyrrole was brominated to XII, the structure of which is assumed by analogy as is usual in such cases.[12] The 5- and 8-desmethyl derivatives of deuteroporphyrin 9 (III and IV) were obtained by fusing XI with XII and XIII, respectively. The dipyrrylmethenes XII and XIII are more easily available than XI and the product of their self-condensation is easily separated from III or IV. *A priori* it seemed advantageous to use an excess of XII or XIII to increase the yield of III or IV at the expense of XIV and facilitate their separation from the latter.

The isolation of 1-desmethyl-deuteroporphyrin 9 (I) was carried out in the usual way, removing by-products with aqueous resorcinol[13] after esterification. Here the esterified product may not have been completely extracted from insoluble material (see below). The isolation of III and IV required additional steps to remove products of the self-condensation of the dipyrromethenes: 1,3,5,7-tetramethylporphin and 1,5-dimethylporphin-2,3,6,7-tetrapropionic acid (XIV). We were unable to convert XIII into its 5-bromomethyl derivative which with X would lead to IV but not to XIV. The tetramethylporphin was easily recognized and separated by its solubility in chloroform before esterification and non-porphyrin by-products were removed with aqueous resorcinol after esterification. As the porphyrins, like XVI, were incompletely extracted from the insoluble material during esterification with methanolic hydrogen chloride, a subsequent extraction was necessary. The 5- and 8-desmethyldeuteroporphyrins were separated from XIV by countercurrent distribution as the rather insoluble free acids to avoid the possible complications of ester hydrolysis. They were crystallized as their methyl esters.

(11) Compare ref. 6, p. 169.

(12) Reference 6, pp. 62 ff.

(13) S. F. MacDonald and R. J. Stedman, *Can. J. Chem.*, **32**, 896 (1954).

The XIV distributed as a tetrapropionic acid and the melting point of its ester agreed with that of the porphyrin synthesized from XI alone. The structures I, III and IV were confirmed by converting the esters to tribromo derivatives.

Porphin-1,3,5,7-tetrapropionic acid (XVI) was required as a source of 3-(2-carboxyethyl)-maleimide (desmethyl-hematinic acid), which we had failed to obtain from simpler pyrrole derivatives. This porphyrin had been synthesized from the dipyrrylmethene obtained from 2-methyl-5-carboxypyrrole-3-propionic acid and bromine.[14] The analogous bromination of VII failed, but that of VIII led to the isomeric dipyrrylmethene XV, converted as usual to porphin-1,3,5,7-tetrapropionic acid (XVI). The structure of the dipyrrylmethene XV is assumed by analogy[12] and it evidently contained some perbromide.

The straightforward comparison of cytodeuteroporphyrin with the synthetic porphyrins was disappointing. As the infrared spectra of III and IV in carbon disulfide were insufficiently characteristic, useful comparisons were limited to the solids, and differences might then be due to polymorphism. It appeared that either III (m.p. 183–187° and 211–213°)[15] or IV (m.p. 198–202°) but not I (m.p. 230–234°) might be cytodeuteroporphyrin. The melting point of cytodeuteroporphyrin from crystalline cytohemin,[7] m.p. on our stage 194–198° (lit. 188°), was not depressed by IV but its mixed melting point with III was inconclusive, being complicated by solid phase changes. However, the X-ray powder pattern of this cytodeuteroporphyrin was unrelated to that of III and had only few strong lines in common with that of the "normal" form (see below) of IV.

It was still possible that an identity might be established between other solid phases of the porphyrins. As we had not enough of this specimen of cytodeuteroporphyrin (<1 mg.) for an infrared mull spectrum or for much manipulation, we tried to make the synthetic specimens give an X-ray photograph identical with that of cytodeuteroporphyrin. Cytodeuteroporphyrin being derived from two cytochromes, it was not impossible that it was a mixture. When III and IV were crystallized together from chloroform–methanol in the cold and three fractions taken, the second fraction (m.p. 196–200.5°) gave an X-ray photograph identical with that of the cytodeuteroporphyrin. After recrystallization from hot methanol, avoiding fractionation, this fraction gave an X-ray photograph identical with that of the "normal" form of IV crystallized in the same way (see below). Thus cytodeuteroporphyrin was apparently IV and, whether or not III was also involved, polymorphism was.

We then compared the X-ray photographs of IV crystallized under various conditions, hoping to obtain it in the form in which cytodeuteroporphyrin had been characterized or, as some polymorphic forms of porphyrins are not always obtainable at will, in a form which could be obtained consistently. Like cytodeuteroporphyrin, IV had only one m.p. When the chloroform in solutions

(14) H. Fischer, R. J. Doyle and W. Gleim, *Ann.*, **525**, 32 (1936).

(15) Unless otherwise noted discussion refers to the methyl esters.

of IV was displaced by methanol and the latter slowly evaporated hot, X-ray powder photographs showed that IV consistently separated in the "normal" form first observed. From cold methanol two further types of crystals resulted. The X-ray powder photographs of these two (separated manually), of the "normal" form, and of the above second fraction (or cytodeuteroporphyrin), did not reveal which of the four, if any, were single polymorphs, but suggested that the lines of at least three polymorphs were involved. The relationships are less clear cut than we had previously stated.[1] Finally, when the cytodeuteroporphyrin was recrystallized hot by the method leading to the "normal" form of IV, its X-ray powder photograph was identical with that of the latter; cytodeuteroporphyrin was therefore IV. Unlike subsequent comparisons, these were made with cytodeuteroporphyrin prepared from crystalline cytohemin.[7]

Confirmation through further comparisons required more cytodeuteroporphyrin, but fresh horse hearts were not available as a source of crystalline cytohemin.[16] Applying the procedure of Warburg, Gewitz and Volker[7] to fresh beef hearts or to frozen horse hearts led to cytohemin–protohemin preparations whose high lipid content rendered later steps in this procedure ineffective. In some runs modifications were made to raise the cytohemin–protohemin ratio[17] facilitating the eventual separation of the derived deuteroporphyrins. The crude cytohemin (in the chloride form) was then fused with resorcinol and, after removing iron from the hemins, the resulting cytodeuteroporphyrin and deuteroporphyrin 9 were separated by countercurrent distribution between hydrochloric acid and ether. The cytodeuteroporphyrin was crystallized as its ester from hot methanol.

This cytodeuteroporphyrin, from beef or horse heart as specified in the Experimental, was identified with IV by the m.p., mixed m.p., visible and infrared mull spectrum of the "normal" form of the methyl ester, the m.p. and mixed m.p. of its copper complex and by the X-ray powder photographs of these, of the tribromo derivative and of the free acid.

Incidentally, when a mixture of IV and deuteroporphyrin 9 was fractionally crystallized, the X-ray photograph of one fraction was nearly identical with that of IV and those of the other two clearly represented mixtures. In view of this and the separation of IV from III mentioned above, it is unlikely that the formation of mixed crystals (solid solutions) would prevent the detection of any second major component in cytodeuteroporphyrin.

Experimental[18]

3,5,5′-Trimethylpyrromethene Hydrobromide (V).—2-Methylpyrrole[19] (2.67 g.) and 2,4-dimethylpyrrole-5-aldehyde[20] (4.04 g.) were heated to solution in 3 ml. of ethanol and 10.5 ml. of 42% hydrobromic acid added. The product (35%) was filtered off after 3 hours and washed with acetic acid. It formed orange-red needles from acetic acid, m.p. 237° after darkening.

Anal. Calcd. for $C_{12}H_{15}N_2Br$: C, 53.91; H, 5.66; N, 10.49; Br, 29.90. Found: C, 54.06; H, 5.73; N, 10.65; Br, 29.81.

More recently it was prepared in 68% yield by the method given for the hydrochloride below.

3,5,5′-Trimethylpyrromethene Hydrochloride.—2-Methylpyrrole (810 mg.) and 2,4-dimethylpyrrole-5-aldehyde (1230 mg.) were dissolved in 10 ml. of dry ethanol. Dry hydrogen chloride was passed into the solution for 2 minutes to precipitate the product (70%). For analysis it was recrystallized from ethanol, m.p. 187° dec.

Anal. Calcd. for $C_{12}H_{15}N_2Cl$: C, 64.71; H, 6.79. Found: C, 64.90; H, 6.49.

4,3′,4′-Tribromo-3,5,5′-trimethylpyrromethene Hydrobromide.—Bromine (177 mg.) in 98% formic acid (1 ml.) was added to a suspension of 41 mg. of the dipyrrylmethene hydrobromide V in 1 ml. of formic acid. The mixture was heated for 10 minutes on the steam-bath then cooled and the product (90%) filtered off. Recrystallization from formic acid gave red needles, m.p. 205° dec.

Anal. Calcd. for $C_{12}H_{12}N_2Br_4$: C, 28.60; H, 2.40; N, 5.56; Br, 63.45. Found: C, 28.98; H, 2.74; N, 5.42; Br, 63.28.

3,5,8-Trimethylporphin-6,7-dipropionic Acid Dimethyl Ester (1-Desmethyl-deuteroporphyrin 9 Methyl Ester) (I).—A thoroughly ground and dried mixture of the dipyrromethene hydrobromides V (0.93 g.) and VI[21] (1.95 g.) and succinic acid (8.7 g.) was heated for 1.25 hours at 185–195° in four portions. The warm melts were ground with hot water and the combined mixtures brought to *p*H 4 with disodium hydrogen phosphate at 100°, then worked up as usual using 45% aqueous resorcinol.[13] The solution was, however, passed through a second column of alumina before crystallizing the product (76 mg., 4.2%) from chloroform (1 ml.)–methanol. Recrystallization from methanol (thimble) gave plates and needles, m.p. 222–227°, raised to 230–234° by two further recrystallizations.

Anal. Calcd. for $C_{31}H_{32}O_4N_4$: C, 70.97; H, 6.15; N, 10.68. Found: C, 70.61; H, 5.92; N, 10.58.

1,2,4-Tribromo-3,5,8-trimethylporphin-6,7-dipropionic Acid Dimethyl Ester.—Bromine in acetic acid (1.05 ml. of a 15% (w./v.) solution) was added to a warm solution of 35 mg. of I in 8 ml. of acetic acid. The solution was heated for 10 minutes in the steam-bath, cooled, and glistening purple plates of the perbromide (69 mg.) were separated and dried. This was dissolved in 7 ml. of acetone and after 2 hours water was added to complete the precipitation of the porphyrin. It was dissolved in 8 ml. of hot chloroform. The solution was filtered, heated, and 8 ml. of hot methanol added. Next day the product was isolated as needles, m.p. 274–276° dec.

Anal. Calcd. for $C_{31}H_{29}O_4N_4Br_3$: C, 48.89; H, 3.84; N, 7.36; Br, 31.50. Found: C, 49.04; H, 4.12; N, 7.27; Br, 31.62.

2-Methyl-5-carboxy-pyrrole-4-propionic Acid (VII).—The corresponding ester[22] (5 g.) in 20 ml. of ethanol and 30 ml. of aqueous sodium hydroxide (8% w./v.) was refluxed on the steam-bath for 1 hour. Most of the solvent was then distilled off, finally *in vacuo*. The residue was diluted to 50 ml. and the product precipitated with sulfur dioxide at 0°. After washing and drying it was recrystallized from ether (thimble) as nearly colorless needles (60%), m.p. about 132° dec.

(16) The crystallization of cytohemin requires preparations from fresh horse heart and the removal of the bulk of the impurities within one day (private communication from Professor Warburg).

(17) Compare R. Lemberg, B. Bloomfield, P. Caiger and W. H. Lockwood, *Australian J. Exp. Biol. Med. Sci.*, **33**, 435 (1955).

(18) Melting points are uncorrected. Those of porphyrin derivatives indicate the full melting range and were done on a Kofler block requiring a correction, checked periodically, of $<\pm 2°$. Otherwise melting points were determined in capillaries. The infrared spectra were obtained by Dr. R. N. Jones and Mr. R. Lauzon. The X-ray powder photographs were obtained by Dr. W. H. Barnes and by Dr. Maria Przybylska; we are particularly grateful to the former for the interpretation. In all pairs found identical, the patterns had 30 to 36 measurable lines except those of the tribromoporphyrin which had 10.

(19) P. A. Cantor, R. Lancaster and C. A. VanderWerf, *J. Org. Chem.*, **21**, 918 (1956).

(20) H. Fischer and W. Zerweck, *Ber.*, **56**, 519 (1923).

(21) H. Fischer and A. Kürzinger, *Z. physiol. Chem.*, **196**, 213 (1931).

(22) S. F. MacDonald and R. J. Stedman, *Can. J. Chem.*, **33**, 458 (1955).

Anal. Calcd. for $C_9H_{11}NO_4$: C, 54.82; H, 5.62; N, 7.10. Found: C, 54.68; H, 5.81; N, 6.99.

2-Methyl-pyrrole-4-propionic Acid (VIII).—The crude dicarboxylic acid VII was heated and stirred to solution on the steam-bath with 1.25 parts of water. The solution was then quickly brought to boiling, cooled and the product filtered off. After drying it was extracted with anhydrous ether (thimble, a second thimble below the first contained Darco). Colorless needles (65% from the ester), m.p. 79–81°, separated when pentane was added to the concentrated extract.

Anal. Calcd. for $C_8H_{11}NO_2$: C, 62.72; H, 7.24. Found: C, 62.56; H, 7.13.

4,5′-Dimethyl-5-carboxy-pyrromethene-3,3′-dipropionic Acid Hydrobromide (X).—A slurry of 2-formyl-4-methyl-5-carboxy-pyrrole-3-propionic acid[23] (IX, 100 mg.), 2-methylpyrrole-4-propionic acid (VIII, 68 mg.) in 0.2 ml. of methanol was stirred and cooled in an ice–salt-bath. Cold hydrobromic acid (0.7 ml. of 42%) was added dropwise. After 20 minutes the brown micro needles (93 mg., 48%), decomposing at about 160°, were separated and dried.

Anal. Calcd. for $C_{18}H_{21}N_2O_6Br$: C, 49.00; H, 4.79; N, 6.35; Br, 18.11. Found: C, 48.87; H, 4.94; N, 6.35; Br, 17.98.

5,4′-Dibromo-4,5′-dimethylpyrromethene-3,3′-dipropionic Acid Hydrobromide (XI).—Bromine (420 mg.) in 98% formic acid (1.4 ml.) was added to 387 mg. of the dipyrromethene X. Crystals separated when the mixture was heated for 2 minutes on the steam-bath. After 1 hour at 20°, ether was added and the product filtered off as glistening purple-brown micro needles and plates (333 mg.). Their birefringence disappeared completely at 210° without melting.

Anal. Calcd. for $C_{17}H_{19}N_2O_4Br_3$: C, 36.78; H, 3.45; N, 5.05; Br, 43.20. Calcd. for $C_{17}H_{19}N_2O_4Br_5$: C, 28.56; H, 2.67; N, 3.92; Br, 55.90. Found: C, 30.46; H, 2.75; N, 4.89; Br, 51.34.

On adding acetone the substance dissolved then separated as a deep purple microcrystalline powder, behavior on heating unchanged.

Found: C, 37.44; H, 3.59; Br, 42.17.

Much higher yields were obtained when formic acid was removed from the mixture in a vacuum desiccator and the product washed with ether.

1,5-Dimethylporphin-2,3,6,7-tetrapropionic Acid Tetramethyl Ester (XIV).—The pyrromethene hydrobromide XI (223 mg.) was ground, dried, and fused for one hour at 185–195° with 700 mg. of succinic acid. The melt was worked up as for 1-desmethyldeuteroporphyrin 9 above and the product, m.p. 258–259°, crystallized from hot chloroform (1 ml.)–methanol (8 ml). After catalytic dehalogenation[24] it separated as plates (10.5 mg., 7%), m.p. 257–261° after a phase change at 199°.

Anal. Calcd. for $C_{38}H_{42}N_4O_8$: C, 66.86; H, 6.20. Found: C, 68.82; H, 7.46.

2,3-Dimethylpyrrole.—2,3-Dimethyl-4-carbethoxypyrrole-5-carboxylic acid[24] (20 g.) was converted into 2,3-dimethylpyrrole (70%) by the method developed for 2,4-dimethylpyrrole.[25]

3,4′,5′-Trimethyl-3′,4,5-tribromopyrromethene Hydrobromide (XII).—Bromine (2.4 ml.) in 12 ml. of acetic acid was added to 1.09 g. of 2,3-dimethylpyrrole in 20 ml. of acetic acid. After 10 days the amorphous product (61%) was separated and washed with acetic acid, m.p. > 280°.

Anal. Calcd. for $C_{12}H_{12}N_2Br_4$: C, 28.60; H, 2.40; N, 5.56; Br, 63.45. Found: C, 28.07; H, 2.67; N, 5.43; Br, 63.68.

5-Methyl-4,3′,5′-tribromopyrromethene-3,4′-dipropionic Acid Hydrobromide (XV).—2-Methylpyrrole-4-propionic acid (1 g.) was warmed to solution in 4 ml. of acetic acid and the solution cooled to room temperature. Bromine (2 ml.) in 5 ml. of acetic acid was run in with shaking. After cooling, the mixture was seeded, scratched and left in a desiccator at 50 mm. over potassium hydroxide for 4 days. The product (1.62 g.) was stirred, filtered, and washed with acetic acid, then with ether. For analysis it was twice recrystallized from acetic acid; it decomposed without melting.

Anal. Calcd. for $C_{16}H_{16}N_2O_4Br_4$: C, 31.00; H, 2.60; Br, 51.56. Found: C, 29.31; H, 2.69; Br, 54.85.

Porphin-1,3,5,7-tetrapropionic Acid Tetramethyl Ester (XVI).—The pyrromethene hydrobromide XV (15 g.) was ground and dried with 50 g. of methylsuccinic acid. The mixture was heated in one lot for 1.5 hours at 190–200° in a test-tube with slow stirring. The melt was ground with 3 l. of hot water. The insoluble material was filtered off, washed, dried, and left overnight in 150 ml. of 5% methanolic hydrogen chloride, then extracted for 2 hours with fresh methanolic hydrogen chloride (thimble), the porphyrin ester hydrochloride being very slightly soluble. The pigment was then brought into chloroform and worked up as usual, filtering through alumina, washing with 45% aqueous resorcinol, filtering through alumina again and crystallizing from chloroform–methanol. Recrystallized from acetone displacing chloroform, it formed prisms; 174 mg. 2.2%, m.p. 270–271° (lit.[14] 265–266°).

Anal. Calcd. for $C_{36}H_{38}N_4O_8$: C, 66.04; H, 5.85; Br, 0.00; Found: C, 65.52; H, 5.64; Br, 0.93.

1,3,5-Trimethylporphin-6,7-dipropionic Acid Dimethyl Ester (8-Desmethyl-deuteroporphyrin 9 Dimethyl Ester) (IV).—A mixture of 1.545 g. of 5,4′-dibromo-4,5′-dimethylpyrromethene-3,3′-dipropionic acid hydrobromide (XI), 6.855 g. (5 mols.) of 4,3′,5′-trimethyl-3,5,4′-tribromopyrromethene hydrobromide[26] (XIII) and 60 g. of methylsuccinic acid was ground then dried *in vacuo*. This mixture was introduced in portions into a test-tube (1.5″ o.d.) which was held at 195–205° in an oil-bath and fitted with a slow stirrer. Heating was continued for 1 hour after the addition. The melt was freed of methylsuccinic acid as usual with much hot water using disodium hydrogen phosphate to bring the *p*H to 5. The dry powdered insoluble material was extracted with chloroform (thimble) to remove the tetramethyl-porphin.[27] The dried insoluble residue was left overnight at 20° in 5% methanolic hydrogen chloride and undissolved matter extracted with fresh methanolic hydrogen chloride (thimble). The pigment was brought from methanol into chloroform, the concentrated solution (150 ml.) washed with 45% aqueous resorcinol (4 × 25 ml., previously washed with benzene and chloroform) then with water, and the chloroform evaporated. The residue was dissolved in ether, extracted into 4% hydrochloric acid, returned to fresh ether, and the ether evaporated. The residue was dissolved in chloroform, the solution filtered through deactivation alumina, and the solvent evaporated. The esters were hydrolyzed in refluxing aqueous methanolic sodium hydroxide, methanol distilled off, and the porphyrins isolated after precipitation with acetic acid. These were subjected to countercurrent distribution between 0.28% hydrochloric acid and ether, in nine portions to avoid crystallization, the porphyrins in the first five tubes, 10 ml. of each phase per tube, 100 transfers. To determine the relative concentrations, all the pigment was brought into the lower phase with five drops of concentrated hydrochloric acid and the absorption measured at 545 mμ. Two symmetrical bands were centered at tubes 14(K = 0.16)[28] and 64(K = 1.78; K calcd. for 0.45% hydrochloric acid, 0.69). The 8-desmethyl-deuteroporphyrin 9 in tubes 52–74 was brought into ether, the ether evaporated, the residue esterified with methanolic hydrogen chloride, and the ester was brought from methanol into chloroform. The washed and dried (sodium sulfate) solution was concentrated, then hot methanol was added while the solvent was boiled off until crystallization was virtually complete, giving needles (31.4 mg., 2%), m.p. 198–202°; visible spectrum in pyridine–ether (relative densities in parentheses): maxima at 619(0.35), 593(0.08), 571(0.33), 565(0.44), 521(0.57) and 489(1.0) mμ, shoulders at 609, 557, 493 and 484 mμ.

(23) H. Fischer and H. Andersag, *Ann.*, **458**, 117 (1927).

(24) Compare A. H. Corwin and R. H. Krieble, THIS JOURNAL, **63**, 1829 (1941).

(25) A. Treibs and R. Schmidt, *Ann.*, **577**, 110 (1952).

(26) H. Fischer and H. Scheyer, *Ann.*, **434**, 237 (1923).

(27) Alkali did not extract this from chloroform; compare H. Fischer and H. Kirstahler, *Z. physiol. Chem.*, **198**, 50 (1931).

(28) This is evidently the 1,5-dimethylporphin-2,3,6,7-tetrapropionic acid described above. In one run it was isolated as its methyl ester (0.5%), m.p. 256–260° after a phase change at 190–195°. With 0.28% hydrochloric acid the calculated K for a coproporphyrin of hydrochloric acid number 0.09[29] is 0.20.

(29) Reference 6, p. 487.

The product was further characterized by the X-ray powder photography and infrared mull spectrum.

Anal. Calcd. for $C_{31}H_{32}N_4O_4$: C, 70.97; H, 6.15; N, 10.68. Found: C, 70.37, 71.31; H, 6.30, 6.16; N, 10.44.

When this ester and the isomeric ester of III were crystallized together from chloroform–methanol, avoiding fractionation, very small crystals of indeterminate form, m.p. largely 163–175°, resulted.

1,3,5-Trimethylporphin-6,7-dipropionic Acid (8-Desmethyl-deuteroporphyrin 9).—The synthetic dimethyl ester IV was hydrolyzed with alkali and the pigment brought into ether. The free acid crystallized in clusters of parallel rods as the ether solution was concentrated. It was characterized by its X-ray powder photograph.

Copper Complex of 1,3,5-Trimethylporphin, 6,7-Dipropionic Acid Dimethyl Ester.—Methanolic copper acetate was added to a chloroform solution of the synthetic porphyrin ester IV and the chloroform displaced by methanol. On cooling, tiny needles of the copper complex separated, m.p. 236.5–240°, sintering from 233°, characterized by an X-ray powder photograph.

2,4,8-Tribromo-1,3,5-trimethylporphin-6,7-dipropionic Acid Dimethyl Ester.—The synthetic ester IV (6 mg.) was warmed to solution in 1.2 ml. of acetic acid and 0.21 ml. of bromine in acetic acid (15% w./v.) added. The mixture was heated for 10 minutes in the steam-bath. After 4 hours the crystalline perbromide was filtered off and dissolved in 1.2 ml. of acetone. After 2 hours 6 ml. of water was added to complete the precipitation of the porphyrin. It was recrystallized hot from chloroform (1 ml.)–methanol (2 ml.). The product (2 mg.), m.p. 285–287° decomp., crystalline under the polarizing microscope, was characterized by its X-ray powder photograph.

Anal. Calcd. for $C_{31}H_{29}N_4O_4Br_3$: Br, 31.50. Found: Br, 32.11.

1,3,8-Trimethylporphin-6,7-dipropionic Acid Dimethyl Ester (5-Desmethyl-deuteroporphyrin 9 Dimethyl Ester) (III).—A mixture of 1.6 g. of the dipyrromethene hydrobromide XI, 7.2 g. (5 mols.) of XII and 60 g. of methylsuccinic acid was fused and worked up as in the synthesis of the isomer IV. The same by-products were separated, the alkyl porphyrin by chloroform extraction, the tetrapropionic acid in the first 10 tubes after countercurrent distribution using 0.45% hydrochloric acid. The dimethyl ester, from the 5-desmethyl-deuterioporphyrin 9 (K = 0.67), formed plates (17 mg., 1%), m.p. 183–187° and 211–213°, characterized by the X-ray powder photograph and infrared mull spectrum. The lower melting form tended to separate from cold solutions and was converted to the higher melting one at about 200°.

Anal. Calcd. for $C_{31}H_{32}N_4O_4$: C, 70.97; H, 6.15. Found: C, 70.85; H, 6.03.

Copper Complex of 1,3,8-Trimethylporphin-6,7-dipropionic Acid Dimethyl Ester.—Prepared as was that of the isomeric ester of IV, it formed long needles, m.p. 236–238° after a phase change at 206°, also characterized by an X-ray powder photograph.

2,4,5-Tribromo-1,3,8-trimethylporphin-6,7-dipropionic Acid Dimethyl Ester, prepared from III just as its isomer was prepared from IV, formed hair-like crystals, m.p. 284–288° dec.

Anal. Calcd. for $C_{31}H_{29}N_4O_4Br_3$: Br, 31.50. Found: Br, 31.19.

Deuteroporphyrin 9 Methyl Ester.—Hemin was degraded to this ester, m.p. 224–227° (lit.[24] 224–224.5°); spectrum in pyridine–ether (relative densities in parentheses): maxima at 619(0.40), 593(0.09), 572(0.35), 565(0.46), 552.5(0.64) and 489.5 mμ (1.0), shoulders at 610, 557, 494 and 484 mμ. It was also characterized by its X-ray powder photograph and by that of its copper complex, m.p. 235–236° (lit.[30] 234°).

(30) T. C. Chu and E. J. Chu, THIS JOURNAL, **74**, 6276 (1952).

Cytodeuteroporphyrin Methyl Ester. (a) **From Fresh Beef Hearts.**—The mince from eighteen hearts was washed with water, with 0.5% saline, then extracted essentially by the procedure of Warburg, *et al.*[7] The cytohemin was extracted from the third (sodium acetate) precipitate with acetic acid, reprecipitated with heptane and dissolved in acetone–hydrochloric acid. Ether (1 l.) and then water were added to this solution. The ether layer was well washed with 1% hydrochloric acid, concentrated to 150 ml. and left one day at −30°. The ether solution, separated from crystalline protohemin, was evaporated. Cytohemin was extracted from the residue with acetic acid and precipitated with heptane giving a preparation (69 mg.) of cytohemin–protohemin about 1.5:1, containing lipid.

This material (21 mg.) was fused with resorcinol and the iron removed from the hemins in acetic acid.[7] The acetic acid solution was poured into water and the porphyrins brought into ether with sodium acetate. They were extracted from the washed ether solution by 3% hydrochloric acid then returned to fresh ether (300 ml.). The porphyrins in the washed and concentrated ether solution (50 ml. in the first five tubes) were subject to countercurrent distribution to 200 transfers between 0.45% hydrochloric acid and ether. The two fractions had maxima at tubes 53 (deuteroporphyrin 9, K = 0.36[31]) and 81 (cytodeuteroporphyrin, K = 0.68) separated by a very pale minimum at tube 67. The cytodeuteroporphyrin in tubes 69–96 was esterified and its chloroform solution filtered through deactivated alumina. After displacing the chloroform by methanol, cytodeuteroporphyrin dimethyl ester (2.2 mg.) separated hot as needles, m.p. 198–202°, X-ray powder photograph identical with that of the "normal" form of IV; spectrum in pyridine–ether (relative densities in parentheses): maxima at 618.5(0.35), 593(0.08), 571(0.33), 565(0.44), 521(0.57) and 489(1.0) mμ, shoulders at 609, 557, 493 and 484 mμ. This spectrum agrees with that of IV and differs significantly from that of deuteroporphyrin 9 only in relative intensities.

(b) **From Frozen Horse Hearts.**—A cytohemin preparation obtained similarly from frozen horse hearts was extracted with hydrochloric acid in ether.[7] From this extract heptane precipitated a 4:1 mixture of cytohemin and protohemin, again containing lipids. The subsequent steps were as under (a) above. There were clear tubes between the two maxima after the countercurrent distribution. The cytodeuteroporphyrin methyl ester formed needles m.p. 201–202.5°, 201.5–203.5 when mixed with synthetic IV, 165–202° when mixed with III. Its infrared mull spectrum (although better resolved) and its X-ray powder photograph were identical with those of the "normal" form of IV and differed from those of III.

Copper Complex of Cytodeuteroporphyrin Methyl Ester.—Prepared as was the complex of IV, the copper complex of cytodeuteroporphyrin from beef heart formed needles, m.p. 237–239° sintering from 235°, mixed m.p. with the copper complexes of the esters of synthetic III, IV, and of deuteroporphyrin 9, 207–227°, 237–239° and 225–233°, respectively. Its X-ray powder photograph was identical with that of the copper complex of synthetic IV.

Bromo Derivative of Cytodeuteroporphyrin Methyl Ester.—The bromination of cytodeuteroporphyrin methyl ester from beef hearts was analogous to that of IV. The product formed hair-like crystals which lost crystallinity at 283–289° but were not molten at 300° (lit.[7] 298°). Its X-ray powder photograph was identical with that of synthetic 2,4,8-tribromo-1,3,5-trimethylporphin-6,7-dipropionic acid methyl ester.

Cytodeuteroporphyrin (Free Acid).—Cytodeuteroporphyrin ester from horse hearts was hydrolyzed with alkali and the free acid brought into ether. Concentration of the ether solution gave rods of cytodeuteroporphyrin, the X-ray powder photograph of which was identical with that of synthetic IV (free acid).

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(31) Compare S. Granick and L. Bogorad, *J. Biol. Chem.*, **202**, 781 (1953).