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Use of the Aqueous Formic Acid-Chloroform-Dimethylformamide Solvent System for the Purification of Porphyrins and Hemins

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The purification of proto-, meso-, deutero- and hematoporphyrin preparations utilizing the aqueous formic acid-chloroform-dimethylformamide (AFCD) solvent system was investigated. Although the selectivity of this solvent system was lower than that of the aqueous formic acid-chloroform (AFC) system, it was none the less found to be more suitable for preparatory purposes owing to its higher dissolving capacity. A probable reason for the difficulties encountered in purifying hematoporphyrin is its strong tendency to form aggregates. The flexibility of the AFCD system was indicated by application to the further purification of protohemin. The isolation method of Labbe and Nishida yielded protohemin containing probably less than 1 % of impurities, as demonstrated by fractionation employing the AFCD system. This protohemin also resulted in proto- and mesoporphyrins having a high degree of purity. Conversely, a commercial preparation of protohemin yielded protoporphyrin containing 10 – 20 % of a compound thought to be a deuteroporphyrin having RCO-groups at positions 2 and 4.

The use of the aqueous formic acid-chloroform (AFC) solvent system in the separation of free dicarboxylic porphyrins has been previously described.[1] The AFC system was found to be suitable for small-scale separations of the porphyrins since it possessed high selectivity and did not exhibit the disadvantages associated with the ethyl ether-hydrochloric acid system.[2] On the preparatory level, however, the AFC system was rather unsatisfactory due to its low dissolving capacity. Attention was therefore focused upon the tendency of the porphyrins to form aggregates, which appeared to be the principal reason for the difficulties encountered in their purification, especially that of protoporphyrin IX.

In order to eliminate aggregation, a fourth component, dimethylformamide (DMF), was added to the solvent system. This component is reportedly an excellent solvent for porphyrins.[3] As is elucidated in the present article, aqueous formic acid-chloroform-dimethylformamide (AFCD) forms a valuable

solvent system for the purification of dicarboxylic porphyrins and hemins. Utilizing this improved system it was possible to study the partition behaviour of hematoporphyrin IX in greater detail. The method of multiple partition in the purification of protoporphyrin IX was also re-evaluated.

MATERIALS AND METHODS

Protohemin IX. Preparation *1* of the protohemin was isolated from bovine blood according to the method of Labbe and Nishida.[4] The ratio of the volume of blood to that of the extraction solvent was 1:10. From 400 ml of blood, 1.546 g of crystalline protohemin IX were obtained. Preparation *2* of the protohemin was a commercial product of the Sigma Chemical Co.

Protoporphyrin IX. The preparation of protoporphyrin IX from protohemin followed the ferrous sulfate method of Morell and co-workers.[5] Upon the basis of its spectroscopic properties (Table 1, e), the protoporphyrin obtained from protohemin preparation *1* appeared to have a high degree of purity. Protohemin preparation *2*, on the contrary, yielded a product exhibiting absorption maxima, in chloroform, at 632, 606, 577, 543, 508, and 408 nm. These values are slightly larger than the values reported for pure protoporphyrin IX.[6-8]

Mesoporphyrin IX was prepared according to the procedure developed by Baker and co-workers.[9] Protohemin preparation *1*, further purified by means of the partition method described below, was employed as starting material. Spectroscopy was not able to reveal any impurities in the resultant mesoporphyrin preparation.

Deuteroporphyrin IX. Protohemin (preparation *1*) was heated with resorcinol at 190 – 200°C for 15 min.[10] After cooling to room temperature, the reaction mixture was dissolved in 15 ml of concentrated sulphuric acid. Fifty grams of ice and 85 ml of water were then added to the sulphuric acid solution, which was subsequently filtered and diluted with 150 ml of water. Deuteroporphyrin precipitated from the solution upon neutralization with sodium acetate to pH 5. The product was recovered by filtration, washed with water and dried at 50°C for 2 h. No impurities were spectroscopically revealed in the preparation.

Hematoporphyrin IX. A commercial preparation of hematoporphyrin dihydrochloride (Koch-Light & Co.) was utilized. This preparation appeared to be spectroscopically pure.

Solvents. The solvents employed were of analytical grade. Chloroform was washed three times with distilled water immediately prior to use. Diethyl ether was first treated with a concentrated solution of ferrous sulfate and then washed, dried and distilled. Dimethylformamide was further purified by distillation at reduced pressure.

Fractionation by multiple liquid-liquid partition. Partition separations were performed utilizing the Hietala apparatus.[11] Porphyrins were fractionated by means of the aqueous formic acid (of varied molarity)/chloroform(25)-dimethylformamide(1), while, in the separation of hemins, the upper phase consisted of 22.5 M formic acid and the lower phase of chloroform(10)-dimethylformamide(1). The upper phase was employed as mobile solvent in all of the fractionations described in the present article. The phase ratio varied from 0.2 to 0.3 among the different fractionations. A shaking frequency of 22 cycles/min, an amplitude of $\pm 45°$ and a flow rate of 1 – 2 ml/min were utilized. Theoretical distribution curves and partition coefficients were calculated by methods previously described.[1] When considered advantageous, elution upon the basis of a stepwise pH gradient was applied.

Spectroscopy. The absorption spectra of the porphyrins were recorded by means of a Cary Model 15 spectrophotometer, while single absorbances were measured employing a Beckman DU spectrophotometer.

pH values. A Radiometer PHM 4c pH meter was utilized in measuring hydrogen ion concentrations (20°C). The pH meter was calibrated using phthalate buffer.

Table 1. Spectroscopic properties of porphyrins.

Compound	Solvent	Peak positions (nm) and peak ratios (R) I nm	R	Ia nm	R	II nm	R	III nm	R	IV nm	R	S nm
a. Hematoporphyrin IX	Py	622.0	0.200	596.0	0.100	569.0	0.447	532.0	0.595	499.0	1.000	403.0
»	Ee	623.0	0.257	596.0	0.138	569.0	0.457	529.0	0.608	497.5	1.000	397.0
b. 2(4)-Vinyl-4(2)-hydroxyethyl-deuteroporphyrin IX	Ee	627.0	0.280	602.0	0.140	573.0	0.430	532.5	0.720	500.0	1.000	400.5
»	Chl	626.0	0.310	601.0	0.184	572.5	0.526	537.5	0.724	502.5	1.000	405.0
»	Py	626.5	0.312	601.0	0.149	572.5	0.508	537.5	0.762	502.0	1.000	405.5
c. Deuteroporphyrin IX	Ee	620.5	0.317	595.5	0.087	566.5	0.415	523.0	0.585	492.0	1.000	393.5
»	Chl	619.0	0.258	—	—	565.5	0.428	529.0	0.563	496.0	1.000	398.0
d. Mesoporphyrin IX	Ee	622.0	0.412	595.0	0.105	567.5	0.456	525.5	0.725	495.0	1.000	394.0
»	Chl	620.0	0.335	594.0	0.103	566.5	0.473	531.0	0.696	498.0	1.000	398.5
e. Protoporphyrin IX (from hemin)	Ee	631.5	0.408	605.0	0.093	576.0	0.447	535.0	0.781	502.0	1.000	403.0
f. »	Ee	631.5	0.440	605.0	0.097	576.0	0.445	535.0	0.785	502.0	1.000	403.5
»	Chl	629.0	0.349	603.0	0.148	575.0	0.563	539.5	0.850	504.0	1.000	406.0
g. Porphyrin 638	Ee	638.0	0.146	—	—	582.5	0.385	539.0	0.430	506.0	1.000	408.0
»	Chl	638.0	0.114	—	—	583.0	0.371	545.0	0.372	510.5	1.000	412.0

S = Soret band, Py = pyridine, Ee = ethyl ether, and Chl = chloroform.

RESULTS

Purification of hematoporphyrin IX. No impurities had been previously [1] noted in the commercial hematoporphyrin dihydrochloride preparation (Koch-Light & Co.). However, low amounts of other porphyrins may have been present in this preparation, since rather small quantities of feeding material were employed in the partition fractionations. Moreover, the behavi-

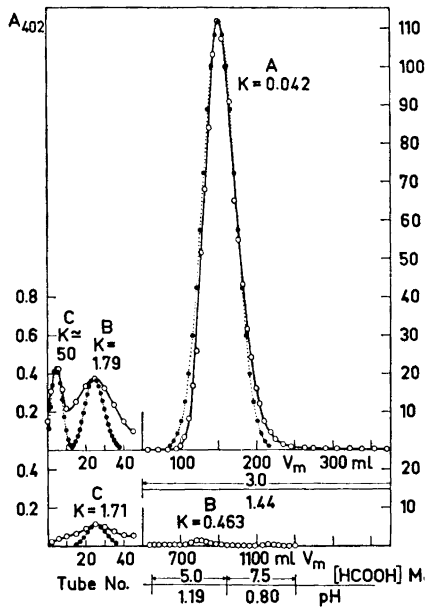

Fig. 1. Purification of the hematoporphyrin IX preparation. Solvent system: HCOOH(3.0, 5.0, and 7.5 M)/$CHCl_3$(25)-DMF(1). Ten milligrams of hematoporphyrin dihydrochloride were dissolved in 1.2 ml of DMF, and to the resulting solution were added 30 ml of $CHCl_3$ + 0.38 ml of HCOOH + 10 ml of H_2O. The mixture was then sampled into tubes $r = 0,1,2$. Number of tubes utilized = $N = 50$. Average volume of mobile phase (HCOOH) in a partition unit = $v_m = 2.64$ ml; average volume stationary phase ($CHCl_3$ – DMF) in a partition unit = $v_s = 10.86$ ml. Total volume of effluent eluted from the apparatus = $V_m = 1300$ ml; flow rate = 1 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring A_{402} of the effluent fractions and of the lower phases in the tubes. A = hematoporphyrin IX (96 %), B = 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX (3 %), and C = protoporphyrin IX (1 %).

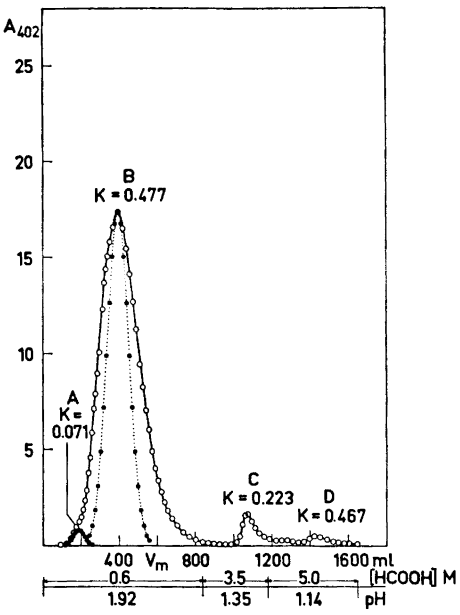

Fig. 2. Refractionation of component A (hematoporphyrin IX) in Fig. 1. Solvent system: HCOOH(0.6, 3.5, and 5.0 M)/$CHCl_3$(25)-DMF(1). The component eluted within the range 100 – 250 ml was dissolved in 20 ml of 0.6 M HCOOH, which was then sampled into tubes $r = 0,...,6$, each containing 10 ml of the lower phase. $N = 50$. $v_m = 3.14$ ml; $v_s = 10.36$ ml. $V_m = 1650$ ml; flow rate = 1 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring A_{402} of the effluent fractions. A, C, and D = various molecular forms of hematoporphyrin, and B = hematoporphyrin dication (70 %).

our of the hematoporphyrin had not been extensively investigated as a function of the pH of the upper phase.

The hematoporphyrin preparation was therefore, in the present study, subjected to careful examination, since Schwartz *et al.*[12] have reported that commercial preparations were found to contain 30 % or more of other porphyrins. According to the above authors, nine distinct fractions can be obtained by countercurrent distribution (CCD), while many more are apparent using column chromatography.

Fig. 1 presents the results of a fractionation which began with 3.0 M formic acid (pH 1.44). The upper portion of the figure discloses the situation at an eluted volume of 550 ml. The absorption spectrum of component A in the effluent appeared to be identical with the di-cation spectrum of hematoporhyrin IX (λ_{max}: 590, 547, and 400 nm). Moreover, the absorption spectrum of the neutral form of component A closely matched the spectroscopic properties of hematoporphyrin IX (Table 1, a). Two further components remained in the apparatus at this stage of elution: component B, having the spectroscopic properties of 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX (Table 1, b), and component C, having those of protoporphyrin IX (Table 1, f). The lower portion of the figure reveals the outcome of continued fractionation, first by eluting with 400 ml of 5.0 M formic acid and then with about 350 ml of 7.5 M formic acid. Component B, in this part of the figure, shows the approximate amount (3 %) of 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin eluted. The amount of protoporphyrin (C) is estimated to be 1 %.

Fig. 2 presents the results of the refractionation of component A in Fig. 1. Fractionation was begun, in this case, with 0.6 M formic acid (pH 1.92). Four separate components (A – D) were detected in the effluent, while one additional component (E, not indicated in the figure), having a diffuse concentration zone, remained in the apparatus. Components A and B exhibited normal di-cation spectra when measured in the effluent. When the absorption spectra of components C and D were measured in the effluent, however, an additional peak at 370 nm was revealed (Fig. 3, Part A). A small amount of concentrated formic acid, when added to this effluent, yielded a normal di-cation spectrum (Fig. 3, Part B). The spectra of the neutral forms of components A to E were identical with the spectrum of the neutral form of hematoporphyrin IX. The absorption spectrum of the hematoporphyrin in distilled water is presented in Fig. 4. This spectrum contains a Soret band at 370 nm and differs also in other regards from the spectroscopic properties reported for the various forms of hematoporphyrin. The spectrum in Fig. 4 is typical of hematoporphyrin in an aggregated state.

The above experimental facts indicate that components A, C, and D in Fig. 2 are different forms of aggregated hematoporphyrin. Component A was ascertained as a separate molecular form of hematoporphyrin by initiating its fractionation with 0.5 M formic acid (pH 2.00). The component was then completely separated from the principal hematoporphyrin zone (B). The possibility of component A being either 2,4-hydroxymethyldeuteroporphyrin or some other hydrophilic porphyrin was excluded by an additional refractionation, in which the feeding material consisted of the pure hematoporphyrin

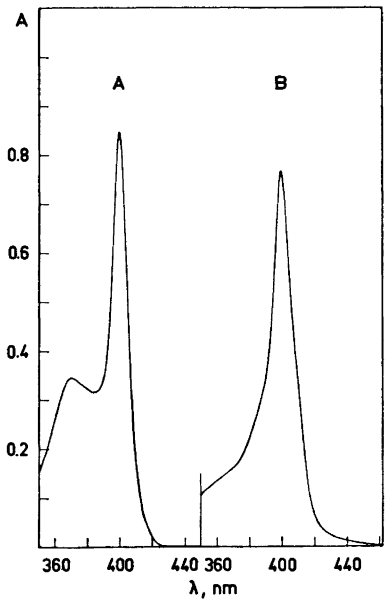

Fig. 3. Visible absorption spectrum of hematoporphyrin IX in the region of the Soret band. Part A: the spectrum of component C (Fig. 2) measured immediately following its elution (at 1105 ml) from the apparatus; part B: the same after the addition of conc. HCOOH (1.0 ml of HCOOH was added to 15 ml of the effluent eluted at 1105 ml).

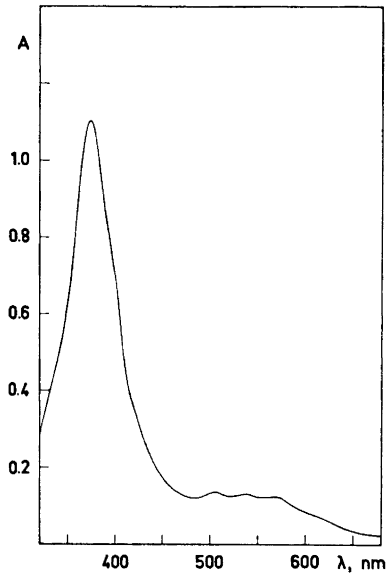

Fig. 4. Visible absorption spectrum of hematoporphyrin IX in distilled water.

fraction (B). In this case, a new fraction A was separated from the primary hematoporphyrin zone.

Purification of protohemin IX. The purification of protohemin preparation *1* utilizing the AFCD solvent system is presented in Fig. 5. Only traces (apparently less than 1 %) of two other hemins, A and B, were separated from the principal protohemin zone C. Presumably, component A represents hematohemin IX, while B is 2(4)-vinyl-4(2)-hydroxyethyldeuterohemin IX. The solvent system employed appears to be useful for the fractionation of hemins. As observed in Fig. 5, deviations from the theoretical values are negligible in the case of protohemin IX. Amounts as great as 200 mg have been purified in a single fractionation according to this method. As described below, protohemin purified by this procedure yields mesoporphyrin having a high degree of purity, when the method of Baker *et al.*[9] is utilized in preparing the latter compound.

Purification of protoporphyrin IX. Fig. 6 presents the results of a fractionation performed upon the protoporphyrin IX obtained from protohemin preparation *1*. A small amount of rapidly migrating material (component A)

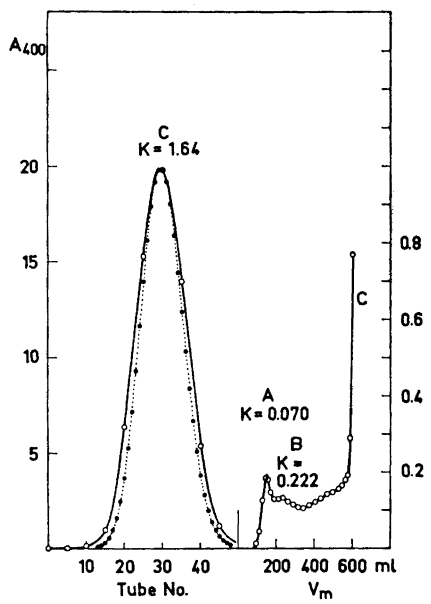

Fig. 5. Purification of protohemin IX preparation *1*. Solvent system: HCOOH-(22.5 M)/$CHCl_3$(10)-DMF(1). Twenty milligrams of the protohemin preparation were dissolved in 5.0 ml of DMF, and to the resulting solution were added 50 ml of $CHCl_3$ + 18 ml of HCOOH + 3.0 ml of H_2O. The mixture was then sampled into tubes $r = 0,...,5$. $N = 50$. $v_m = 2.28$ ml; $v_s = 11.22$ ml. $V_m = 620$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring $A_{400\,nm}$ of the effluent fractions and of the lower phases in the tubes. A = hematohemin IX, B = 2(4)-vinyl-4(2)-hydroxyethyldeuterohemin IX, and C = protohemin IX.

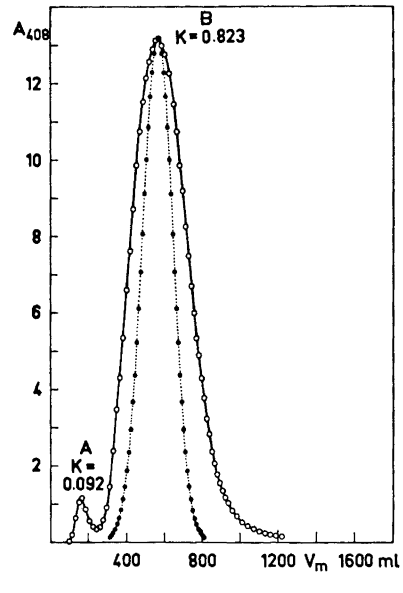

Fig. 6. Purification of protoporphyrin IX obtained from protohemin preparation *1*. Solvent system: HCOOH(8.0 M)/$CHCl_3$(25)-DMF(1). Eleven milligrams of the protoporphyrin preparation were dissolved in 2.0 ml of DMF and to the resulting solution were added 50 ml of $CHCl_3$ + 4.6 ml of HCOOH + 10.4 ml of H_2O. The mixture was then sampled into tubes $r = 0,...,4$. $N = 50$. $v_m = 3.14$ ml; $v_s = 10.36$ ml. $V_m = 1222$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring A_{408} of the effluent fractions. A = 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX (1 – 2 %) and B = protoporphyrin IX (98 – 99 %).

separated from the principal porphyrin zone (component B). Spectroscopically, fraction A appeared to consist primarily of 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX. When the protoporphyrin obtained from fraction B was refractionated utilizing the same solvent system, additional quantities of hydrophilic porphyrins appeared. The indication is that the vinyl groups of the protoporphyrin slowly become hydrated under the prevailing acidic conditions. This result is in accord with the investigations of Falk and coworkers.[7]

When protoporphyrin IX obtained from protohemin preparation *2* was subjected to partition fractionation, the result presented in Fig. 7 was achieved. Small amounts of hydrophilic porphyrins (components A and B) were also

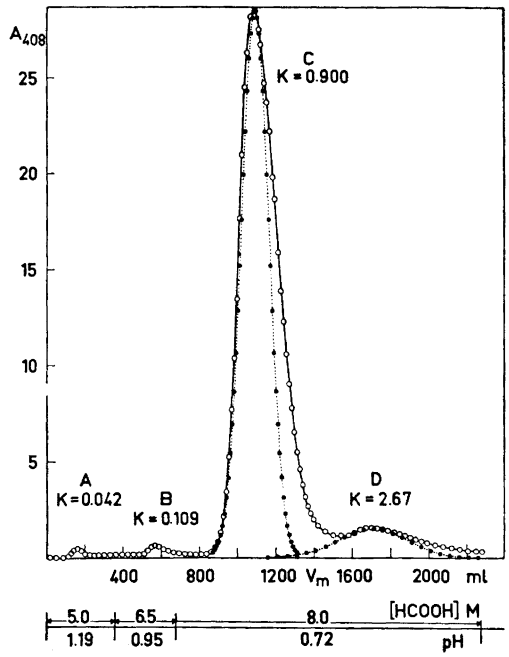

Fig. 7. Purification of protoporphyrin IX obtained from protohemin preparation *2*. Solvent system: HCOOH(5.0, 6.5, and 8.0 M)/$CHCl_3$(25)-DMF(1). Eighteen milligrams of the protoporphyrin preparation were dissolved in 4.0 ml of DMF and to the resulting solution were added 100 ml of $CHCl_3$ + 5.7 ml of HCOOH + 24.3 ml of H_2O. The mixture was then sampled into tubes $r = 0,...,9$. $N = 50$. $v_m = 2.88$ ml; $v_s = 10.62$ ml. $V_m = 2280$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring $A_{408\ nm}$ of the effluent fractions. A = hematoporphyrin IX + 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX, B = 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX, C = protoporphyrin IX (≃80 %), and D = "porphyrin 638" (≃15 %).

observed in this separation. Spectroscopically, fraction A appeared to be a mixture of hematoporphyrin IX and 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX, while fraction B consisted predominantly of the latter porphyrin. However, the principal impurity in this case was a porphyrin (component D) which differed distinctly from protoporphyrin according to its visible absorption spectrum (Table 1, g). Evidently, component D is identical to the compound previously observed (Fig. 6 in Ref. 1) as being an impurity in protoporphyrin prepared from the same commercial protohemin. The spectroscopic properties of component D, hereafter denoted as "porphyrin 638", are most similar to those of 2,4-methoxycarbonyldeuteroporphyrin IX, although the properties of 2,4-acetyl- and 2,4-propionyldeuteroporphyrin IX also fit rather well.[8] The di-cation spectrum of "porphyrin 638" exhibited absorption maxima at 607, 564, and 418 nm. These values closely approximate those reported for the di-cation of 2,4-acetyldeuteroporphyrin IX.[13] "Porphyrin 638" appeared to be stable in the employed solvent system. When the frac-

tions eluted within the range 1400 – 2250 ml were refractionated, the amounts of protoporphyrin IX and "porphyrin 638" within these fractions remained unchanged (Fig. 8). These characteristics suggest that "porphyrin 638" is a deuteroporphyrin having RCO-groups at positions 2 and 4, where R may be either the methyl, ethyl, methoxy, or ethoxy radical.

The possibility that "porphyrin 638" was formed from photoprotoporphyrin under the acidic conditions prevalent during fractionation was eliminated in the following manner: 10 mg of protoporphyrin IX, obtained from protohemin

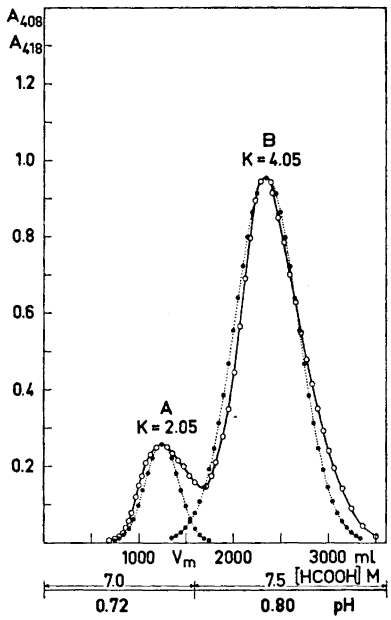

Fig. 8. Refractionation of component D ("porphyrin 638") in Fig. 7. Solvent system: HCOOH(7.0 and 7.5 M)/$CHCl_3$(25)-DMF(1). The component (eluted within the range 1400 – 2250 ml) was dissolved and sampled into the apparatus as in Fig. 6. $N = 50$. $v_m = 2.28$ ml; $v_s = 11.22$ ml. $V_m = 3500$ ml; flow rate = 2 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring A_{408} of the effluent fractions up to a total volume of 1600 ml and A_{418} of the effluent fractions eluted thereafter. A = protoporphyrin IX (20 %) and B = "porphyrin 638" (80 %).

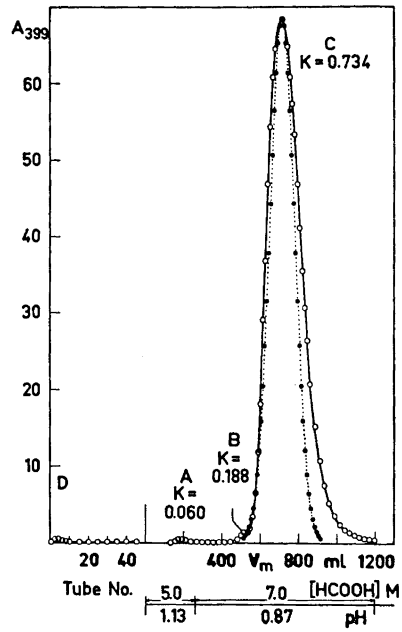

Fig. 9. Purification of mesoporphyrin IX obtained from further purified protohemin preparation *I*. Solvent system: HCOOH(5.0 and 7.0 M)/$CHCl_3$(25)-DMF(1). Twenty milligrams of mesoporphyrin, prepared according to the method of Baker *et al.*, were dissolved in 2.0 ml of DMF and to the resulting solution were added 50 ml of $CHCl_3$ + 2.85 ml of HCOOH + 12.2 ml of H_2O. The mixture was then sampled into tubes $r = 0,...,5$. $N = 50$. $v_m = 3.04$ ml; $v_s = 10.46$ ml. $V_m = 1195$ ml; flow rate 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring A_{399} of the effluent fractions and of the lower phases in the tubes. A = hematoporphyrin IX, B = formyldeuteroporphyrin IX (?), C = mesoporphyrin IX (99 %), and D = unknown porphyrin(s).

preparation *1* and dissolved in a neutral solvent (ethyl ether), were exposed to daylight and atmospheric oxygen. The appearance of an absorption peak at 669 nm indicated the formation of photoprotoporphyrin.[14,15] This protoporphyrin preparation, contaminated by a small amount of photoprotoporphyrin, was then subjected to partition fractionation. The result was visually similar to that of Fig. 6, with the exception that fraction A now contained, in addition to 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin IX, a second hydrophilic porphyrin which exhibited, in ethyl ether, an absorption peak at 650 nm. This serves as an indication that the alteration product of photoprotoporphyrin in aqueous acidic solution is probably other than "porphyrin 638".

Purification of meso- and deuteroporphyrin IX. The mesoporphyrin, obtained from protohemin preparation *1* which had been further purified by partition fractionation, contained only traces of other porphyrins, as shown by the results presented in Fig. 9. Spectroscopically, fraction A appeared to consist of hematoporphyrin IX, while fraction B possessed a Soret band at 421 nm and may be a formyldeuteroporphyrin. A small quantity of an unknown porphyrin(s) (fraction D) remained at the sampling end of the apparatus. The spectroscopic properties of fraction C eluted within the range 600 – 1000 ml closely resembled those of mesoporphyrin IX (Table 1, d).

Fig. 10 presents the results of further purification of the deuteroporphyrin IX preparation. Fraction A consisted of negligible amounts of two porphyrins exhibiting red absorption peaks, in diethyl ether, at 623 and 641 nm, respectively. A small quantity of unknown prophyrin material (fraction C) remained at the sampling end of the apparatus after this fractionation as well. The principal fraction (B) yielded deuteroporphyrin IX having the spectroscopic properties presented in Table 1, c.

DISCUSSION

Flexibility is a valuable property of the AFCD solvent system. The ratios of the solvent components may be varied. Thus, if larger amounts of porphyrins than those stated in the present work need to be purified, then the volume fraction of dimethylformamide may be increased. The fact that the AFCD system can be applied to the fractionation of hemins is an indication of its flexibility.

The selectivity of the AFCD system employed for fractionation of the porphyrins appears in Fig. 11. The slope of lines B and E, referring to 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin and protoporphyrin, respectively, is estimated as 2.6, while the slope of lines A, C, and D, representing hemato-, deutero-, and mesoporphyrin, respectively, is 2.2. The corresponding values for the AFC solvent system are 3.2 and 2.7.[1] The selectivity of the AFCD system is therefore less than that of the AFC system. The separation factor ($\beta = (k_1/k_2) \geq 1$) for the AFCD system is 3.0 between proto- and mesoporphyrins (pH 1.0), 3.6 between meso- and deuteroporphyrins, and 75 between deutero- and hematoporphyrins. The corresponding values for the AFC system were reported as 4.5, 6.8 – 7.4, and 75 – 80.[1]

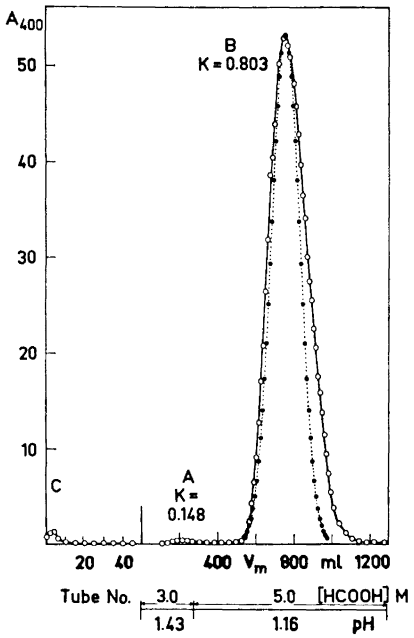

Fig. 10. Purification of deuteroporphyrin IX obtained from protohemin preparation *1*. Solvent system: HCOOH(3.0 and 5.0 M/$CHCl_3$(25)-DMF(1). Twentythree milligrams of the deuteroporphyrin preparation were dissolved in 2.0 ml of DMF and to the resulting solution were added 50 ml of $CHCl_3$ + 2.5 ml of HCOOH + 16.2 ml of H_2O. The mixture was then sampled into tubes $r = 0,...,5$. $N = 50$. $v_m = 2.44$ ml; $v_s = 11.06$ ml. $V_m = 1278$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring A_{400} of the effluent fractions and of the lower phases in the tubes. A = hematoporphyrin IX + unknown porphyrin, B = deuteroporphyrin IX (99 %), and C = unknown porphyrin(s).

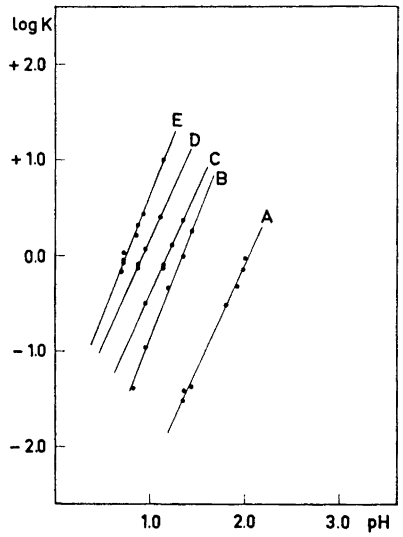

Fig. 11. Log K as a function of upper phase pH. A = hematoporphyrin, B = 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin, C = deuteroporphyrin, D = mesoporphyrin, and E = protoporphyrin.

For small-scale fractionations, the AFC system may be a better choice, owing to its high selectivity. However, a compromise must be achieved between the selectivity and the dissolving capacity of the solvent system when it is to be employed on the preparatory level. Otherwise, aggregation may seriously restrict the method and may lead to considerable confusion and misinterpretation of the results. This was clearly indicated by the present investigation into the behavior of hematoporphyrin. It now appears obvious that aggregation is at least one probable reason for the difficulties encountered

in the purification of this porphyrin.[12,16,17] Evidently, the high percentage of impurities observed to be present in six commercial preparations is due to the strong tendency of hematoporphyrin to from aggregates.

In light of the investigations herein described, the utilization of the multiple liquid-liquid partition technique for purifying protoporphyrin must be revaluated. This method is undoubtedly useful for the purification of crude protoporphyrin preparations. However, since hydration of the vinyl groups occurs readily under the acidic conditions employed, the technique never yields protoporphyrin of a high degree of purity. Protoporphyrin purified in this manner will always be contaminated by small quantities of 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin. Therefore, it would appear of greater advantage to first carefully purify the protohemin, since the vinyl groups of this compound seem to be more resistant towards hydration than those of the protoporphyrin. Highly-purified protohemin yields protoporphyrin having a comparable degree of purity, provided that the method of Morell and co-workers [5] is utilized for the removal of iron. Protoporphyrin preparations contaminated by 2(4)-vinyl-4(2)-hydroxyethyldeuteroporphyrin may be purified in the following manner. The preparation is first dissolved in a sufficient volume of chloroform(25)-dimethylformamide(1) (aggregation must be avoided). The resulting solution is rapidly extracted in a separatory funnel with successive volumes of 5.0 M aqueous formic acid, washed several times with distilled water and finally evaporated to dryness at reduced pressure.

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