to 0.1 M have been employed to compute the activity coefficient, relative partial molal heat content and heat capacity of the acid in this solvent.

3. The accuracy of the experimental results is of the order of ± 0.1 mv., leading to an error of about ± 0.001 in the relative activity coefficient and ± 30 cal. in the relative heat content. A greater source of error in these values may reside in the uncertainty of the extrapolation. A general discussion of these thermodynamics properties will be reserved until the investigation of the 82% dioxane–water mixtures of dielectric constant of approximately 10 is completed.

NEW HAVEN, CONN. RECEIVED JUNE 21, 1938

[CONTRIBUTION FROM THE DIVISION OF BIOCHEMISTRY, MAYO FOUNDATION, AND THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF MINNESOTA MEDICAL SCHOOL]

The Distribution Coefficients of Porphyrins between Ether and Hydrochloric Acid and Applications to Problems of Quantitative Separation

BY ANCEL KEYS AND JOACHIM BRUGSCH[1]

Differential solubility in various solvents is the basic means of separating porphyrins in mixtures.[2-6] The most useful solvents are ether, chloroform and hydrochloric acid.[7,8] These three reagents have been used to effect separation of biological porphyrins by a procedure involving repeated crystallizations.[9,10] No proof of the quantitative accuracy of this method has been given; in any case it suffers from three limitations: (1) it is very time-consuming; (2) it serves only to isolate the principal porphyrin in the mixture; (3) it is stated that at least 50 micrograms of porphyrin is needed, but the work reported has generally involved much more; these amounts are not always available in biological work.

The porphyrins known or believed to occur in the normal or pathological human metabolism are: protoporphyrin ($C_{32}H_{32}N_4(COOH)_2$), coproporphyrin ($C_{32}H_{34}N_4(COOH)_4$), mesoporphyrin ($C_{32}H_{36}N_4(COOH)_2$) deuteroporphyrin ($C_{28}H_{28}N_4(COOH)_2$), and uroporphyrin ($C_{32}H_{30}N_4(COOH)_8$). All but the last named (uro-) are soluble in ether acidified by glacial acetic acid. Except in extremely rare cases, uroporphyrin occurs naturally only in the most minute amounts. Traces of other unidentified porphyrins have been reported in biological materials but these questionable substances may be neglected for the present.

Most of the natural porphyrins are soluble in chloroform, but repeated trials convinced us that chloroform extraction is not suitable for quantitative separation of porphyrins from natural mixtures unless extraordinary precautions are taken. Traces of impurities, especially colloids, may enable some of the porphyrins to go into colloidal solution in chloroform, prevent their extraction or cause loss by adsorption on the walls of the vessels used.

Willstätter and Stoll[11] made a qualitative separation of the ether-soluble porphyrins by extraction of the ethereal solution with hydrochloric acid. Willstätter[12] has characterized as the "HCl number" that concentration of hydrochloric acid which will extract two-thirds of the porphyrin from an equal volume of porphyrin solution. The available information on hydrochloric acid solubility of the porphyrins is in terms of Willstätter's "HCl number."

Since the naturally occurring porphyrins are reported to differ widely from one another in their hydrochloric acid numbers, a quantitative separation on this basis should be feasible, provided: (1) the hydrochloric acid solubility is a fixed characteristic, (2) the hydrochloric acid solubility for a given porphyrin is independent of

(1) Fellow in Medicine in the Mayo Foundation.

(2) H. Fischer, *Z. physiol. Chem.*, **132**, 15 (1924); **137**, 228 (1924).

(3) H. Fischer and R. Duesberg, *Arch. exp. Path. Pharm.*, **166**, 95 (1932).

(4) C. J. Watson, *Z. physiol. Chem.*, **204**, 57 (1932).

(5) A. E. Garrod, *J. Physiol.*, **17**, 349 (1894).

(6) A. E. Garrod, "Inborn Errors of Metabolism," 2d ed., London, p. 136.

(7) H. Fischer and A. Treibs, in "Tabulae Biologicae," Vol. III, 1926, pp. 339, et seq.

(8) A. Kirstahler, *ibid.*, Vol. VII, 1931, p. 49, et seq.

(9) K. Dobriner, *J. Biol. Chem.*, **113**, 1 (1936).

(10) K. Dobriner, W. H. Strain and S. A. Localio, *Proc. Soc. Exptl. Biol. Med.*, **36**, 752 (1937).

(11) R. Willstätter and A. Stoll, "Untersuchungen über Chlorophyll," Berlin, 1913.

(12) R. Willstätter, in Abderhalden, "Handbuch der biologischen Arbeitsmethoden," Abt. I, Teil 3, pp. 1–70.

the presence of other porphyrins, (3) suitable hydrochloric acid concentrations are chosen so as to effect the separation in a reasonable number of extractions and (4) a satisfactory means of estimating the amount of porphyrin in the extracts is at hand.

We can begin by stating that the last-named requisite is fulfilled. All the porphyrins with which we are concerned are fluorescent in ultraviolet light and, over a limited range of concentration, the intensity of the fluorescence under standard conditions is directly related to the concentration. The present paper is a study on the partition between ether and various concentrations of hydrochloric acid of the following pure crystalline porphyrins: copro-, hemato-, meso-, deutero-, protoporphyrin and phylloerythrin ($C_{33}H_{34}N_4O_3$).

Preparation of Pure Porphyrins.—Our pure porphyrins were checked by their absorption spectra in ether and in hydrochloric acid solution.[13] In the following paragraphs the wave lengths of the principal bands are given in mμ. The values in parentheses are taken from Kirstahler.[8]

Preparation of Protoporphyrin.—Fresh blood is run into ten times its volume of 0.25% hydrochloric acid, shaken gently and after an hour the brown hydrochloric acid pigment is taken up in glacial acetic acid–ether. The ether is reduced to a small quantity, preferably in a vacuum. With the addition of 4% hydrazine sulfate in strong acetic acid solution the complete conversion to protoporphyrin is brought about when the solution is boiled down to about a fourth the original volume. The protoporphyrin is separated by filtration, and shaken with acetic acid ether in a separatory funnel. By this procedure a part of the protoporphyrin goes over into the ether along with some impurities. From this solution the protoporphyrin is recovered by extraction with 10% hydrochloric acid. The rest is taken up in hydrochloric acid, concentrated and preferably precipitated as the sodium salt by the addition of sodium hydroxide. Purification is readily brought about by washing, re-solution and reprecipitation. Protoporphyrin prepared in this way has the following characteristic absorption bands:

In 5% Hydrochloric Acid
I, 608–594, *602* (601)
II, 575–545, *560* (557)

In Ether Solution
I, 643–629, *636* (633)
II, 594–574, *584* (585)
III, 549–533, *541* (537)
IV, 514–494, *504* (502)

Preparation of Mesoporphyrin.—Crystalline mesoporphyrin was prepared from protoporphyrin by the method of Fischer and Kögl (1924) in which the vinyl groups are reduced by the action of iodic acid. The mesoporphyrin had the following absorption bands:

In 5% Hydrochoric Acid
I, 598–591, *594* (593)
II, 562–544, *553* (549)

In Ether Solution
I, 626–621, *623* (623)
II, 571–567, *569* (577?)
III, 532–523, *528* (529)
IV, 506–489, *496* (495)

Hematoporphyrin.—We used the crystalline preparation made by the Nordmark Company which is known commercially as "Photodyn." This hematoporphyrin had the following bands in the absorption spectrum:

In 5% Hydrochloric Acid
I, 595–588, *592* (596)
II, 559–540, *550* (552)

In Ether Solution
I, 626–623, *625* (625)
II, 599–594, *597* (598)
III, 571–567, *569* (569)
IV, 536–526, *531* (531)
V, 507–487, *497* (497)

Preparation of Coproporphyrin.—We used crystalline synthetic coproporphyrin I supplied by the kindness of Prof. Hans Fischer, as well as a crystalline preparation from human excreta. The latter preparation involved extraction with acetic acid ether, transfer to 5% hydrochloric acid, transfer to ether and repeated fractionation by means of ether and 0.25% HCl. Our preparation had the following absorption bands:

In 5% Hydrochloric Acid
I, 594–578, (594, 575)
II, 553–543, *548* (551)

In Ether Solution
I, 626–621, *624* (624)
II, 598–594, *596* (597)
III, 571–567, *569* (568)
IV, 532–524, *528* (529)
V, 504–486, *495* (498)

Preparation of Phylloerythrin.—We prepared phylloerythrin from ox gallstones in the course of the preparation of bilirubin by the method of H. Fischer (1911). The combined washings were carried over into ether and after purification were taken up by 10% HCl which was then neutralized and the phylloerythrin was carried back to ether, from which typical crystals were obtained. The phylloerythrin had the following absorption spectra:[14]

In 10% Hydrochloric Acid
I, 628–604, *621* (620)
II, 577–565, *571* (568)

In Ether Solutions
I, 638–635, *637* (637)
II, 597–579, *588* (593, 584)
III, 564–554, *559* (561)
IV, 528–513, *521* (521)

(13) We are indebted to Dr. Charles Sheard for assistance in the determination of the absorption spectra.

(14) Comparison values in parentheses given by H. Fischer, in Oppenheimer, "Handbuch der Biochemie," 1933, 2d ed., Suppl. Vol. I (1), pp. 247, et seq.

Preparation of Deuteroporphyrin.—Deuteroporphyrin was prepared from deuterohemin by the action of hydrazine sulfate. We were unable to obtain sufficient material for recrystallization, but our preparation had the typical absorption spectra of deuteroporphyrin. Through the kindness of Dr. C. J. Watson we were able to check several of the hydrochloric acid solubility points with a solution of pure synthetic deuteroporphyrin from the laboratory of Prof. H. Fischer. However, since our preparation was not recrystallized, we prefer to term it simply "deutero type" porphyrin.

Measurement of Porphyrin Concentration in Hydrochloric Acid Solution

All our measurements of porphyrin concentration have been made in terms of the fluorescence in ultraviolet light. The intensity of fluorescence of a porphyrin solution in hydrochloric acid is stated to be linearly proportional to the porphyrin concentration in extreme dilution but not otherwise.[15–17] We have found this relation to be quantitative over the range 0.05 to 2 micrograms per ml. The fluorescence, however, is markedly affected by *p*H and, to a smaller extent, by temperature.[18] With the exception of phylloerythrin, we have measured the fluorescence of all our porphyrins in 5% HCl. With phylloerythrin we found it necessary to use 10% HCl. We have observed no effect of ordinary variations in room temperature, even in summer when some measurements were made at 30°.

The fluorescence spectra of the porphyrins studied here are not identical,[19] so the relative intensity of the fluorescence of the different porphyrins at equal concentration would be expected to differ slightly according to the system used for measurement. All of our measurements have been made by visual comparison, in some cases with the Zeiss "stufenphotometer."

Fluorescence is excited by the light from a quartz mercury vapor lamp. The ultraviolet light is passed through filters to cut out all radiation of a wave length longer than about 3800 Å. The "uviol" or Wratten No. 18A (ultraviolet) filters are satisfactory, especially when used as the windows of a copper sulfate cell. Wratten Nos. 15G and 18A make an excellent combination but a very powerful ultraviolet lamp must be used. The fluorescence of the porphyrin solutions may be studied in any type of quartz absorption vessels or ordinary glass vessels, provided these latter have relatively thin walls and possess no fluorescence of their own. Lacking more specialized apparatus, satisfactory comparisons can be made with matched test-tubes placed in the path of filtered ultraviolet light from an ordinary clinical ultraviolet lamp.

Standard coproporphyrin solution in 5% HCl was the standard of reference and all results here are expressed in terms of fluorescence equivalent to coproporphyrin. In general, the most useful concentrations for standard solutions are 0.2, 0.1 and 0.05γ of porphyrin per ml.

(15) R. Fikentscher, *Biochem. Z.*, **249**, 257 (1932).
(16) A. A. H. van den Bergh, W. Grotepass and R. E. Revers, *Klin. Wochsch.*, **11**, 1534 (1932).
(17) J. T. Brugsch, *Z. ges. exp. Med.*, **95**, 471 (1935).
(18) H. Fink and W. Hoerburger, *Z. physiol. Chem.*, **202**, 8 (1931).
(19) C. Dhéré, in Abderhalden, "Handbuch der biologischen Arbeitsmethoden," Abt. II, Teil 3, Heft 4, Lieferung 400, pp. 3097, et seq.

In the preparation of ethereal porphyrin solutions of known concentration dissolve from 2 to 20 micrograms of the pure crystalline material in about 30 ml. of 5% HCl. The exact concentration of porphyrin in the hydrochloric acid is determined, in terms of intensity of red fluorescence, by comparison with a stock standard solution of coproporphyrin. As soon as this measurement is completed, the acid solution is neutralized with solid sodium acetate and extracted with successive 25-ml. portions of ether until the aqueous phase no longer shows red fluorescence in ultraviolet light. It may be necessary to add a few drops of acetic acid in this process to keep the mixture slightly on the acid side of neutrality. The combined ethereal solution contains all the porphyrin originally dissolved in the 5% HCl.

For the purposes of quantitative analysis it is necessary that the partition between ether and hydrochloric acid be measured under conditions which are reproducible and easily applied. We add the hydrochloric acid to the ethereal solution in a separatory funnel and slowly shake back and forth by hand. Violent or prolonged agitation is unnecessary and may result in loss or oxidation of some of the porphyrin. The partition does not seem to be affected by the ordinary variations in room temperature in the range 20 to 26°. After the extraction, the hydrochloric acid extract was drawn off, the concentration brought up to 5% HCl and the fluorescence intensity measured at once. Finally, the ether was extracted with 5% HCl until all porphyrin had been removed from the ether. The fluorescence intensity was measured in these final extractions so that the total recovery of porphyrin from the ether was measured.

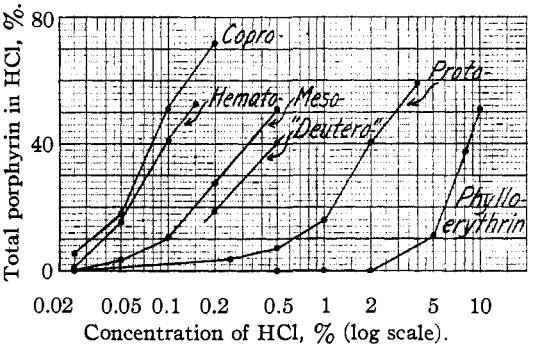

Fig. 1.—Extraction of porphyrins from ethereal solution by various concentrations of hydrochloric acid.

Figure 1 shows the results obtained when the volume relations were 1 part hydrochloric acid solution to 3 parts ethereal solution. Distribution coefficients for the several porphyrins over the useful range of hydrochloric acid concentration are given in Figs. 2 and 3. Most of these values were obtained using the volume relation 1 part hydrochloric acid to 3 parts ether. In a number of cases, however, the constancy of the distribution coefficient was checked by determinations made with the volume relation 1 part hydrochloric acid to 1 part ether.

Included in Fig. 2 are two points from an unknown porphyrin which occurs in very minute amounts in human feces. This porphyrin was soluble in chloroform and

was purified so far as possible with repeated fractionation by means of 0.25% HCl, ether, 5% HCl and chloroform. As can be seen from the distribution coefficient this porphyrin appears to be a separate entity. This appearance is fortified by the fact that the distribution coefficient did not change during six successive extractions.

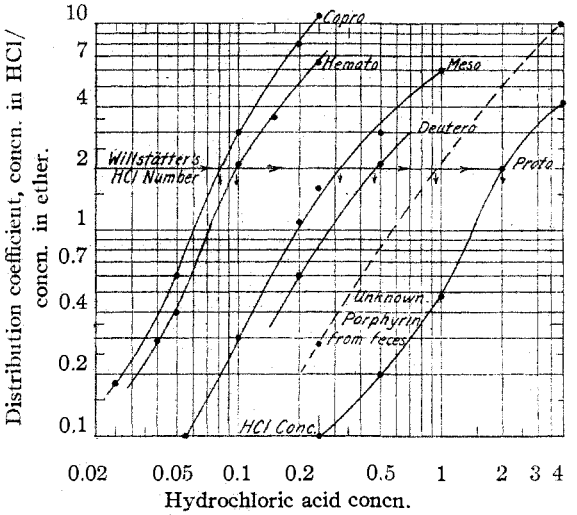

Fig. 2.—Distribution coefficients for the partition of porphyrins between ether and various concentrations of hydrochloric acid.

The distribution coefficient is simply the ratio of the concentration of the solute in the acid phase to that in the ether phase. If we let a = the percentage of the solute in the acid, and v the volume of the acid as percentage of the total volume, the distribution coefficient is

$$K = \frac{a/v}{(100 - a)/(100 - v)} \text{ and}$$

$$a = \frac{100\,Kv}{100 + Kv - v} \qquad (I)$$

Willstätter's hydrochloric acid number corresponds to a distribution coefficient of 2.0. In Table I we have given the previous values for the HCl number and the values calculated from the present results.

Quantitative analysis using distribution coefficients necessarily must involve successive extractions. It is requisite then that the experimental results with successive extractions conform to theory. General principles applicable to successive extractions were formulated by Herz[21] (1909). Let b_1 = the amount, in percentage terms, unextracted after the first extraction; *i. e.*, $b_1 = 100 - a$. Then

$$b_1 = 100\left(\frac{100 - v}{100 - v + Kv}\right)$$

(20) H. Fischer and A. Treibs, in Oppenheimer, "Handbuch der Biochemie," 2d ed., Suppl. vol. 30, 1930, p. 72.

(21) Herz, "Der Verteilungssatz," Stuttgart, 1909, p. 5.

TABLE I

HYDROCHLORIC ACID NUMBERS OF PORPHYRINS

Concentration of HCl which will extract two-thirds of the porphyrin from ether solution when equal volumes of HCl and ether are used in a single extraction. "Previous Values" taken from A. Treibs,[22] Fischer,[14] Fischer and Treibs[20] and A. Kirstahler.[8]

Porphyrin	Previous values	Present values	Uncertainty
Proto-	2	2.0	±0.1
Deutero-	0.3–0.4	0.47	± .05
Meso-	0.5	.32	± .05
Hemato-	0.1	.098	± .003
Copro-	0.08	.081	± .003
Phylloerythrin	about 9	8.5	± .5
Unknown porphyrin[a] from human feces	...	0.94	...

[a] This porphyrin is recovered in very small amounts from human feces by extraction in the following manner: extract with acetic ether, transfer to 5% HCl, transfer back to ether, extract with 0.25% HCl, neutralize, extract with chloroform, transfer to 5% HCl, carry back to ether, extract with HCl.

After n extractions the amount remaining in the ether (unextracted by the acid) will be

$$b_n = 100\left(\frac{100 - v}{100 - v + Kv}\right)^n \qquad (II)$$

Figure 4 shows the correspondence between experiment and theory for three successive extractions, using 3 volumes of ether and 1 volume of acid for each extraction. For this case equation II becomes

$$b_3 = 100\left(\frac{3}{3 + K}\right)^3 \qquad (IIA)$$

In Fig. 4 the lines have been calculated from equation IIA and Figs. 2 and 3; the points are experimental observations.

Figure 4 shows that the agreement is good except at the highest hydrochloric acid concentrations where the extraction tends to be more complete than predicted. This is probably due to the fact that a small amount of hydrochloric acid is carried over in the ether to the succeeding extraction so that the hydrochloric acid is somewhat increased. This error is greatest with phylloerythrin and least with coproporphyrin.

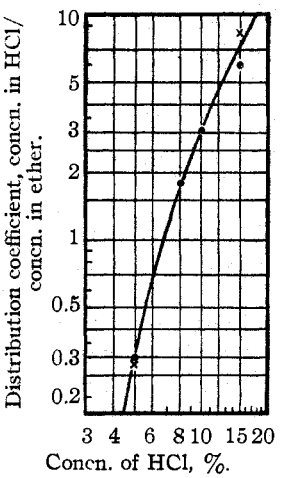

Fig. 3.—Distribution coefficient for the partition of phylloerythrin between ether and hydrochloric acid.

(22) A. Treibs, in Oppenheimer, "Handbuch der Biochemie," 2d ed., Ergänzungsband, 1930, pp. 104–116.

Accordingly it appears that successive extractions with the lowest effective hydrochloric acid concentration should be satisfactory.

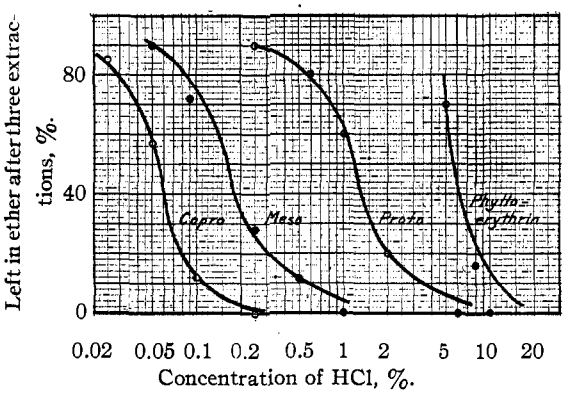

Fig. 4.—Constancy of the distribution coefficient in successive extractions. Lines calculated from distribution coefficients obtained in single extractions, points are experimental results with three successive extractions, using one volume hydrochloric acid to three volumes ether in each.

Finally, it is necessary to know whether the extraction of one porphyrin proceeds in quantitative independence of the presence of a second porphyrin. This question was studied with synthetic mixtures of porphyrins of known composition in terms of fluorescence equivalent to coproporphyrin. The results with two mixtures—copro- and protoporphyrin, and copro- and mesoporphyrin—are given in Fig. 5. In Fig. 5 the circles represent the experimental observations, the heavy lines were calculated from the distribution coefficients. The general agreement is good but there is a tendency to extract less than expected in some of the extractions. In all cases, however, the error is less than 4% of the total porphyrin.

The apparent small discrepancy between theory and observation with mixed porphyrins possibly may mean that the fluorescence intensities per unit concentration for the several porphyrins are not identical. It is generally assumed that the fluorescence intensities of the porphyrins studied here are equivalent per molecule. Our own studies indicate that this is approximately true when measurements are made in 5% HCl, but, owing to the excessively minute amounts of the materials available, an uncertainty of the order of 10% must be admitted.

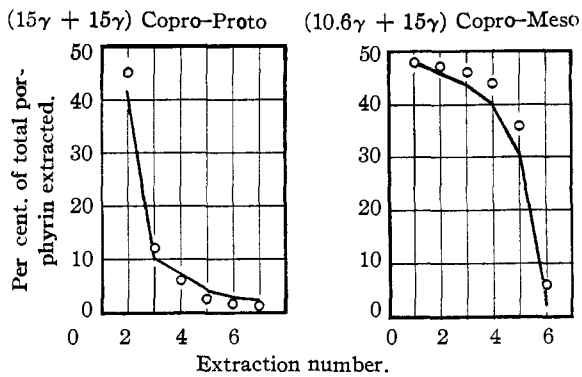

Fig. 5.—Successive extractions of mixtures of porphyrins. Extraction of ethereal solution by 0.25% HCl, one volume of hydrochloric acid to three volumes ether in each extraction. Points experimentally determined, lines calculated from distribution coefficients (Fig. 2).

Summary

The following pure porphyrins were prepared and studied: copro-, hemato-, meso-, deutero- and protoporphyrin, and phylloerythrin.

Distribution coefficients for the porphyrins between ether and hydrochloric acid were determined by fluorescence measurements, over the range 0.025 to 10% HCl.

For each of the porphyrins the distribution coefficient at a given hydrochloric acid concentration is constant over a range of porphyrin concentration from 0.05 to 2000 micrograms per ml.

The results of successive extractions of ethereal solution by hydrochloric acid are quantitatively predictable from the distribution coefficients, but the error becomes significant when over 85% of the total porphyrin has been extracted.

The results of extractions of mixed porphyrins conform approximately to expectations from the distribution coefficients, but the error in any single extraction may be as great as 5% of the total porphyrin.

The use of successive extractions with appropriate concentrations of hydrochloric acid provides a satisfactory basis for at least semiquantitative separation of the ether-soluble porphyrins when as much as 0.01 mg. of total porphyrin is available.

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