Anal. Calcd. for $C_{14}H_{14}N_2S$: N, 11.57. Found: N, 11.01.

Ring Cleavage by Piperidine.—A reaction mixture of 1 g. of III (R = R′ = H) and 25 ml. of piperidine was refluxed for 24 hours. It was cooled, poured into dilute hydrochloric acid and was then extracted with ether. The ethereal solution gave upon evaporation colorless crystals, which upon crystallization from ethyl alcohol melted at 171°; identified as phenylpiperidylurea (m.p. and mixed m.p.[17]).

Piperidinium Adducts.—A mixture of 1 g. of the appropriate 5-arylidene-3-aryl-2,4-thiazolidinedione and 1 ml. of piperidine in 30 ml. of dry benzene was kept aside for 48 hours, during which time the solid substance dissolved gradually. The benzene was evaporated slowly, and the residue was triturated with cold ethyl alcohol. The solid, so obtained, was crystallized from benzene or from hot ethyl alcohol.

The piperidinium adducts VIII (see Table III) give colorless crystals, which decompose above their melting points to give the corresponding arylidene derivative.

GIZA, CAIRO, EGYPT (U.A.R.)

The Thermodynamic Stability of Porphyrinogens

BY D. MAUZERALL

Uroporphyrinogen rapidly isomerizes in hot acid solution to a random mixture of isomers, yet the reduced porphyrin can be recovered in high yield. An explanation for the thermodynamic stability of this macrocycle based on the chelate effect is offered.

The porphyrinogens are hexahydro porphyrins in which the four pyrrole residues are linked through methylene bridges. These macrocycles were first studied by Fischer and co-workers[1] and have recently been shown to be the intermediates on the biosynthetic pathways of both heme and chlorophyll.[2] It has now been found that porphyrinogens isomerize readily in hot acid. Fischer and Rotthaus had previously noted that the reduction of mesoporphyrin with zinc dust in hot acetic acid, followed by autoxidation, produced porphyrins having one to three carboxyl groups per molecule.[3] Reduction in the cold or in alkaline solution did not form such mixtures. This isomerization offered a unique opportunity to study the thermodynamic stability of a macrocyclic compound. Uroporphyrinogen was chosen for this study because of its solubility properties, and because of its ready formation from porphobilinogen, its biosynthetic precursor. The isomers of uroporphyrinogen (or of the porphyrin) are composed of the four possible arrangements of the four similar pyrrole residues having two different substituents in the β,β'-positions (Fig. 1).

The isomerization of uroporphyrinogen in hot acid will reach equilibrium only if several conditions are met. First, the mixture of isomers should be a completely random one, since inspection of models of the four isomeric porphyrinogens shows no detectable hindrance among the side chains. Second, the same mixture must be obtained from any isomer. And third, this mixture should be independent of time. These conditions are met, and an explanation of the observed stability of this macrocycle is discussed in the first section. Some aspects of the mechanism of this isomerization are discussed in the second section.

Experimental

Materials.—Uroporphyrin III was obtained from the copper chelate found in turacao features,[4] uroporphyrin I

and coproporphyrin I from the urine of a porphyric bull,[5] and coproporphyrin III from a fraction of broth used in the preparation of diphtheria toxin. In isolating uroporphyrin III *via* the porphyrinogen, acid was avoided to eliminate any possibility of isomerization. The porphyrinogen was titrated with iodine at pH 7, thus avoiding the green by-product formed during autoxidation in alkaline solution.[2,4] Coproporphyrin II was obtained by quantitative decarboxylation of uroporphyrin II.[6] Opsopyrroledicarboxylic acid, uroporphyrin II and a mixture of uroporphyrin isomers were synthetic products and the generous gifts of Dr. S. F. MacDonald. The porphyrins were purified by repeated chromatography on alumina and crystallization of the methyl esters. Porphyrinogens were prepared by reducing the porphyrins with sodium amalgam, as described.[2] Formaldehyde-C^{14} was obtained from Nuclear Chicago, activity $\sim$ 1 curie/mole. Other chemicals were of reagent grade.

Methods.—Spectra were measured with a Beckman model DU spectrophotometer equipped with a photomultiplier or with a Cary model 11. Melting points are corrected and were taken on a heated microscope stage, using polarized light to observe birefringence. Formaldehyde was assayed by the chromotropic acid method[7] and stock solutions standardized by the dimedon method.[8] The free porphyrins were measured spectrophotometrically in 1 M hydrochloric acid, and the esters in freshly distilled chloroform. The extinction coefficients are given in Table I.

TABLE I

MOLAR EXTINCTION COEFFICIENTS OF UROPORPHYRIN

Octamethyl ester in chloroform

λ_{max}, mμ	406	502	536	572	627
$\epsilon \times 10^{3a}$	215	15.8	9.35	6.85	4.18
λ_{min}, mμ	458	522	554	598[b]	608
$\epsilon \times 10^3$	1.9	3.3	1.4	1.4	0.85

Uroporphyrin in 1 M HCl

λ_{max}, mμ	406	552	593
$\epsilon \times 10^3$	505	17.5	6.15
λ_{min}, mμ	520[b]	570[b]	584
$\epsilon \times 10^3$	2.9	6.1	3.9

^{a} $\epsilon = (l/\text{cm.} \times \text{mole per liter}) \log I_0/I$; slit widths $\sim$ 0.01/mm. ^{b} Shoulder.

(1) H. Fischer and A. Stern, "Die Chemie des Pyrrols," Leipzig, 1940, Vol. II, pt. 2, p. 420 ff.

(2) D. Mauzerall and S. Granick, *J. Biol. Chem.*, **232**, 1141 (1958).

(3) H. Fischer and A. Rotthaus, *Ann. Chem.*, **484**, 85 (1930).

(4) R. E. H. Nicholas and C. Rimington, *Biochem. J.*, **50**, 194 (1951).

(5) T. K. With, *ibid.*, **68**, 715 (1958).

(6) P. R. Edmondson and S. Schwartz, *J. Biol. Chem.*, **205**, 605 (1953).

(7) E. Bremanis, *Z. Anal. Chem.*, **130**, 44 (1949).

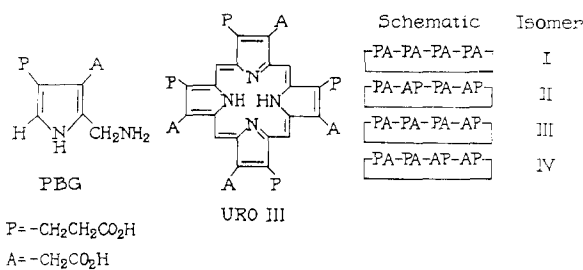(8) D. Spencer and T. Henshall, *Anal. Chim. Acta*, **11**, 428 (1954).

Fig. 1.

Radioactivity was measured at infinite thinness with a gas flow Geiger counter equipped with a very thin window. The efficiency, using polystyrene-C^{14}, was about 40%. The porphyrin esters thin-plated very well, but the formaldehyde–dimedon derivative tended to crystallize. Chloroform was a good plating solvent and plates were prepared at several concentrations (2–50 μg./cm.^{2}) to check infinite thinness.

Preparation and Analysis of Reaction Mixture.—One to five mg. of porphyrinogen was used in these experiments. Additions of the reagents to seal-off tubes were frozen as added, and the contents deaerated by carrying them through two cycles of freeze–thawing under vacuum (<0.02 mm.), with intermediate flushing with high purity nitrogen. The tubes were sealed while under vacuum, and heated in the dark for the required time. Except where otherwise specified, three conditions were used: (1) acid: 1.0 M hydrochloric acid, 0.5 hour at 98°; (2) neutral: 0.25 M sodium phosphate pH 7.6, 21 hours at 60°; (3) alkaline: 0.10 M sodium hydroxide, 2 hours at 98°. The pH's mentioned in this paper refer to the solution at 25°. Ovens or baths were used with the temperature constant to ±0.5°. The reactions were stopped by freezing. An aliquot removed from the tube immediately after opening and thawing was rapidly diluted with 1 M hydrochloric acid. The absorbancy at 406 mμ was followed for 1–3 minutes, then the visible spectra were measured as a function of time. This allowed extrapolation of absorbancies back to the time of sampling, and the determination of the amount of porphyrin, porphomethenes and other pigments present in the sealed tube. The solution could then be photoöxidized and the yield of porphyrin determined by suitable dilution and reading of the Soret band. The photoöxidation of uroporphyrinogen in acid is almost quantitative.[2] The remainder of the solution (kept frozen) was rapidly brought to neutrality if needed and the theoretical amount of iodine added. The porphyrin bands appeared immediately. After suitable dilution the yields of porphyrin and other pigments were determined and this verified the yields obtained by photo-oxidation. These yields agreed within 5% of one another. The yields have not been corrected for the observed instability of uroporphyrinogen under these conditions. Occasionally the product was spectrophotometrically titrated with iodine: the method is accurate to 2% with uroporphyrinogen.[2] The uroporphyrin was purified by absorbing on talc at pH 3.5, washing with dilute buffer, eluting with ammonia, drying, esterifying with methanol–sulfuric acid and chromatographing the ester on alumina, using chloroform–ligroin (b.p. 65–75°) as eluent. Although the yields given are based on spectra it must be noted that all of the above operations plus a crystallization of the ester can be carried out with over 90% recovery on milligram amounts of the porphyrin. Since the isomers differ in some properties, e.g., I and II are less soluble than III and IV, possible fractionation was avoided by using quantitative techniques and determining yields at each step. Chemical purity was determined by the ratios of the intensities of the four visible bands of the uroporphyrin ester in chloroform, and by paper chromatography. The uroporphyrin methyl esters which analyzed as $\sim$ $^3/_4$ isomer III–IV by chromatography, melted over the range 260–270°.

Formaldehyde was determined in the aqueous supernatant and washings, following absorption of the uroporphyrin on the talc, and the dimedon derivative (isolated by adding carrier formaldehyde if necessary) was crystallized to constant activity. The melting points were identical with that of a known sample.

The isomer content of the product was determined by the uroporphyrin ester method of Falk and Benson.[9,10] This is a relative method, and depends on the standards used. It differentiates only isomers I + II from III + IV, and is not reliable for ratios of $^1/_8$ or less. For quantitative estimation, therefore, a simplification of the method distinguishing coproporphyrin isomers was used.[11] The free uroporphyrin was decarboxylated to coproporphyrin in >95% yield by heating for 4 hours at 180° in 1 M hydrochloric acid, which had been thoroughly deaerated by freeze–thawing and sealing under vacuum. One mμmole of the coproporphyrin was chromatographed by the descending method at 24 ± 1°, in the dark. The solvent was 10 parts (v./v.) 2,6-lutidine and 7 parts 0.7 M aqueous ammonia. The isomers separate into three groups with R_f II > III + IV > I. Standard isomer mixtures containing ratios slightly greater and slightly less than the unknown were always placed alongside the unknown and the determinations were repeated an average of five times. The limit of detection is about $^1/_{32}$ for each of the groups, and the accuracy of the determination is about ±$^1/_{16}$ for small amounts of isomers I and II and about ±$^1/_8$ for more equal ratios of isomers I and III.

Since the isomer analysis method was critical to this work, the technique was verified by condensing opsopyrroledicarboxylic acid with various concentrations of formaldehyde in acid, neutral and alkaline solutions. All of the uroporphyrins produced (13 expts., 4 determinations each) when analyzed by the uroporphyrin ester method, averaged $^1/_8$ ± $^1/_8$ isomers I + II. With mixtures of 1 mole of formaldehyde per mole of pyrrole, the isomer mixture, analyzed by the coproporphyrin method (3 expts., 5 determinations each) following oxidation and decarboxylation, was the random mixture within experimental error. This result therefore serves as a quantitative test of the method.

As a further check, coproporphyrinogen I was also isomerized under the same conditions used for uroporphyrinogen and the coproporphyrin formed on reoxidation was found to be a random mixture of isomers (Table II, expt. 9).

Results and Conclusions

1. **Equilibrium.**—The random mixture of the four isomers of uroporphyrin contains $^1/_2$ III, $^1/_4$ IV, $^1/_8$ I and $^1/_8$ II.[12] Analysis showed that the mixture obtained after heating uroporphyrinogen I in acid was composed of $^3/_4$ III + IV, $^1/_8$ I and $^1/_8$ II, the experimental error being ± 1/16 (Table II, expt. 5). The same mixture was obtained on heating uroporphyrinogen III (expt. 6). The small amounts of uroporphyrin II and IV available were conserved for use as standards in the analysis. A time study showed that theo ismerization was complete in about 10 minutes and that the ratio of the isomers in the mixture remained constant for about 20 hours in 1.0 M hydrochloric acid at 98° in the absence of air. However, the uroporphyrinogen is not chemically stable under these conditions and decomposes with a half-life of about 8 hours. One of the products of this decomposition is uroporphyrin, which is completely unaffected by the hot acid. A final yield of approximately 25% of the randomized, oxidized porphyrins was thus obtained on long heating. This relatively slow decomposition would not seriously disturb the equilibria. The random equilibrium among the isomers indicates that the driving force for this isomerization must be the entropy of mixing.

After 0.5 hour in acid at 98°, the recovery of porphyrin was 85–90%, and this yield was unaffected by dilution from 10^{-3} to 6×10^{-5} M. Spectral measurements showed this product to be

(9) J. E. Falk and A. Benson, *Biochem. J.*, **55**, 101 (1953).

(10) T. C. Chu and E. J. H. Chu, *J. Biol. Chem.*, **227**, 505 (1957).

(11) J. E. Falk, E. I. B. Dresel, A. Benson and B. C. Knight, *Biochem. J.*, **63**, 87 (1956).

(12) G. H. Cookson and C. Rimington, *ibid.*, **57**, 476 (1954).

TABLE II

UROPORPHYRINOGEN ISOMERIZATION AND INCORPORATION OF HC^{14}HO

Expt.	Compound	Condition[a]	% URO preoxid.[b]	% URO yield	Isomer[c] I	Isomer[c] II	Ratio molar activities, URO: formaldehyde
1	URO'gen I + formaldehyde[d]	Acid	51	25	1/8	1/8	1.00 ± 0.05
2	URO'gen I + formaldehyde[d]	Neutral	1.3	83	1[e]	..	0.0093 ± 0.001
3	URO'gen I + formaldehyde[d]	Alkaline	2.5	61	1[e]	..	.0021 ± .0004
4	URO I + formaldehyde[d]	Acid	..	100	1	0	.0004 ± .0004
5	URO'gen I	Acid	1.0	87	1/8	1/8	....
6	URO'gen III[f]	Acid	0.2	81	1/8	1/8	....
7	URO'gen I + dimedon[g]	Acid	7.1	34	1/8[e]	..	....
8	URO'gen I + dimedon[g]	Neutral	1.3	84	1[e]	..	....
9	COPRO'gen I[h]	Acid	24	29	1/8	1/8	....

[a] See Experimental section for details; the concentration of uroporphyrin (URO) or uroporphyrinogen (URO'gen) was 10^{-3} M. [b] Calculated on porphyrin recovered. [c] Determined following decarboxylation to coproporphyrin. The remainder is isomers III + IV; error ± 1/16. [d] The molar ratio of HC^{14}HO to URO'gen or URO was 1.0. The molar activity of the HC^{14}HO was 87 millicuries per mole. [e] Sum of isomers I + II; analyzed as URO esters only; error ± 1/8. [f] This sample contained ≤ 1/16 isomer I and no isomer II; heating time 1 hour. [g] The molar ratio of dimedon to URO'gen was 44. [h] The concentration was 3.5×10^{-4} M.

more than 95% cyclized uroporphyrinogen, with 1% or less in the form of fully oxidized porphyrin. The remaining few per cent. were in the form of porphomethenes,[2] the di- and tetrahydro reduction levels between porphyrins and porphyrinogens. Similar recoveries were obtained when the cooled mixtures were opened to the air and photo-oxidized in acid at 25° for about 0.5 hour or when they were neutralized and instantaneously oxidized with iodine within 1 minute after opening. It is therefore unlikely that these operations interfered with the isomer or yield of uroporphyrin obtained. A sample isomerized in acid was also titrated with iodine at pH 7, yielding a reduction equivalent of 6.5 hydrogens, as compared to 6.1 for freshly prepared uroporphyrinogen.

Further evidence for this equilibrium both among the isomers and between the cyclized linear polypyrrylmethanes was obtained by condensing porphobilinogen (Fig. 1) in 1 M hydrochloric acid at 98° for 0.5 hour, anaerobically. The yield of uroporphyrin was 78% and the isomer content was identical to that obtained on isomerizing uroporphyrinogen. Over 95% of the product was in the totally reduced form: 0.1% was in the porphyrin form, and less than 5% in the porphomethene form. Titration of the crude product with iodine showed it to contain 6.3 hydrogens.

The equilibria among the isomers, together with the high yield of porphyrinogen, implies that this macrocycle is thermodynamically preferred to the wide variety of possible linear polypyrrylmethanes. The choice will be between cyclization and higher linear polypyrrylmethanes since this type of condensation polymerization involving the elimination of water or ammonia goes forward under a wide variety of conditions. It is likely that the driving force is the increase in bond energies, which is about 12 kcal. per mole of polymer bond formed. The lack of dilution effect on the yield over the twentyfold concentration range suggests that most of the apparent loss of uroporphyrinogen is due to the decomposition mentioned above. The lack of material prevented the study of the isomerization of uroporphyrinogen at concentrations greater than 10^{-3} M. Higher concentrations were approached indirectly by the condensation of opsopyrroledicarboxylic acid and formal-

dehyde in acidic solution (see Expt. section on isomer analysis). The condensation of these compounds between 5×10^{-4} to 5×10^{-1} M. gave uroporphyrinogen in yields monotonically decreasing from 80 to 50%. Even over this 1000 fold range of dilution, the effect is still small.

Macrocycles with structures similar to the porphyrinogens have been prepared in high yields at much higher concentrations ($\sim$1 M) than those used in this work ($10^{-3}M$). However, since the product often crystallizes out of solution in these preparative experiments, equilibrium may not be attained. These macrocycles include acetone-pyrrole (cyclo-tetra-α-isopropylidenepyrrole, formed in 88% yield),[13] acetone-furan (cyclotetra-α-isopropylidenefuran, 63% yield)[14] and cyclic tetranucleic p-substituted phenol-formaldehyde polymers.[15,16] Ott and Zinke have explained the stability of the latter compounds as due to internal hydrogen bonds.[15] This, however, could not apply to the cyclic tetrafuran obtained in 63% yield. Also, the question of hydrogen bonding between pyrrole molecules is still under discussion.[17] Infrared studies show that a 1 M solution of pyrrole in carbon tetrachloride is only half associated. This association in aqueous solution would be reduced by competition with water molecules. We therefore prefer an argument similar to that proposed to explain the extra stability of metal chelates over their non-cyclic analogs.[18] In linear condensation polymerization, the number of free molecules remains the same, whereas in the cyclization step one free molecule is gained and this is favored by the increase in translational entropy. A similar argument can be made for addition polymerization. The calculated value depends on the choice of standard states. The actual value must be lower than that calculated for the gain of a completely free molecule because of restricted motions in the cyclic form, and a good

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(14) R. G. Ackman, W. H. Brown and G. F. Wright, J. Org. Chem., 20, 1147 (1955).

(15) R. Ott and A. Zinke, Osterr. Chem. Ztg., 55, 156 (1954).

(16) R. F. Hunter and C. Turner, Chemistry & Industry, 72 (1957).

(17) For references, see L. J. Bellamy, "The Infra Red Spectra of Complex Molecules," 2nd ed., J. Wiley and Sons, Inc., New York, N. Y., 1958, p. 253.

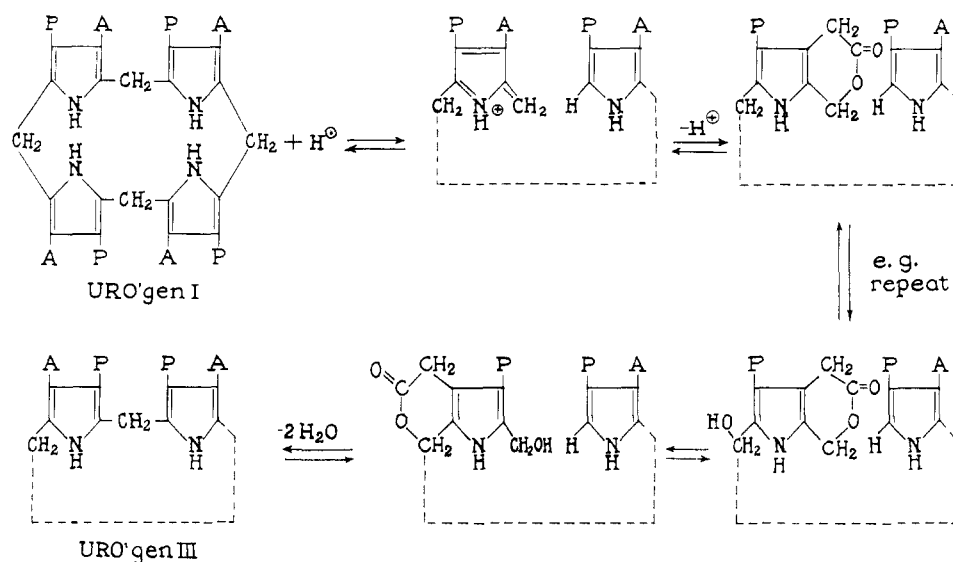(18) M. Calvin and R. Bailes, THIS JOURNAL, 68, 949 (1946).

Isomerization of URO'gen in acid

Fig. 2.

model of these effects has been described and applied to metal chelates by Westheimer and Ingraham.[19] Solvation effects will also contribute to the value of the entropy, and since they may be fairly large and of either sign, little more can be said quantitatively.

This equilibrium argument implies that the smallest possible cyclic polymer will be formed. Models show that the cycle composed of three pyrrole nuclei is highly strained, whereas that with four nuclei is quite free of strain. The possibility that some cycles with more than four nuclei may be present in small amounts merits investigation. Oxidation should give heterocycles stabilized by aromatic resonance by analogy to porphyrins, since planar models of these can be constructed when methine hydrogens are allowed to point inward into the ring. The disfavoring of the cyclization due to restricted motions will be correspondingly smaller if the motions of the linear molecule are previously restricted due to bulky groups. This may well occur with the pyrrylmethanes considered here and moreover helps to explain the well known ring stabilizing feature of alkyl or aryl groups.[20]

No detectable isomerization occurred when uroporphyrinogen was heated under neutral, anaerobic conditions for 42 hours at 98°. The half-life of the uroporphyrinogen under these conditions is 15–20 hours. No conclusion can therefore be drawn concerning the thermodynamic stability of uroporphyrinogen in neutral solution.

2. **Mechanism.**—On isomerizing the porphyrinogen in the presence of formaldehyde, considerable incorporation was found to occur (expt. 1). The formaldehyde caused a decrease in yield of product, and a greatly increased percentage of the product was in the oxidized, i.e., porphyrin form. Simultaneously a large increase in pyr-

rylmethene-like by-products absorbing at ∼480 mµ occurred. Had the formaldehyde completely exchanged with the methylene groups of the uroporphyrinogen, the ratio of the molar activity of the product uroporphyrin to that of the added formaldehyde would have been 0.81. The agreement with the observed value of 1.0 is only fair, due to the extensive decomposition of the uroporphyrinogen. Very little incorporation of formaldehyde occurs in neutral solution (expt. 2), and none in alkaline solution (expt. 3). Uroporphyrin neither isomerizes nor incorporates formaldehyde in hot acid (expt. 4).

The addition of dimedon to uroporphyrinogen I in hot acid caused the yield of recovered porphyrin to decrease markedly (expt. 7). Since the uroporphyrin was still largely isomer III (IV) either the rate of attack of the dimedon was slower than the isomerization of the uroporphyrinogen, or equilibria was reached with formaldehyde distributed between the porphyrinogen and the dimedon derivative. The data obtained on varying the concentration of dimedon could be made to fit either of these alternatives. In neutral solution dimedon does not affect the yield of uroporphyrin (expt. 8), although it reacts far more rapidly with formaldehyde under these conditions.[8]

The following mechanism (Fig. 2) is consistent with these observations. It is similar to that postulated by Treibs and Fritz to explain the "exchange" reactions of dipyrrylmethanes.[21]

Following the initial splitting of the ring, many pathways are possible, only one of which is shown here. The lactone, favored only on the side of the pyrrole carrying the acetic acid side chain, would stabilize the onium ion or formaldehyde residue. The hydroxymethyl group on the P side would be more susceptible to elimination as formaldehyde, which could condense with an α-hydrogen of a pyrrole residue or could lead to by-products by oxidizing some of the methane links to methenes

(19) F. H. Westheimer and L. L. Ingraham, J. Phys. Chem., 60, 1668 (1956).

(20) F. S. Dainton and K. J. Ivin, Quart. Revs., 12, 82 (1958).

(21) A. Treibs and G. Fritz, Ann. Chem., 611, 162 (1958).

which are far less reactive to electrophilic attack. This oxidation could occur either by hydrogen transfer or by condensation to give a dipyrrylethylene with subsequent tautomerization to a meso-substituted dipyrrylmethene of the type studied by Treibs, et al.[22] The observation (expt. 9) that the yield of recovered coproporphyrin is but one-third (30%) that of uroporphyrin when coproporphyrinogen is isomerized under identical conditions, coupled with the far larger preoxidation, can be interpreted as a measure of the importance of this lactone. The formation of this lactone is not essential to the rearrangement, since the isomerization proceeds as readily with coproporphyrinogen. Its presence reduces the concentration of free formaldehyde at equilibrium and so decreases the observed side reactions.

The isomerization of porphyrinogens weakens the arguments based on the formation of isomer "III" of coproporphryin[23] and of uroporphyrin[24]

(22) A. Triebs, E. Hermann, E. Meissner and A Kuhn, Ann. Chem., 602, 170 (1957).

when appropriately substituted α-acetoxymethyl or α-aminomethyl pyrroles are condensed in hot acid solution.

The incorporation of formaldehyde into a porphyrinogen may be used to prepare uroporphyrin or coproporphyrin highly and specifically labeled in the methine groups.

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(24) A. Treibs and W. Ott, Ann. Chem., 615, 137 (1958).

The Condensation of Porphobilinogen to Uroporphyrinogen[1]

BY D. MAUZERALL

The condensation of porphobilinogen gives high yields of uroporphyrinogen. The isomer and yield of uroporphyrinogen and the incorporation of formaldehyde when the condensation is carried out under neutral or alkaline conditions support a mechanism involving attack by pyrryl-$CH_2NH_3^+$ (or an equivalent lactone) with the elimination of either H^+ or $CH_2=NH_2^+$ from the second pyrrole. The reaction in hot acid solution is complicated by the isomerization and reactivity of the macrocyclic product.

The isolation of porphobilinogen[2] and the proof of its structure[3] were major steps in the identification of the intermediates in porphyrin biosynthesis.[4] This pyrrole forms uroporphyrin in high yields under mild experimental conditions. Early work indicated that the product was largely the naturally occurring isomer III[1b] showing that a rearrangement had taken place. A very reasonable mechanism was proposed by Cookson and Rimington to explain these facts.[3] This paper clarifies the question of the identity of the isomers and the reduction level of the product, and gives some evidence for the mechanism of the condensation.

Experimental

Materials.—The porphobilinogen was isolated from urine of porphyric patients by the method of Cookson and Rimington,[3] and purified just prior to this series of experiments by crystallizing the HCl salt. This pyrrole was kept at −20°. It was analyzed as PBG·H₂O. Calcd. for $C_{10}H_{16}O_5N_2$: C, 49.14; H, 6.60. Found: C, 48.77; H, 6.84. The

source and method of purification of the porphyrins and other materials are given in the preceding paper.[5]

Analytical Methods.—Porphobilinogen was determined colorimetrically with a modified Ehrlich reagent.[6] The method of isomer analysis and other procedures are given in the preceding paper.[5] Due to the inherent difficulties of determining radioactivity by the "infinitely thin" technique, several products were checked by counting in solution.[7] This is a convenient and highly reproducible method of obtaining homogeneous infinitely thick counting with very small amounts of material. However, accuracy requires a high specific activity. Formamide, di-n-butyl phthalate, diphenyl ether and mixtures of these compounds are useful as non-volatile solvents. The comparison with the plating technique is good (Table I) and serves to justify this simpler, more sensitive technique. The counting error was easily held to less than 1% except for the very weakly labeled products. As a result of errors in determining the concentration and activity of added highly labeled formaldehyde, these ratios are only accurate to ±10%. About one-half of the uroporphyrin ester samples were rechromatographed on silica gel and re-assayed to check radioactive purity. The molecular activities were constant to ±5%. Various fractions from the band of the chromatographic column also had roughly constant activities. One sample was crystallized twice between chromatographs and also had constant activity. In addition, two uroporphyrin samples were chemically decarboxylated[8] and the molar activity of the coproporphyrin was also found to be constant (Table I).

(1) (a) Part of this work was presented at the IVth International Congress of Biochemistry, Vienna, Sept., 1958; abstracts, p. 4. (b) Formulas of the various compounds and isomers mentioned are to be found in the preceding paper.[5]

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