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The Biosynthesis of Chlorobium Chlorophylls-660. The Isolation and Purification of Porphyrins from *Chlorobium thiosulfatophilum*-660*

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ABSTRACT: Concentrated suspensions of *Chlorobium thiosulfatophilum*-660, when incubated with the tetrapyrrole precursors glycine and 2-ketoglutarate or δ-aminolevulinic acid, have produced coproporphyrin III, uroporphyrins I and III, traces of metalloporphyrins, porphyrins with 2, 3, 5, 6, and 7 carboxyl groups, and tetracarboxylic porphyrins with phyllo type spectra.

We have also studied the effect of ethionine on such incubations. A new and useful type of chromatography has been developed, whereby porphyrins are eluted from powdered polyethylene columns according to the number of carboxyl groups, the most highly carboxylated being removed first. This polyethylene chromatography has been used in conjunction with countercurrent distribution for separation and purification of the excreted porphyrins. No *meso*-alkylation of the tetrapyrrole nucleus has occurred before the coproporphyrinogen III stage during the biosynthesis of chlorobium chlorophyll-660 by this organism.

Although a great deal of work has appeared concerning the biosynthesis of chlorophyll *a* (Bogorad, 1960; Granick and Mauzerall, 1961) and bacteriochlorophyll (Lascelles, 1961, 1964), relatively little has appeared on the biosynthesis of the chlorobium chlorophylls. Recently, structures have been assigned to the chlorophylls of the green sulfur bacterium, *Chlorobium thiosulfatophilum* (Holt *et al.*, 1962, 1963; Mathewson *et al.*, 1963a,b). A unique feature of the chlorophylls of the 660 series is that in addition to the γ-substituent involving the cyclopentenone ring E, there is a *meso*-alkyl (methyl or ethyl) substituent on the tetrapyrrole nucleus.[1] We have been interested in determining the point in the biosynthetic sequence of production of the chlorobium chlorophylls-660 at which the *meso*-alkyl substituent is attached to the macrocycle.

Porphyrins have been detected in the regular growth medium of *C. thiosulfatophilum* (Lascelles and Cooper, 1955; Lascelles, 1955; Erokhin and Krasnovskii, 1963; Uspenskaya and Kondrat'eva, 1964). Also, although *C. thiosulfatophilum* cannot grow on media containing organic compounds without the normal inorganic nutrients being present (Larsen, 1953; Moshentseva and Kondrat'eva, 1962) the biomass and porphyrin excretion is increased if precursors of tetrapyrroles are included along with the regular growth medium (Uspenskaya, 1965a,b).

We have also observed porphyrin excretion by *C. thiosulfatophilum*-660 when the organism was cultured under normal growth conditions. However, much larger quantities of porphyrins were excreted if the bacteria were first grown under normal conditions and then concentrated and incubated with the tetrapyrrole precursors. Following work done with purple bacteria by Lascelles (1955, 1956) and Gibson *et al.* (1962), working with *Rhodopseudomonas spheroides*, and by Cooper (1963), working with *R. capsulata*, we have identified the main porphyrins excreted by *C. thiosulfatophilum*-660 under similar conditions as coproporphyrin III and uroporphyrins I and III. In addition we have detected traces of metalloporphyrins, porphyrins with 2, 3, 5, 6, and 7 carboxyl groups, and tetracarboxylic porphyrins with phyllo type spectra (for explanation of spectral types see Falk, 1963); however, protoporphyrin was not specifically identified as the dicarboxylic

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[1] Holt *et al.* (1962, 1963) have assigned the *meso*-alkyl substituents to the δ-position. However, on the basis of the exchange properties of the pheophorbide (Mathewson *et al.*, 1963a) and the chlorophyll (Mathewson *et al.*, 1963b), we have assigned the *meso*-alkyl substituents to either the α- or β-position.

porphyrin. Hence it may be stated that up to the stage of coproporphyrinogen III, no *meso*-alkylation of the tetrapyrrole nucleus has taken place in *C. thiosulfatophilum*-660.

Experimental Procedures

Organism. The strain of *C. thiosulfatophilum* (strain PM; Stanier and Smith, 1960) was obtained from Dr. R. Y. Stanier, and was maintained in liquid growth medium. The bacteria produced one chlorobium chlorophyll-660 as a main product (*ca.* 70%; Mathewson *et al.*, 1963a).

Growth Medium. The growth medium was dissolved in distilled water and was essentially the synthetic medium of Larsen (1953) except that it contained $NaHCO_3$, 27.0 mM; $Na_2S \cdot 9H_2O$, 2.8 mM; and no $Na_2S_2O_3$.

Normal Growth Conditions. The bacteria were grown anaerobically, with continued stirring, at 30°, in 22-l. flasks. The flasks were illuminated by two 250- or 300-w reflector flood lamps placed 10–20 cm away, and cooled internally by a stainless steel cooling coil regulated by a thermister. Reinoculations were made by a 1:10 dilution of a fully grown (2–3 days) culture with fresh growth medium.

Harvesting of Cultures. The 22-l. cultures were centrifuged at 30,000 rpm after 2–3 days of growth using a continuous flow Sharples centrifuge. The yield of cells was about 2.0–2.5 g (dry wt)/flask, determined after removal of water *in vacuo* over anhydrous $CaCl_2$. The bacteria (wet pack) were suspended in 200 ml of fresh growth medium (minus $FeCl_3$) and stored at 2–3°.

Incubation Media. MEDIUM A. Normal growth medium (see above) minus $FeCl_3$; plus: glycine, 0.01 M; 2-ketoglutaric acid, 0.01 M; disodium succinate, 0.02 M; pH 7.0–7.2.

MEDIUM B. Mixture I of Lascelles (1956) which contained inorganic salts and glycine, 0.01 M; 2-ketoglutaric acid, 0.01 M; but disodium succinate, 0.02 M in place of disodium fumarate; pH 6.8.

MEDIUM C. Mixture A of Cooper (1963) which contained inorganic salts and glycine, 0.01 M; disodium succinate, 0.04 M; DL-methionine, 0.002 M; but no nicotinic acid; pH 7.0.

MEDIUM D. Mixture II of Lascelles (1956) which contained inorganic salts and δ-aminolevulinic acid, 0.002 M; pH 6.9.

ADDITIONS. The following substances were added as indicated in Table II, at the following concentrations: $FeCl_3$, 8.7 μM; DL-methionine, 0.002 M; DL-ethionine, 0.002 M; disodium succinate, 0.02 M; NaOH, 1.0 M, was added to all media for pH adjustment.

Incubation Conditions. SMALL SCALE. For the incubations reported in Table II, 6-ml aliquots of the freshly suspended harvest were centrifuged for 20 min at 3200 rpm, were resuspended in the desired medium, and were incubated anaerobically, with occasional stirring, in 60-ml bottles at the indicated temperature and time. The approximate bacteria concentration was 1 mg (dry wt)/ml. Each bottle was illuminated by one 75-w reflector flood lamp placed 10–20 cm away, and cooled by an externally controlled water bath. For experiments at more dilute bacteria concentrations, less of the normal culture growth was harvested and resuspended.

LARGE SCALE. For large-scale production of porphyrins for the purpose of isolation and characterization, two incubation sizes were employed: (a) for medium D (plus succinate and iron) and medium C (with ethionine in place of methionine), 100-ml aliquots of the freshly suspended harvest were centrifuged and resuspended as with the small-scale incubations, and were incubated anaerobically, with occasional stirring, for 2 days at 50°, and 7 days at 25°, respectively, in 1-l. Roux bottles. Each bottle was illuminated by one 250-w reflector flood lamp placed 10–20 cm away, and cooled by an externally controlled water bath; (b) for medium A, the bacteria from three to four fully grown 22-l. flasks were centrifuged and resuspended in one 22-l. flask containing medium A (bacteria concentration 0.3–0.4 mg (dry wt)/ml) and were incubated anaerobically, with continual stirring, for 7–8 days at 30°. The flask was illuminated and cooled as with the normal growth conditions. It was found that medium A was preferable to medium B in these long-term incubations because the bacteria did not deteriorate in the presence of sulfide ion. There was no apparent difference in the porphyrins produced, however.

Porphyrin Estimation. The total porphyrin content of incubations reported in Table II was estimated by measurement of the absorbance of the Soret peak (*ca.* 396 mμ) in the neutral (pH 7–8) incubation medium after centrifugation at 17,000 rpm for 20 min. The molar extinction coefficients employed in estimation were, for the methyl esters in neutral solution (Falk, 1963): runs 1–8 (mostly coproporphyrin), $\epsilon = 1.80 \times 10^5$; runs 9–15 (mostly uroporphyrin), $\epsilon = 2.17 \times 10^5$. The approximate percentages of porphyrins were estimated from fractions from polyethylene chromatography and countercurrent distribution.

Large-Scale Extraction of Porphyrins. After removal of cells by centrifugation, the large-scale 22-l. incubation media were brought to pH 3.5 with 85% phosphoric acid and extracted in 4-l. lots with 500 ml of ethyl acetate and then three times with 250 ml of ethyl acetate. The combined 4-l. lots were then brought to pH 1.8 with 85% phosphoric acid and extracted once with 800 ml of cyclohexanone by stirring rapidly for 1 hr in a large container fitted with a drain at the bottom. About 1–1.5 kg of NaCl was added to facilitate separation of the layers. The ethyl acetate extracts were combined and evaporated *in vacuo* and the precipitated porphyrins, cell debris, and water residue was added to a sintered glass filter (9 × 9 cm) filled with powdered polyethylene.[2] The porphyrins were washed from the polyethylene with acetone until no more fluorescence was being removed, and the acetone was evaporated *in vacuo* after the addition of an equal volume of aqueous base (1 M NaOH or ammonia). The water

[2] Dow Chemical Company, Experimental Resin QX 2187.

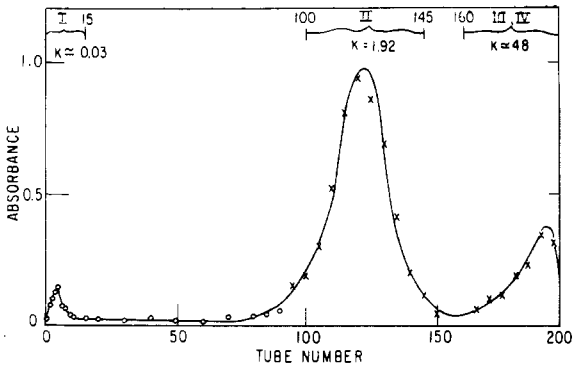

FIGURE 1: Countercurrent distribution diagram for separation of major (tetracarboxylic) porphyrins produced from incubations of *C. thiosulfatophilum*-660 with medium A. The phases were ethyl acetate–aqueous HCl (pH 1.45); transmittance of aqueous (O) or ethyl acetate (×) phases were measured by a Fisher photometer with a 425-mμ filter.[3]

TABLE 1: Optimum Solvents and pH for Elution of Various Carboxylated Porphyrins from Powdered Polyethylene Columns.

No. of Free Carboxyl Groups[a]	Paper Chromatography R_F Values[b]	Solvent (%, v/v)	pH
8	0.09	Aq 0.1 M KH_2PO_4–2,6-lutidine	6.7
7	0.15	Same	7.0
6	0.28	Same	7.2
5	0.46	Same	7.3
4	0.54	Same or	7.4
		Aq 2,6-lutidine (0.25–0.5)	9.0–9.2
3	0.60	Same (1)	9.5
2[c]	0.70	Same (5–10)	9.8–10.0

[a] Porphyrins were free acid and partially esterified samples of uro-, copro-, and hematoporphyrin. [b] Lutidine–water (5:3)–ammonia vapor system of Eriksen (1953). [c] Bands with 1 and 0 carboxyl groups left on column may be removed by acetone.

residue was employed for polyethylene chromatography. The cyclohexanone extract, which also contained ethyl acetate extracted from the water, was extracted three times with about 0.25 the volume of 1 M aqueous ammonia. Cyclohexanone was removed from the ammonia solutions by extraction two times with 0.5 the volume of peroxide-free ether and the ammonia solutions were employed for polyethylene chromatography.

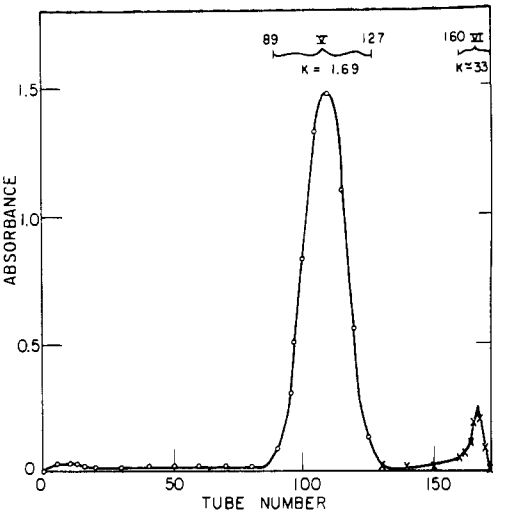

FIGURE 2: Countercurrent distribution diagram for separation of major (tetracarboxylic) porphyrins produced from incubations of *C. thiosulfatophilum*-660 with medium C (with ethionine, 2 mM, in place of methionine). The phases were ethyl acetate–aqueous H_3PO_4 (pH 1.40); transmittance of aqueous (O) or ethyl acetate (×) phases measured by a Coleman photometer with a 390-mμ filter. Tubes 2–25 contained a nonporphyrin material with absorption at 460 mμ.

The 1-l. incubation media were extracted in much the same way except proportionately more ethyl acetate and cyclohexanone was used to extract the porphyrins: four ethyl acetate extracts of 250 ml and three cyclohexanone extracts of 100 ml were employed. In the case of medium D, more cyclohexanone extracts were required (from 5 to 8) and the basic extracts from both the ethyl acetate and cyclohexanone solutions were combined before polyethylene chromatography.

Polyethylene Chromatography. Powdered polyethylene[2] was packed dry into 7.5 × 40 cm columns and washed with 1 l. each of acetone, methanol, and distilled water. The aqueous base solutions from the porphyrin extracts were brought to pH 4–5 (almost to the point of precipitation of the porphyrins) with 85% phosphoric acid, and the solutions were added to a prepared polyethylene column, and washed with 0.1 M phosphate buffer, pH 4–5. For extracts containing less porphyrin, smaller (3.5 × 30 cm) columns were used. It was found that acid up to 5 M HCl did not desorb the porphyrins but that basic solvents selectively desorbed the porphyrins according to the number of carboxyl groups, the more highly carboxylated porphyrins being desorbed first. Small, clean bands were formed using various buffered and dilute solutions of aqueous 2,6-lutidine. The solvent was not changed until each band was eluted. Table I shows optimum solvents for elution of porphyrins with from 8 to 2 carboxyl groups, determined on free acid and partially esterified samples of uro-, copro-, and hematoporphyrin.

Countercurrent Distribution. The apparatus em-

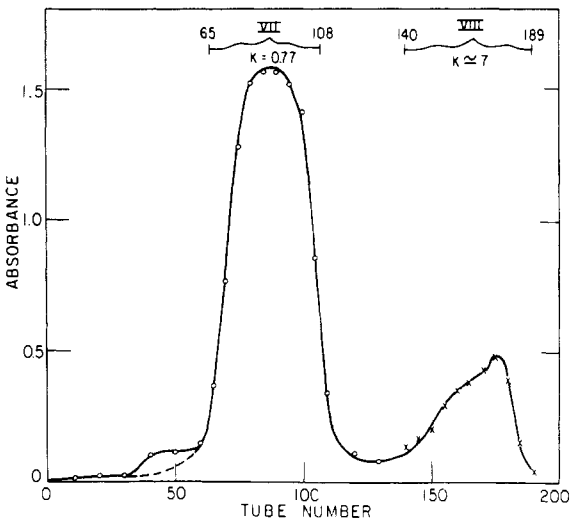

FIGURE 3: Countercurrent distribution diagram for separation of major (octacarboxylic) porphyrins produced from incubations of *C. thiosulfatophilum*-660 with medium D (with succinate, 20 mM; and $FeCl_3$, 8.7 μM). The two phases were formed by equilibration of cyclohexanone, acetone, and aqueous HCl (0.3 M) in the ratio 2:1:2; transmittance of aqueous (O) or organic (×) phases measured by a Coleman photometer with a 390-mμ filter. Tubes 35–60 contained an unidentified impurity.[4]

ployed was an automatic 200-tube Craig machine and was used in a manner similar to that described by Granick and Bogorad (1953) except that the solvents which formed the two phases were: for coproporphyrin, ethyl acetate and aqueous acid (HCl or H_3PO_4, pH 1.4) in a ratio of 1:1; for uroporphyrin, cyclohexanone, aqueous HCl (0.3 M), and acetone in a ratio of 2:2:1. For the latter system, the acetone was added to prevent emulsion formation; during equilibration of the phases it partitioned approximately equally between the cyclohexane and aqueous phases. The distribution diagrams (Figures 1–3) were constructed after measuring the transmittance, T, with a Coleman (390-mμ filter) or a Fisher (425-mμ filter) photometer and converting to absorbance by the formula: $A = (-\log T)C$, where C is a correction factor equal to $K + 1$, if the aqueous phase was measured, and $(K + 1)/K$, if the organic phase was measured. [K is the distribution coefficient, equal to $N/(n - N)$, where N is the tube of maximum concentration of the band and n is the total number of transfers.] An additional countercurrent separation[3] employed 0.3 M HCl with ethyl acetate but acid concentrations greater than 0.3 M led to noticeable hydrolysis of the ethyl acetate.

Paper Chromatography. FREE PORPHYRINS. The number of carboxyl groups on the free porphyrins in the ethyl acetate and cyclohexanone extracts after polyethylene chromatography was determined by the 2,6-lutidine–water–ammonia vapor method of Eriksen (1953).

PORPHYRIN ESTERS. After purification by polyethylene chromatography and countercurrent distribution, the porphyrin esters were prepared by treatment of the free acid for at least 24 hr with 5% (v/v) concentrated H_2SO_4 in anhydrous methanol (Falk, 1963). The paper chromatographic methods of Chu *et al.* (1951) and Falk and Benson (1953) were employed for copro- and uroporphyrin, respectively, and also the modifications of both systems reported by Chu and Chu (1957). Better development of the spots was obtained if the Chu and Chu (1957) methods were modified to include a chloroform equilibration atmosphere in the first solvent tank instead of a kerosene atmosphere.

HCl Number Determination. The HCl number is defined and given as 0.08 for coproporphyrin by Falk (1963). A plot was constructed of the hydrogen ion concentration (measured by pH meter) squared *vs.* the ratio of the concentration of porphyrin in the acid layer to that in the ether layer [determined by measurement of the absorbance of the Soret peak in both layers and assuming the following molar extinction coefficients (Falk, 1963): $\epsilon_{acid} = 4.89 \times 10^5$ and $\epsilon_{ether} = 1.80 \times 10^5$]. The HCl number, expressed as per cent (w/v) of HCl, was obtained from the acid concentration corresponding to a ratio of 2 on the plot.

Instrumental Measurements. Visible spectra were recorded on a Cary Model 14 spectrophotometer; infrared spectra were recorded on a Perkin-Elmer 421 spectrophotometer; nuclear magnetic resonance spectra were recorded on a Varian A-60 spectrometer; pH measurements were taken with a Beckman battery-operated Model G or line-operated Zeromatic pH meter.

Materials. SYNTHESIS OF δ-AMINOLEVULINIC ACID (HCL SALT). The procedure was essentially Route B of Neuberger and Scott (1954). The product (mp 142–145° dec) had a nuclear magnetic resonance spectrum in D_2O (δ-values in ppm; TMS = 0) consisting of a two-proton singlet at 4.29 (δ-methylene protons) and a four-proton multiplet at 2.95 (α- and β-methylene protons). The methyl ester (mp 104–106°) had in addition a three-proton singlet at 3.81.

PORPHYRINS. Copro- and hematoporphyrin were purchased from Mann Research Lab., Inc., New York, N. Y.; uroporphyrin was purchased from K and K Labs, Inc., Plainview, N. Y.

SOLVENTS. All solvents were distilled before use. Ether was distilled from solid ferrous sulfate and washed with 10% aqueous ferrous sulfate just before use to remove peroxides. The kerosene used for paper chromatography was a fraction with bp 40–80° at 1 mm.

[3] Component III′ was noticed in place of III and IV when the mixture of porphyrins was heated to 45° in strong HCl before countercurrent distribution. Tubes 160–197 were further purified by countercurrent distribution: phases, ethyl acetate–aqueous HCl (0.3 M); III, $K \simeq 0.05$; IV, K very large (end tubes). In addition partially esterified derivatives of coproporphyrin III appeared at $K = 0.18$ (tricarboxylic) and $K = 0.84$ (dicarboxylic), along with traces of true di- and tricarboxylic porphyrins if the prior polyethylene chromatography was omitted.

TABLE II: Porphyrin Production by Incubation of *C. thiosulfatophilum*-660 with Organic Media.[a]

Expt No.	Medium	Incubation Medium and Conditions: Compounds Deleted[b]	Compounds Added[c]	$FeCl_3$ Added[d]	Temperature	Time (hr)	Total Porphyrin (μM)	Porphyrin[e] Composition (%): URO	COPRO	Other
1	A	none	none	−	30°	40	9.1	1	90	9
2	B	none	none	−	30°	35	4.5	9	82	9
3	B	none	none	+	30°	35	2.7	1	90	9
4	C	methio	none	−	25°	81	1.5	..	..	.
5	C	none	none	−	25°	81	<1	..	..	.
6	C	methio	ethio	−	25°	81	4.7	37	63	1
7	C	none	ethio	−	25°	81	<1	..	..	.
8[f]	D	δ-ALA	succ	−	30°	38	0.7	..	..	.
9[f]	D	none	none	−	30°	38	5.1	..	..	.
10[f]	D	none	succ	+	30°	38	21.8	..	..	.
11[g]	D	none	succ	+	50°	46	86.4	90	2	8
12	D	none	succ	+	25°	74	67	..	..	.
13	D	none	succ and ethio	+	25°	74	100	..	..	.
14	D	none	succ and methio	+	25°	74	64	..	..	.
15	D	none	succ, ethio and methio	+	25°	74	90	..	..	.

[a] All incubations in 60-ml bottles at bacteria concentration of 1 mg (dry wt)/ml except where noted. [b] Methio is DL-methionine; δ-ALA is δ-aminolevulinic acid. [c] DL-Methionine (methio) and DL-ethionine (ethio) added at 2 mM; disodium succinate (succ) added at 20 mM. [d] $FeCl_3$ added at 8.7 μM. [e] URO is uroporphyrin; COPRO is coproporphyrin; see Tables III and IV for other porphyrins. [f] Bacteria concentration 0.4 mg (dry wt)/ml. [g] One-liter incubation bottle.

Results

Incubations with Organic Compounds. The results of incubations with the various organic media are presented in Table II. It can be seen from the table that $FeCl_3$ at 8.7 μM represses porphyrin production to only 60% of the level attained without iron, when *C. thiosulfatophilum*-660 was incubated with glycine, 2-ketoglutarate, and succinate (medium B, expt 2 and 3). Ethionine (expt 6) caused an increase in porphyrin production when incubated with glycine and succinate (medium C, expt 4), whereas methionine (expt 5) did not. Methionine reversed the effect of ethionine, however (expt 7). In all of the above cases the predominant porphyrin excreted was coproporphyrin III. When δ-aminolevulinic acid (medium D, expt 9) was incubated with *C. thiosulfatophilum*-660, however, the predominant porphyrin excreted was uroporphyrin I. If succinate and $FeCl_3$ were included in the incubation mixture a significant increase in porphyrin production was noticed (expt 10–12). The effect of ethionine with medium D (expt 13) was similar to that noticed with medium C, but methionine (expt 15) was not as active in inhibiting the effect of ethionine.

In none of expt 1–7 (media A–C) was there any indication of the presence of significant amounts of porphyrinogen. No increase in fluorescence or absorbance was noticed by bubbling oxygen into freshly opened bottles or by treating extracts with dilute iodine solutions. However, there was a very dramatic increase in fluorescence when oxygen was bubbled into freshly opened bottles of medium D (δ-aminolevulinic acid) containing succinate and iron (expt 10–15), indicating considerable uroporphyrinogen formation. An intermediate with absorption at 500 mμ which gradually was oxidized to a porphyrin (increase in the Soret absorption) by standing in the air was also observed, and was similar to the intermediate described by Cooper (1963) which he attributed to a dipyrrylmethenetetrapyrrole intermediate between coproporphyrinogen and coproporphyrin.

Characterization of Porphyrins Produced. Large-scale incubations similar to expt 1, 6, and 11 (Table II) were carried out and extracted as described in the experimental procedure. The number of carboxyl groups of the major and also of minor spots visible on paper chromatograms (Eriksen, 1953) was determined after the ethyl acetate and cyclohexanone extracts were subsequently purified by polyethylene chromatography. The minor (2, 3, 5, 6, and 7 carboxyl groups) porphyrin bands observed during polyethylene chromatography were treated with NaOH (1 M or stronger) and re-examined by paper chromatography, to assure that these were not due to partially esterified derivatives of uro- and coproporphyrin. The paper chromatographic spots visible after these procedures were employed were (number of carboxyl groups in parenthesis): (a) medium A: ethyl acetate extract, major band (4), minor bands (3 and 8); cyclohexanone extract, major band (8); (b)

TABLE III: Visible Spectra of Countercurrent Distribution Components.[a]

Component	Type[b]	$\lambda_{max}^{1MNH_4OH}$, mμ					
		Red	I	II	III	IV	Soret
I	Phyllo	...	625 (0.16)	573 (0.56)	538 (0.56)	504 (1.00)	375
III[c]	Phyllo	640 (0.43)	617 (0.45)	568 (0.85)	540 (0.80)	505 (1.00)	385
III'[c]	Phyllo	...	625 (0.20)	573 (0.52)	537 (0.49)	508 (1.00)	375
II, V (COPRO III)	Etio	...	615 (0.32)	563 (0.60)	537 (0.75)	502 (1.00)	392
VII (URO I)	Etio	...	613 (0.30)	562 (0.59)	537 (0.81)	502 (1.00)	397
IV[d]	Metallo	620–640 (0.55)	595 (0.58)	560 (1.00)	523 (0.86)	...	396–400
IV (after H_2SO_4)	Etio	...	615 (0.35)	565 (0.63)	535 (0.72)	503 (1.00)	392
VI[e]	Metallo	...	...	560 (1.00)	525 (0.46)	...	402
VIII[f]	Metallo	650–660 (0.06)	608 (0.06)	562 (1.00)	526 (0.66)	...	397
VIII[f] (after H_2SO_4)	Etio	640 (0.08)	614 (0.22)	562 (0.53)	537 (0.72)	502 (1.00)	399
		$\lambda_{max}^{3MaqHCl}$, mμ					
I	Acid	...	603 (0.30)	561 (1.00)	525(sh) (0.18)	...	414[g]
III	Acid	625 (0.34)	588 (0.57)	548 (1.00)	...	...	404
II, V (COPRO III)	Acid	...	590 (0.39)	548 (1.00)	...	...	403
VII (URO I)	Acid	...	590 (0.42)	552 (1.00)	...	...	407[h]

[a] For countercurrent distribution components, see Figures 1–3; relative absorption in parentheses. [b] For explanation of spectral types, see Falk (1963). [c] See footnotes 3 and 6. [d] Spectrum taken in ethyl ether. [e] Spectrum taken in ethyl acetate. [f] After further polyethylene purification.[4] [g] Soret maximum very dependent upon acid concentration and varied from 412 to 420 mμ with increasing acid concentration. [h] Value in 1 M HCl.

medium C (with ethionine, 2 mM, in place of methionine): ethyl acetate extract, major band (4); (c) medium D (with succinate, 20 mM, and $FeCl_3$, 8.7 μM): combined ethyl acetate and cyclohexanone extracts, major band (8), minor bands (4, 5, 6, and 7). The visible spectra of components I–VIII observed after purification of the major bands from polyethylene chromatography by countercurrent distribution (Figures 1–3) are shown in Table III. The spectra of the metalloporphyrins IV and VIII[4] after these components were treated with concentrated H_2SO_4, neutralized, and extracted back into organic solvents are also shown in Table III, and it can be seen that the spectra are of the etio type. Whereas iron was detected in the sulfate salt after treatment of IV, the nature of the metal in VIII could not be determined. Paper chromatography of the porphyrin esters showed II, V, and IV (after removal of the metal) to be pure coproporphyrin III, whereas VII and VIII (after removal of the metal) were mostly uroporphyrin I with traces of the III isomer. Comparison by paper chromatography with our commercial porphyrin esters showed that both commercial coproporphyrin and uroporphyrin (from animal urine) were mostly the I with traces of III isomer. An HCl number plot of II yielded an HCl number of 0.087 which compares well with the HCl number for coproporphyrin of 0.08 reported by Falk (1963).

The infrared spectrum in CCl_4 of II was similar to that of commercial coproporphyrin (mostly the I isomer). However, the assignment of the coproporphyrin isolated from *C. thiosulfatophilum*-660 as the III

[4] Tubes 140–189 were further purified by polyethylene chromatography and yielded a metalloporphyrin (VIII) and a material with a chlorin-like spectrum (major absorption 636 and 413 mμ).

isomer was confirmed by the absence of strong bands at 1049, 1232, and 1277 cm^{-1}, which were present in the commercial sample. This observation corresponded with the distinction between the isomers reported by Falk and Willis (1951) and to the spectra of Gray *et al.* (1950). These literature data were obtained from Nujol mulls which obscured C–H absorption; but bands at 2947, 2922, and 2853 cm^{-1} (C–H stretch) and 1450 (w), 1430, and 1420 (w) cm^{-1} (C–H bend), observed in both commercial and natural coproporphyrin (I and III isomers, respectively), were also reported by Craven *et al.* (1952) for the CCl_4 solutions, although these authors could not observe the distinction between the isomers reported by Falk and Willis (1951). The infrared spectra of uroporphyrins I and III are very similar (Falk and Willis, 1951); however, decreased absorption at 1250 cm^{-1} reported for uroporphyrin I but not for the III isomer (Falk and Willis, 1951) was observed with both component VII and the commercial uroporphyrin, and hence confirmed the chromatographic data that these were both predominantly the I isomer.

The nuclear magnetic resonance spectrum (δ-values, TMS = 0) of component II (*ca.* 0.14 M in $CDCl_3$) showed several singlet peaks for the ring methyl (3.25, 3.35, and 3.45), ester methyl (3.58 and 3.60), and *meso* (9.39 and 9.50) protons similar to the reported spectrum (Becker *et al.*, 1961) for coproporphyrin III, and confirmed the presence of the unsymmetrical substitution of the III isomer in component II. On the other hand, the nuclear magnetic resonance spectrum of VII (*ca.* 0.10 M in $CDCl_3$) showed only one singlet peak for propionic ester methyl (3.81), acetic ester methyl (3.88), acetic ring methylene (5.08), and *meso* (10.03) protons, and triplet peaks for propionic α-methylene (3.42) and propionic ring methylene (4.42) protons similar to the reported spectrum (Becker *et al.*, 1961) of uroporphyrin I, and confirmed the presence of the symmetrical substitution of the I isomer in component VII.

Although examination of porphyrins which appeared in only minor quantities was limited, these porphyrins, after separation by polyethylene chromatography, were examined by paper chromatography of the methyl esters. The major (octacarboxyl) band obtained from the cyclohexanone extract of medium A was examined along with porphyrins containing 7, 6, 5, and 4 carboxyl groups produced during incubation with δ-aminolevulinic acid, succinate, and iron (medium D). In addition, the three phyllo porphyrins (components I, III, and III′) observed during countercurrent distribution of the major (tetracarboxyl) band obtained from the ethyl acetate extract of medium A (Figure 1) were similarly examined. The results are presented in Table IV.

Discussion

Discussion of Experimental Procedures. Our primary interest in the examination of the porphyrins produced by *C. thiosulfatophilum*-660 was to determine if there had been any *meso*-alkylation of the tetrapyrrole nucleus. Even though uroporphyrins I and III and coproporphyrin III could be detected as the principal excretion products in rather crude extracts, isolation and purification of the excreted porphyrins on a large scale was necessary in order to be able to detect small quantities of unknown porphyrins which might otherwise have gone unnoticed. We found that a combination of polyethylene chromatographic and countercurrent distribution purifications was quite satisfactory because of the distinctly different principles involved in the two methods. Polyethylene chromatography gives separation on the basis of the number of carboxyl groups whereas countercurrent distribution employs the principle of separation on the basis of differences in acid number, *i.e.*, on the basis of basicity. Hence a *meso*-alkylated porphyrin formed after coproporphyrin III (and containing four carboxyl groups) would appear in the coproporphyrin fraction from polyethylene chromatography, but would be separable from coproporphyrin by countercurrent distribution because of differences in acid number.

The extraction procedure described in the experimental section differs from reported procedures (Schwartz and Wikoff, 1952; Lascelles, 1956; Dresel and Falk, 1956; Sano and Rimington, 1963) in that it includes no strong acid extraction of ethyl acetate solutions of porphyrins. We found that such a procedure led to considerable porphyrin ester formation by transesterification with ethyl acetate and caused confusion in paper chromatographic determinations of the number of carboxyl groups of the extracted porphyrins and during subsequent purification steps.

Previous column procedures (Falk, 1961), including a recent paper by Baker *et al.* (1964), have reported separation of porphyrins according to the number of carboxyl groups present. Polyethylene purification has previously been employed for chlorophyll purification (Anderson and Calvin, 1962; Mathewson *et al.*, 1963a,-1963b); however, the present polyethylene method is the first application of a reverse phase column method to the purification of porphyrins, whereby the more highly carboxylated porphyrins are desorbed first. In addition to the clean separation of porphyrins containing different numbers of carboxyl groups, there was evidence for separation of porphyrins, including isomers of the same porphyrin, containing the same number of carboxyl groups, although this separation was far less distinct. Metalloporphyrins have been observed running at the front of free porphyrin bands. Also multiple bands were observed during chromatography of the minor porphyrins produced during δ-aminolevulinic acid incubations. Although these could not be separated cleanly by elution from the column, it was apparent by examination of early and later fractions of these bands (Table IV, and below) that they were due to isomer separation on the polyethylene column.

Countercurrent distribution methods previously reported have included separation of dicarboxylic porphyrins (Granick and Bogorad, 1953), of coproporphyrin from dicarboxylic porphyrins (French *et al.* 1964), and of various *meso*-alkylated octaalkyl por-

TABLE IV: Paper Chromatographic Data for Methyl Esters of Porphyrins Produced by Incubation of *C. thiosulfatophilum*-660 with Organic Substrates.

Medium	Porphyrin Designation[b]	No. of COOH[c]	Paper Chromatographic Systems (R_F Values[a]) System A 1st Solvent	System A 2nd Solvent	System B 1st Solvent	System B 2nd Solvent	System C 1st Solvent	System C 2nd Solvent
Coproporphyrin I			0.80	0.00	0.62	0.00	0.90	0.76
Coproporphyrin III			0.85	0.43	0.62	0.23	0.90	0.76
A	I	4 COOH	0.79	0.33[d]	...	...	...	...
A	III	4 COOH	0.84	0.41[d]	...	...	...	...
A	III′	4 COOH	0.84	0.46[d]	...	...	...	...
D[e]	...	4 COOH	0.84	0.02, 0.41	0.62	0.00, 0.24	0.89	0.77
D[e]	...	5 COOH	0.78	0.02, 0.32	0.56	0.04, 0.17	0.89	0.72
D[e]	...	6 COOH	0.75	0.23[f]	0.53	0.09	0.89	0.66
D[e]	...	7 COOH	0.76	0.07	0.48	0.04	0.89	0.58
D[e]	...	8 COOH	0.66	0.00	0.47	0.00	0.88	0.00, 0.51
A	...	8 COOH	...	...	...	...	0.88	0.00, 0.49
Uroporphyrin I			...	...	...	...	0.88	0.00
Uroporphyrin III			...	...	...	...	0.88	0.49

[a] The following systems were employed: system A, Chu and Chu (1957) modification of Chu *et al.* (1951) method, except chloroform was used as the second solvent tank equilibration atmosphere instead of kerosene. After 1 hr of development, the second solvent front had developed 3.1 times as far as the first; system B, same as A, except the first solvent front was carried further and after 1 hr of development the second solvent front had developed only 1.1 times as far as the first; system C, Falk and Benson (1953) method; first solvent R_F values measured from the origin and the second solvent R_F values measured from the first solvent spot. [b] See Table III and Figure 1. [c] As determined by paper chromatography of free acid by the method of Eriksen (1953). [d] These compounds showed dark red fluorescence, whereas all other spots showed bright pink fluorescence. [e] Medium D plus succinate, 20 mM; and $FeCl_3$, 8.7 μM. [f] Broad spot indicating the possibility of two isomers.

phyrins (Abraham *et al.*, 1963). The present procedures are similar, but the use of ethyl acetate as the organic phase for coproporphyrin purification and the use of the cyclohexanone–aqueous HCl–acetone system for uroporphyrin purification allow distribution coefficients near unity to be obtained for these two porphyrins.

Discussion of Results. The determination of coproporphyrin III as the major (*ca.* 90%) porphyrin produced by incubation of *C. thiosulfatophilum*-660 with glycine, succinate, and 2-ketoglutarate resembles the findings of Lascelles (1955, 1956) for *R. spheroides* and Uspenskaya and Kondrat'eva (1964) and Uspenskaya (1965a,b) for *Chloropseudomonas ethylicum* and *C. thiosulfatophilum*. Lascelles (1955, 1956) reported yields in terms of the concentration of porphyrin excreted into the incubation medium. Our concentrated suspensions contained a similar biomass (dry weight) of bacteria per milliliter but the concentration of excreted coproporphyrin was about $^1/_{10}$ the yield reported for the purple bacterium (Lascelles, 1955, 1956), but more than that reported for green bacteria by Uspenskaya (1965a) who did not concentrate the bacteria. When our data were calculated on a μg of porphyrin/g biomass (dry wt) of bacteria our results corresponded quite closely to the data for *Ch. ethylicum* but showed higher yields than those reported for *C. thiosulfatophilum* (Uspenskaya, 1965a). However, we found that the dry weight of centrifuged *C. thiosulfatophilum*-660 always included quite a bit of precipitated elemental sulfur and hence reflected a higher bacteria concentration than was actually present. Such may not have been the case with the purple bacterium.

We have also detected both uroporphyrins I and III, but have failed to detect any coporporphyrin I with media A, B, and C. Analogous to the findings of Uspenskaya (1965b) with *C. thiosulfatophilum*, we find only traces of a dicarboxylic porphyrin which was detected during countercurrent distribution of fractions which were not first purified by polyethylene chromatography.[5] However, we were unable to confirm that this was protoporphyrin.[5] In addition, we observed traces of a tricarboxylic porphyrin.

The results of the inclusion of 8.7 μM ferric ion with medium B do not correspond to the observation of Lascelles (1955, 1956) who found almost complete

[5] Uspenskaya (1965b) has reported considerable production of a dicarboxylic porphyrin, which is assumed to be protoporphyrin, by the green bacterium *Chloropseudomonas ethylicum* (which produces chlorobium chlorophyll-660). However, no definitive establishment of the structure of this porphyrin has been reported.

suppression of porphyrin excretion with *R. spheroides* when 10 μM ferric ion was included. Ferric ion inhibits the δ-aminolevulinic acid synthetase enzyme system in purple bacteria after combination with protoporphyrin to form hemin (Lascelles, 1964). We have not been able to confirm this effect with *C. thiosulfatophilum*-660. However, other results (W. R. Richards and H. Rapoport, 1965, unpublished data) during growth of this organism under iron-deficient conditions indicate that we may not have been able to exclude traces of iron impurities from the supposedly iron-free control media.

There is no indication whether the metalloporphyrin and phyllo type porphyrins observed have any biological significance or whether they were merely artifacts of the isolation and purification procedures;[6] however, the possibility remains that a *meso*-alkylation step may occur shortly after the coproporphyrinogen III stage.

When *C. thiosulfatophilum*-660 was incubated with δ-aminolevulinic acid (medium D) the level of porphyrin production was also about one-tenth of that reported by Lascelles (1955, 1956) for *R. spheroides*. When succinate and ferric ion were included in medium D, instead of the slight reduction in porphyrin production reported for the purple bacterium, a significant increase in porphyrin production resulted, and the level of total porphyrin excretion approached that reported for the purple bacterium. However, instead of an increase in protoporphyrin production (to *ca.* 20–30% in *R. spheroides;* Lascelles, 1955, 1956), the principal porphyrin excreted (*ca.* 90%) was uroporphyrin I. We are unable to explain why the addition of succinate and ferric ion should have caused such a large increase in uroporphyrin I production. Ferric ion may catalyze the enzymatic conversion of coprophyrinogen III to portoporphyrin in the presence of a dicarboxylic acid in purple bacteria (Lascelles, 1964). However, our results do not indicate this effect for ferric ion in *C. thiosulfatophilum*-660.

In addition to uroporphyrin I, uroporphyrin III and both coproporphyrins I and III were detected along with at least two different isomers of a pentacarboxylic porphyrin, (probably) two isomers of a hexacarboxylic porphyrin, and a heptacarboxylic porphyrin. Porphyrins with 5, 6, and 7 carboxyl groups have been reported before in hemolysates of chicken red blood cells (Batlle and Grinstein, 1964a,b), and it is probable that the corresponding porphyrinogens are biosynthetic intermediates between uro- and coproporphyrinogen. From the paper chromatographic R_F values (Table IV) it is likely that there are both III-type and I-type isomers of the pentacarboxylic and probably the hexacarboxylic porphyrins (solvent systems A and B, Table IV); *i.e.*, these isomers would yield coproporphyrins III and I, respectively, upon decarboxylation of the remaining acetic acid residues. In this respect, it is significant that both coproporphyrins I and III were found (*cf.* 4 COOH porphyrins, medium D, solvent systems A and B, Table IV). Only one spot corresponding to a heptacarboxylic porphyrin was visible and it was more likely that it was an isomer of the III-type (solvent system C, Table IV). The enzyme(s) uroporphyrinogen decarboxylase is active for both uroporphyrinogens I and III. The significant occurrence (*ca.* 7%) of these porphyrins with 5, 6, and 7 carboxyl groups confirms that this enzymatic reaction occurs in discrete steps, possibly each catalyzed by a different enzyme. Metalloporphyrins of both uroporphyrins I and III were observed (component VIII, Figure 3) but no porphyrins of the phyllo type were found, and all of the porphyrins described above had "etio" type spectra.

The predominance of uroporphyrin I over the III isomer was the same if the incubation was carried out at 25 or 50°. Heating to 50° deactivates the enzyme uroporphyrinogen III cosynthetase (formerly uroporphyrinogen isomerase; Bogorad, 1958a,b; Granick and Mauzerall, 1958; Hoare and Health, 1959) and would lead to a predominance of porphyrins of the I isomer series only. However, some other factor which we are unaware of must cause the predominance of the I isomer during incubations at 25°.

We found that, in contrast to the results of Cooper (1963), working with the purple bacterium *R. capsulata*, medium C was not an efficient medium for the production of porphyrins. However, if ethionine was employed in place of methionine, porphyrin production exceeded that of medium C with or without methionine. A similar stimulatory effect of ethionine was noted by Gibson *et al.* (1962) who included ethionine in Lascelles' mixture I (with succinate in place of 2-ketoglutarate) during incubations with *R. spheroides*, and by Uspenskaya (1965a) who grew *Ch. ethylicum* and *C. thiosulfatophilum* in the presence of ethionine. The effect of ethionine was ascribed by Gibson *et al.* (1962, 1963) to a competitive inhibition of the enzyme responsible for the methylation of magnesium protoporphyrin which allows intermediates prior to this stage to accumulate. This effect was reversed by the addition of methionine. Since we have observed the same reversal of the effect of ethionine by methionine with *C. thiosulfatophilum*-660, a similar methylation step may be occurring in this organism, although we, as well as Uspenskaya (1965b), have been unable to find any indication of the presence of the monomethyl ester of magnesium protoporphyrin in green bacteria. Also our results support the theory (Gibson *et al.*, 1962) that an intermediate after mag-

[6] The spectra of some metalloporphyrins and phyllo type porphyrins contained additional absorption in the red portion of the spectrum (Table III) which may have been similar to material with absorption near 638 mμ described by Lascelles (1956) after incubation of *R. spheroides* with glycine, 2-ketoglutarate, and fumarate, and again with δ-aminolevulinate under aerobic conditions in the dark. We noticed this material with both media A and D but not during incubations with ethionine (medium C). Allison and Becker (1963) have attributed bands at 640 mμ to interactions of metalloporphyrins with nitrogenous bases such as ammonia. We have found, however, that most of the red absorption associated with VIII could be separated by further polyethylene purification.[4] Treating metalloporphyrin IV (see spectra before and after sulfuric acid treatment, Table III) and phyllo porphyrin III (see spectra of III and III′, Table III) with strong acid also removed most of the 640 mμ absorption.

nesium protoporphyrin may be inhibiting the δ-aminolevulinic acid synthetase system. In addition, we observed a significant stimulatory effect of ethionine when added to medium D, corresponding to similar results reported by Gibson *et al.* (1962) for *R. spheroides*. However, there was no analogous effect for methionine, whereas Gibson *et al.* (1962) had noted that both ethionine and methionine were about equally effective in increasing porphyrin production from medium D. In contrast to the results with medium C, methionine was much less effective for the inhibition of the effect of ethionine. Hence, our results with *C. thiosulfatophilum*-660 confirmed the observation (Gibson *et al.*, 1962) that the effect of ethionine in the presence of δ-aminolevulinic acid is different in nature from its effect in the presence of glycine and succinate. However, we were unable to determine the nature of this difference.

It should be emphasized that while there was evidence for the presence of considerable uroporphyrinogen excretion by *C. thiosulfatophilum*-660 when incubated in medium D with succinate and ferric ion, no indication of the corresponding porphyrinogen was observed when the principal porphyrin excreted was coproporphyrin III (media A–C). Lascelles (1961) has postulated that the conversion of a porphyrinogen to a porphyrin before excretion may be partially catalyzed by specific enzymes. Whereas *R. spheroides* excretes mainly the coproporphyrin (Lascelles, 1955, 1956), *R. capsulata* accumulates mainly the coproporphyrinogen (Cooper, 1963). Hence, there may be an enzyme in *C. thiosulfatophilum*-660 which converts coproporphyrinogen III to coproporphyrin III before excretion, but none for uroporphyrinogen I, which is not, however, a normal biosynthetic intermediate. It has been amply shown that uro- and coproporphyrinogen III and not the corresponding porphyrins are the true biosynthetic intermediates in plants (Bogorad, 1958c), animals (Neve *et al.*, 1956; Mauzerall and Granick, 1958; Sano and Granick, 1961), and purple bacteria (Hoare and Heath, 1958, 1959).

The results of our studies allow us to establish the fact that the biosynthesis of chlorobium chlorophylls-660 proceeds initially through the normal pathway of heme, chlorophyll *a*, and bacteriochlorophyll biosynthesis: by the condensation of glycine with succinyl-coenzyme A to form δ-aminolevulinic acid, and the subsequent conversion of the δ-aminolevulinic acid to porphobilinogen (presumably), uroporphyrinogen III, and coproporphyrinogen III. The fact that no phyllo type porphyrins were observed in the uroporphyrin fraction, but were present in traces in coproporphyrin fractions, may indicate that a *meso*-alkylation step occurs between coproporphyrinogen III and protoporphyrin.[5] However no *meso*-alkylation has occurred prior to the coproporphyrinogen III stage. Since non-*meso*-alkylated protoporphyrin is necessary for the formation of cytochromes which are of the *c*-type in *C. thiosulfatophilum* (Lascelles, 1964), it is more probable that *meso*-alkylation takes place after the protoporphyrin stage.

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Enzymic Synthesis of Dimethyl Selenide from Sodium Selenite in Mouse Liver Extracts*

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ABSTRACT: The enzymic synthesis of dimethyl selenide from sodium selenite was studied with mouse liver extracts as a model system for the reductive utilization of selenium. Dimethyl selenide was identified as the major volatile product in doubly labeled studies with carbon-14 and selenium-75. Both the 165,000 × *g* supernatant fraction of liver and washed microsomes synthesize dimethyl selenide, but neither fraction is as active as a 9000 × *g* supernatant fraction. Activity is proportional to protein concentration, and heated extracts are inactive. Optimal conditions were determined for the over-all reaction. The crude system has a specific requirement for glutathione that cannot be eliminated by various thiols or by dithiothreitol. *S*-Adenosyl-L-methionine is the probable methyl donor. Reduced triphosphopyridine nucleotide, coenzyme A, adenosine 5′-triphosphate, and magnesium are also required for optimal activity. Incubation under nitrogen increases the yield of dimethyl selenide approximately tenfold compared to incubation in air, apparently by preventing the oxidation of labile reduced forms of selenium. The crude system is inhibited 50% by 10^{-6} M arsenite in the presence of a large excess of thiols and is also inhibited by cadmium. The possible role of glutathione derivatives of selenium in the synthesis of organoselenium compounds is discussed.

The trace element selenium prevents a number of nutritional diseases at dietary concentrations of 0.1 ppm or less, including liver necrosis in rats, exudative diathesis in chicks, and white muscle disease in ruminants. This subject is treated in detail in a recent monograph by Rosenfeld and Beath (1964). Although the specific biological role of selenium is not established, selenium is known to be most active in the form of organoselenium compounds such as Factor 3, an incompletely characterized substance isolated from pig kidneys by Schwarz and Foltz (1957). The forms of selenium used in most nutritional studies and veterinary applications have been inorganic salts such as sodium selenite and sodium selenate. These compounds are less active per mole of selenium but are convenient to use and give full protection against the deficiency symptoms, indicating that animals are able to synthesize the biologically active selenium compounds.

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