Ovothiols, a Family of Redox-Active Mercaptohistidine Compounds from Marine Invertebrate Eggs†

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ABSTRACT: We have previously reported a novel thiol compound, 1-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine, or ovothiol, present at high concentration in the eggs of the sea urchin *Strongylocentrotus purpuratus* [Turner, E., Klevit, R., Hopkins, P. B., & Shapiro, B. M. (1986) *J. Biol. Chem. 261*, 13056–13063]. Here we report two related compounds, 1-methyl-N^{α}-methyl-4-mercaptohistidine, or ovothiol B, from the scallop *Chlamys hastata*, and 1-methyl-4-mercaptohistidine, or ovothiol A, from the starfish *Evasterias troschelii*. These two compounds, as well as the *S. purpuratus* compound now designated ovothiol C, were isolated from eggs or ovarian tissue by S-carboxymethylation with [^{3}H]iodoacetic acid, ion-exchange chromatography and ion-pairing high-pressure liquid chromatography. The structures of *S*-(carboxymethyl)ovothiols A and B were determined by ^{1}H NMR, and that of ovothiol A was confirmed by comparison with authentic methylhistidine samples after desulfuration with Raney nickel. In the ovary of each species, the predominant methylation form of ovothiol accounts for at least 80% of the total 4-mercaptohistidine. The ovothiol concentration of the ovary far exceeds that of the testis or somatic tissues. The ovothiol C content of unfertilized *S. purpuratus* eggs is 1.14 μmol/10^6 eggs, equivalent to approximately 4.3 mM average concentration; the glutathione (GSH + GSSG) content is 0.9 μmol/10^6 eggs. In this species, high ovothiol levels persisted for the first 2 weeks of embryonic development. Ovothiol and glutathione account for virtually all of the trichloroacetic acid soluble –SH groups in the egg; these results are compared to several previous studies. Ovothiol A was also isolated without prior alkylation; as previously reported for ovothiol C, it readily oxidizes in air to a disulfide dimer, and the reduced form confers NAD(P)H–O_2 oxidoreductase activity on ovoperoxidase, an enzyme exocytosed at fertilization. Several experimental observations make it unlikely that the ovoperoxidase/ovothiol oxidase activity accounts for the "respiratory burst" and H_2O_2 generation that follow fertilization in *S. purpuratus*; an alternate possibility is that ovothiol functions in the control of H_2O_2 toxicity.

Since the initial work by Rapkine (1931) in which the acid-soluble sulfhydryl groups of the sea urchin embryo were said to fluctuate with the cell cycle, the sulfhydryl contents of the echinoderm egg and developing embryo have been subjects of interest and controversy. Sakai and Dan (1959) showed that fluctuations in acid-soluble sulfhydryls in *Pseudocentrotus depressus* were probably not due to GSH but to acid-soluble proteins; Neufeld and Mazia (1957) obtained a similar result. This work suggested that GSH accounted for most of the non-protein –SH groups of the egg. Fahey et al. (1976), using more specific enzymic assays, also found GSH to be the predominant low molecular weight thiol in unfertilized eggs of *Strongylocentrotus purpuratus*, although an unidentified thiol was more abundant than GSH in *Lytechinus pictus*.

More recently the 4-mercaptohistidines, a new family of low molecular weight thiols, have been reported from echinoderm eggs. The compound 3-methyl-4-mercaptohistidine was reported present in 60-fold greater concentration than glutathione from eggs of *Paracentrotus lividus* (Palumbo et al., 1984). A related compound, 1-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine, or ovothiol, has been isolated from the eggs of *S. purpuratus*. Its intracellular concentration was estimated as several millimolar (Turner et al., 1986), comparable to previous estimates for egg GSH from the same species (Fahey et al., 1976). Unlike well-characterized mercaptohistidines such as ergothioneine (Jocelyn, 1972) that contain sulfur at the C-2 position, ovothiol readily undergoes oxidation by atmospheric oxygen or H_2O_2 to form a disulfide. Ovothiol has been shown to confer NAD(P)H–O_2 oxidoreductase activity on ovoperoxidase (Turner et al., 1985, 1986), a heme peroxidase exocytosed at fertilization that cross-links the extraembryonic protein coat (fertilization membrane) with dityrosyl cross-links (Deits et al., 1984; Foerder & Shapiro, 1977).

Here we report a useful new method for the isolation and identification of 4-mercaptohistidines by ion-pairing HPLC,[1] following S-carboxymethylation with iodoacetic acid. This technique is used to identify two previously unreported mercaptohistidine compounds from starfish and scallop that differ from the *S. purpuratus* compound in the extent of N^{α}-methylation. High levels of mercaptohistidine are shown to persist throughout early development of the sea urchin embryo, and mercaptohistidine and glutathione levels are compared in unfertilized eggs and developing embryos.

Ovothiol A, like ovothiol C, confers NAD(P)H–O_2 oxidoreductase activity on ovoperoxidase; this activity previously suggested a role for ovothiol in the "respiratory burst" of cyanide-insensitive oxygen consumption and H_2O_2 production that follow fertilization in *S. purpuratus* (Turner et al., 1985,

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; MFSW, Millipore-filtered seawater; NOE, nuclear Overhauser effect; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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1986). However, aminotriazole, a potent inhibitor of the ovoperoxidase oxidoreductase activity (Turner et al., 1986), is shown to have no effect on the respiratory burst. Also, eggs treated with iodoacetamide, which renders ovothiol redox inactive in vivo, are able to form and cross-link fertilization membranes. This evidence suggests that the ovoperoxidase/ovothiol system does not play a role in the burst respiration; a possible alternate role of ovothiol in the control of H_2O_2 toxicity is discussed.

Materials and Methods

NADH, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol, aminotriazole, iodoacetic acid, iodoacetamide, glutathione reductase, GSH, GSSG, 1-methylhistidine, 3-methylhistidine, and Dowex resin were obtained from Sigma. D_2O (99.96% D) was obtained from KOR Isotopes. [^{3}H]-Iodoacetic acid and [^{14}C]iodoacetamide were obtained from New England Nuclear. Other chemicals were of reagent grade or the highest grade commercially obtainable.

Specimens of *S. purpuratus* were gathered intertidally in the Strait of Juan de Fuca. Animals were spawned by intracoelomic injection of 0.5 M KCl. Other tissues were obtained by dissection of fresh animals; gut tissue was obtained from animals fasted approximately 30 days. For embryo culture 2.8×10^7 eggs (50 mL) were fertilized with 5×10^9 sperm in 2 L of Millipore-filtered seawater (MFSW). After 30 min, eggs were allowed to settle, and excess sperm were decanted. Embryos were then resuspended with gentle stirring and aeration in 20 L of MFSW containing 0.2 mM EDTA. Embryos were harvested by low-speed centrifugation at appropriate time points and immediately frozen at −80 °C. Specimens of the bay scallop *Chlamys hastata* were caught off the coast of Vancouver Island and purchased alive from a local source, and *Evasterias troschelii* were collected intertidally from Puget Sound. Tissues were dissected from fresh specimens with care to avoid contamination with gonadal material.

NAD(P)H–O_2 oxidoreductase activity was measured by following the oxidation of reduced pyridine nucleotides spectrophotometrically at 360 nm in a Gilford 420 spectrophotometer. The off-peak wavelength of 360 nm for reduced pyridine nucleotides allowed the use of higher concentrations in a 1.0-cm path length cuvette. Assays were conducted in a 1-mL volume containing 50 mM NaCl, 20 mM Hepes, and 20 mM Tris, at pH 8.0 and 25 °C, to which was added for routine assays 0.4 mM NADH, 0.5 mM $MnCl_2$, 1 mM NaCN, 134 nM ovoperoxidase, and the appropriate amount of ovothiol preparation. One unit of activity is defined as the amount of ovothiol needed to stimulate consumption of 1.0 nmol of NADH/min. The dissolved O_2 concentration of the buffer system at 20 °C was 305 μM. Assays were initiated with NADH unless otherwise specified. Ovothiol A disulfide and ovothiol C disulfide were quantitatively reduced for enzymatic studies by incubation for 10 min with a 2-fold molar excess of dithiothreitol, followed by separation on Dowex 50×8 ion-exchange resin, NH_4^+ form, under N_2 as previously described (Turner et al., 1986).

Glutathione assays were done by a modification of the glutathione reductase method of Griffith (1980) for total GSH plus GSSG. Sample or standard was added to a cuvette containing 100 mM sodium phosphate, 5 mM EDTA, 0.21 mM NADPH, 0.6 mM DTNB, and 0.5 unit of glutathione reductase at pH 7.5 and 30 °C in a final volume of 1 mL. Change in A_{412} due to the formation of 5-thio-2-nitrobenzoic acid was monitored and the rate of change between 1.0 and 2.0 OD calculated; optimal rates were obtained with 0.5–5 nmol of GSH. Samples were prepared by incubation of tissue homogenate with 1.0 mM DTT for 10 min to reduce mixed disulfides, followed by addition of 100% trichloroacetic acid to 5% final concentration and centrifugation in a microfuge for 3 min. Up to 50 μL of supernatant could be used in the assay without significantly lowering the pH. Internal and external standards of GSH were used to produce a standard curve for each set of assays; GSSG gave identical results. Inclusion of DTT in the sample preparation had no effect on the standard curve. Ovothiol did not react in the glutathione assay and did not interfere with the assay of glutathione standards when added at equimolar concentration.

NMR Spectroscopy. Proton NMR spectra were obtained at 500 MHz on a Bruker WM-500. All spectra were collected at ambient temperature. Specific acquisition parameters used are given in the figure legends. All chemical shifts were measured relative to an external standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). Des-*S*-ovothiol A was prepared by the Raney nickel method previously described for ovothiol C (Turner et al., 1986).

High-Pressure Liquid Chromatography. All HPLC was performed on a Beckman Model 334 liquid chromatograph. Ion-exchange HPLC was performed as previously described (Turner et al., 1986) with a Waters protein-pak SP-5PW column; a 30-cm Waters Bondapak C-18 column preceded the ion-exchange column and served to desalt the sample and provide adequate back pressure at a flow rate of 1.0 mL/min. Chromatograms were developed with a linear gradient of 0.05–2.0 M ammonium formate, pH 2.5 (2.0 M formic acid, adjusted to appropriate pH with ammonium hydroxide).

Ion-pairing HPLC utilized a 15-cm Altex ultrasphere-ODS C-18 column and standard precolumn (Beckman). Chromatograms were developed with a linear gradient of 0–50% methanol in a buffer system containing 20 mM phosphoric acid and 5 mM sodium heptanesulfonate, adjusted to pH 2.5 with ammonium hydroxide, at a flow rate of 1.6 mL/min.

Scintillation counting was performed in a Beckman LS-230 liquid scintillation counter. Samples in aqueous solution were mixed with H_2O to a total volume of 0.5 mL, followed by 8 mL of Aquasol. Counting efficiency for tritium in this system was 50%.

Assay of Ovothiols by S-Carboxymethylation and Preparation of Standards. Ovothiols A, B, and C were detected quantitatively by treating tissue homogenates with [^{3}H]iodoacetic acid, followed by isolation of the *S*-carboxymethyl derivatives of the ovothiols by Dowex 50 ion-exchange chromatography and HPLC. For routine assays, tissues were homogenized with 4 volumes of H_2O in a Virtis 45 tissue homogenizer and/or Teflon pestle homogenizer. One milliliter of tissue homogenate was adjusted to pH 8, mixed with 1 μmol of DTT, incubated 10 min, and then mixed with 2.5 μmol of [^{3}H]iodoacetic acid containing 2×10^6 cpm of tritium/μmol. For tissues such as egg and ovary that are especially rich in ovothiol, 0.25 mL of 20% homogenate was diluted to 1 mL before derivatization. After a 30-min incubation, samples were precipitated with 10% trichloroacetic acid and protein was pelleted by microcentrifugation for 3 min. The supernatant solution was applied to a disposable plastic column (Kontes) containing 1.0-mL packed bed volume of Dowex 50×8, 100–200 mesh, ammonium form. The column was preequilibrated in 0.5 M formic acid and washed with 10 mL of the same following sample application. The bound ovothiol was eluted with 10 mL of 0.5 M ammonium formate, pH 4.5 (0.5 M in NH_4^+, adjusted to pH 4.5 with formic acid, 88%), and the eluant was twice lyophilized from H_2O, then redissolved

in H_2O, and applied to the ion-pairing or ion-exchange HPLC systems described under High-Pressure Liquid Chromatography. Samples were mixed with 0.2 volume of 1 M phosphoric acid and 0.25 M sodium heptanesulfonate and adjusted to pH 2.5 with 6 N HCl prior to ion-pairing HPLC; the pH only was adjusted to the same value prior to ion-exchange HPLC. The isolation procedure consistently gave >90% recovery of ovothiol added prior to derivatization.

Standards of [^{3}H]-*S*-(carboxymethyl)ovothiols A and B were prepared essentially by scaling up the procedure above. For ovothiol B, 30 g of *C. hastata* ovarian tissue containing 2.3 g of protein was thoroughly homogenized with H_2O to a total volume of 120 mL and boiled for 10 min in a water bath. The protein precipitate was removed by centrifugation (25000*g*) for 30 min, and 75 mL of the clear supernatant was treated with 0.4 mM dithiothreitol for 10 min, followed by 2.5 mM [^{3}H]iodoacetic acid containing 1×10^6 cpm/μmol of tritium for 30 min. The sample was then acidified with 5% trichloroacetic acid and applied to a column containing 30-mL packed bed volumes of Dowex 50×8, 200–400 mesh, ammonium form, preequilibrated with 0.5 M formic acid. The column was washed with 300 mL of 0.1 M formic acid and eluted in batch with 0.5 M ammonium formate, pH 4.5. Fractions of 1 mL containing >100 000 cpm were pooled and twice lyophilized from H_2O. The eluant was redissolved in 1 mL, and aliquots of 150 μL were applied to ion-exchange HPLC as described above. The principal peaks of eluted radioactive material from several separations were pooled and relyophilized. Yield was approximately 4 μmol of *S*-(carboxymethyl)ovothiol B. The identity of the material was confirmed by ^{1}H NMR as described under Results. An essentially identical procedure applied to the ripe gonads of the starfish *E. troschelii* yielded a preparation of homogeneous *S*-(carboxymethyl)ovothiol A.

S-(Carboxymethyl)ovothiol C was prepared from material isolated in the underivatized disulfide form by solvent extraction, ion-exchange chromatography, and HPLC as previously described (Turner et al., 1986). Ten micromoles of ovothiol C disulfide was incubated with 30 μmol of dithiothreitol in 0.2 mL of 100 mM sodium bicarbonate for 20 min; subsequently 0.1 mL of 1 M [^{3}H]iodoacetic acid, 5×10^5 cpm/μmol, was added. After 30 min the mixture was acidified with 10% trichloroacetic acid and applied to 2 mL of Dowex 50×8, 100–200 mesh, ammonium form, which had been preequilibrated in 0.5 M formic acid. The column was washed with 10 mL of 0.5 M formic acid, and [^{3}H]-*S*-(carboxymethyl)ovothiol C eluted with 10 mL of 0.5 M ammonium formate, pH 4.5 (0.5 M in ammonium ion). Yield approached 100% (20 μmol) of the expected amount of product.

RESULTS

Isolation of S-(Carboxymethyl)ovothiols A, B, and C and Separation of Standards. Figure 1 shows the isolation of *S*-(carboxymethyl)ovothiol C from *S. purpuratus* egg homogenate by ion-pairing HPLC following iodoacetic acid derivatization and Dowex 50 chromatography as described under Materials and Methods. *S*-(carboxymethyl)ovothiol C accounts for the principal peak of radioactivity and UV absorbance eluted from the column. Figure 2 shows ion-pairing HPLC separation of 2.0 nmol each of *S*-(carboxymethyl)-ovothiols A, B, and C standards prepared as described under Materials and Methods. Base line resolution of the three methylation forms is achieved, with the elution order of ovothiol C, ovothiol B, and ovothiol A.

Underivatized ovothiol A disulfide was isolated as previously described for ovothiol C (Turner et al., 1986), with the exception that the final step of ion-exchange HPLC was replaced by repeat chromatography on Dowex 50, giving a product which showed only trace organic impurities on ^{1}H NMR. As with ovothiol C, air oxidation of the compound to the disulfide form during the purification was essentially complete. The disulfide was then reduced for enzymatic studies of its activity as an oxidase cofactor by treatment with dithiothreitol followed by Dowex 50 chromatography under N_2 as previously described (Turner et al., 1986). The oxidized and reduced ultraviolet spectra of ovothiol A showed characteristic differences similar to those previously reported for ovothiol C.

Structural Identification. The structure of ovothiol C has been previously elucidated by UV spectrophotometry, mass spectroscopy, and ^{13}C and ^{1}H NMR, including comparison by ^{1}H NMR of des-*S*-ovothiol C with commercially obtained 1-methyl- and 3-methyl-N^{α},N^{α}-dimethylhistidine (Turner et

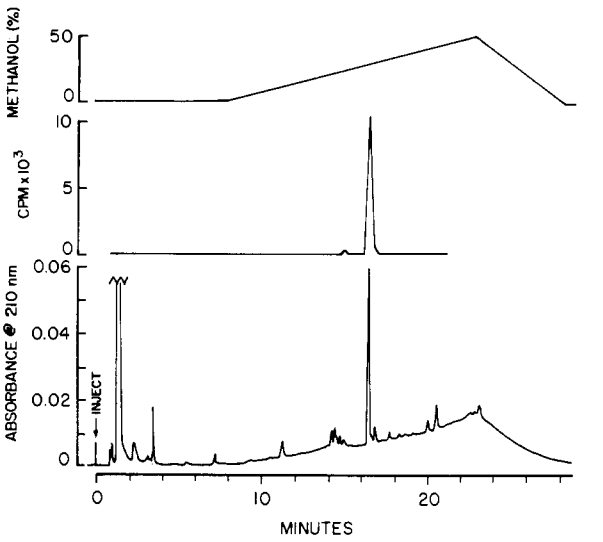

FIGURE 1: Ion-pairing HPLC separation of an egg extract from *S. purpuratus*. Egg homogenate was derivatized, fractionated on Dowex 50×8, applied to ion-pairing HPLC as described under Materials and Methods, and developed with a linear gradient of 0–50% methanol. The trace represents an extract of approximately 5×10^3 eggs.

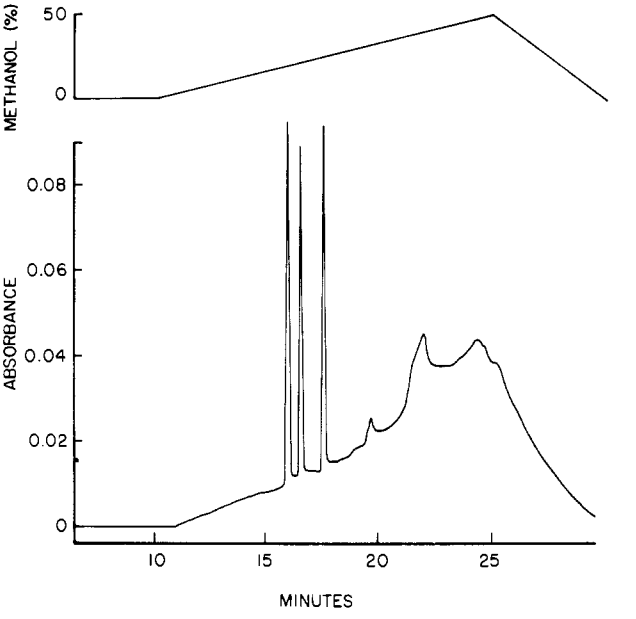

FIGURE 2: Ion-pairing HPLC separation of *S*-(carboxymethyl)ovothiols A, B, and C standards. Two nanomoles of each standard was applied in a total volume of 20 μL. Elution with a methanol gradient was performed as described under Materials and Methods and Figure 1.

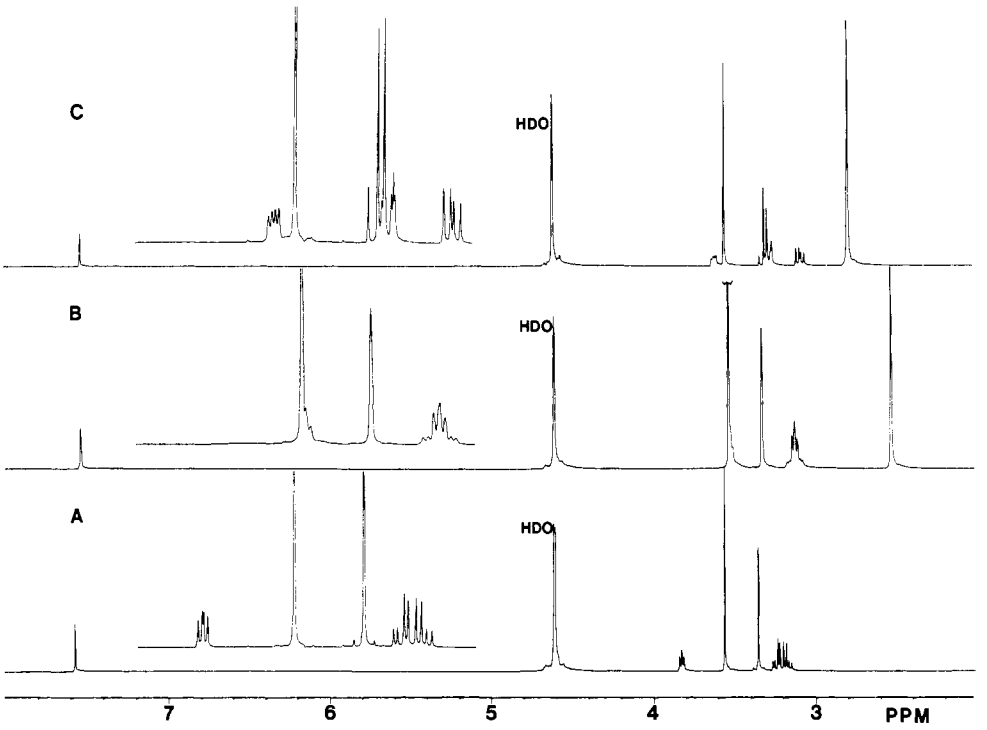

FIGURE 3: 500-MHz ^1H NMR spectra of *S*-(carboxymethyl)ovothiols C (top), B (middle), and A (bottom); inset detail is from 3 to 4 ppm. Spectra of D_2O solutions were obtained with a spectral width of 5000 Hz (10 ppm), acquisition time of 1.6 s, and a relaxation delay of 4 s. The pD, uncorrected, was 6.5–7.5 for all spectra.

al., 1986). Nuclear Overhauser effect (NOE) studies also confirmed the N-1 position as the site of imidazole ring methylation. Figure 3 shows the ^1H NMR spectra of the *S*-carboxymethyl derivatives of ovothiols A, B, and C at neutral pH. Comparison of the spectrum of derivatized ovothiol C with that of the underivatized molecule revealed that the carboxymethyl proton resonance appears at 3.3 ppm, overlapping with the downfield $C^{\beta}H$ resonance. Integration of the spectrum shown in Figure 3 (top) confirms this assignment, as the complex group of peaks at approximately 3.3 ppm contains the intensity of three protons. The other resonance assignments for ovothiol C are identical with those already published: imidazole C-2 H, 7.52 ppm; $C^{\alpha}H$, 3.59 ppm; $C^{\beta,\beta'}H$, 3.3, 3.06 ppm; N-1 CH_3, 3.53 ppm; N^{α}-$(CH_3)_2$, 2.77 ppm. The spectra of ovothiol A and ovothiol B are very similar to that of ovothiol C. The major differences in the spectra are in the upfield singlet peak. This appears as a six-proton resonance at 2.77 ppm in ovothiol C and as a three-proton resonance at 2.52 ppm in ovothiol B and is absent in the ovothiol A spectrum. Thus ovothiols C, B, and A represent dimethyl, monomethyl, and demethyl forms of the molecule. Resonance assignments for the ovothiol A and B spectra could be made by comparison with the ovothiol C spectrum, although nuclear Overhauser experiments were also performed to confirm the assignments and to determine the position of the ring *N*-methyl group (described below). The assignments for ovothiol A are imidazole C-2 H, 7.56 ppm; $C^{\alpha}H$, 3.81 ppm; ring N-CH_3, 3.55 ppm; S-CH_2, 3.34 ppm; $C^{\beta,\beta'}H$, 3.2 ppm. The assignments for ovothiol B are imidazole C-2 H, 7.53 ppm; ring N-CH_3, 3.52 ppm; $C^{\alpha}H$, 3.5 ppm; S-CH_2, 3.31 ppm; $C^{\beta,\beta'}H$, 3.11 ppm; N^{α}-CH_3, 2.52 ppm. Proposed structures of the three forms of ovothiol based on these data appear in Figure 4.

The coupling patterns observed for the S-CH_2 resonances in all three spectra and for the $C^{\beta}H$, $C^{\beta'}H$ resonances of ovothiols A and B are not those predicted by first-order analysis (i.e., two doublet resonances for the carboxymethyl

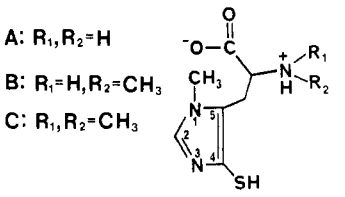

FIGURE 4: Structure of ovothiols A, B, and C, reduced form.

protons and two doublet-of-doublet peaks for the C^{β} protons). The patterns observed occur under conditions where $(\nu_A - \nu_B)/J$ is small, where $\nu_A - \nu_B$ is the difference in chemical shift for the two coupled protons and J is the coupling constant between them.

Further conformation of the structure of ovothiol A was obtained by removal of sulfur from the compound by the Raney nickel catalyst as previously reported for ovothiol C (Turner et al., 1986), followed by comparison of the 500-MHz NMR spectrum of the des-*S*-ovothiol A product with samples of authentic 1-methyl- and 3-methylhistidine. Upon mixing with 3-methylhistidine, two peaks were clearly resolved for the *N*-methyl and two aromatic protons. When des-*S*-ovothiol A was mixed with 1-methylhistidine, only single peaks were observed, indicating that the compounds were identical. Replacement of sulfur with hydrogen by Raney nickel also confirmed C-4 as the site of the sulfur atom in ovothiol A. In des-*S*-ovothiol A a new aromatic proton appeared at 6.75 ppm, upfield from the existing proton at 7.5 ppm; based on the chemical shifts of the aromatic protons of the parent compound imidazole, this is consistent only with an original proton located at C-2 and a sulfur at C-4.

Because an authentic standard for des-*S*-ovothiol B was not available, nuclear Overhauser effect (NOE) studies were performed on *S*-(carboxymethyl)ovothiol B. Reference spectra for NOE were taken at pD 12 (Figure 5), which allowed resolution of the α-methine proton and N-1 methyl groups that overlapped in the neutral pD spectrum (Figure 3, middle). NOE experiments were run as automatic difference spectra,

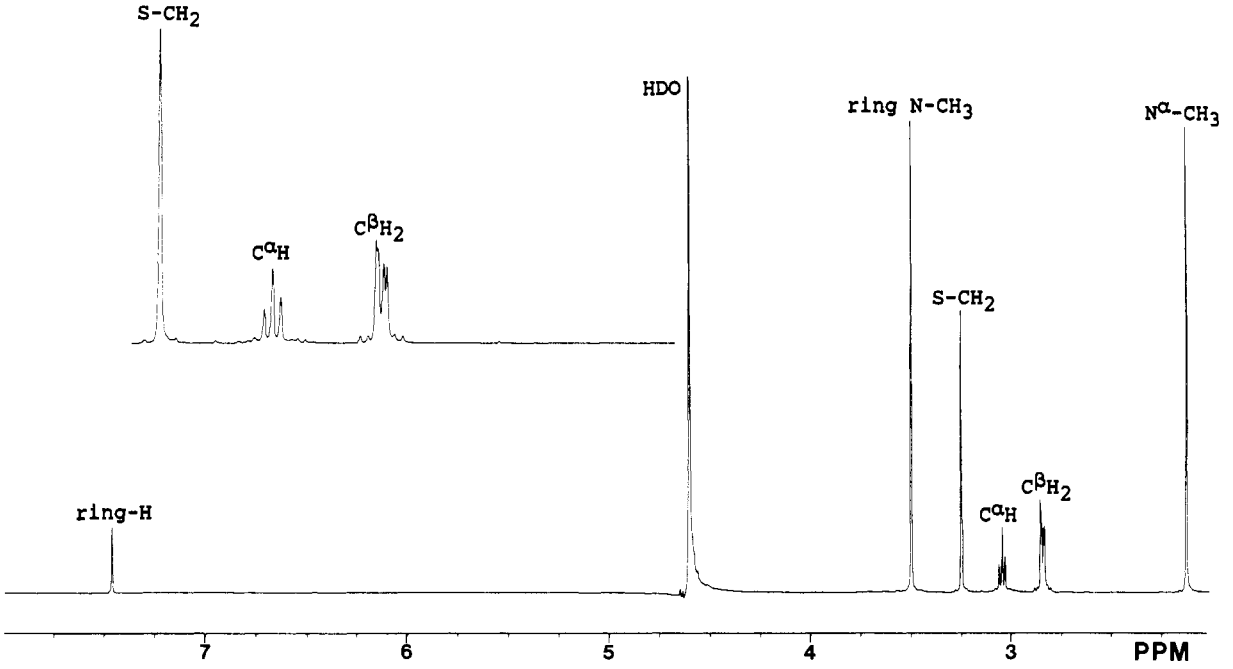

FIGURE 5: ^{1}H NMR spectrum of *S*-(carboxymethyl)ovothiol B. This reference spectrum for NOE was taken at a pD of approximately 12. Inset detail is from 2.3 to 3.3 ppm. Acquisition parameters are the same as in Figure 3.

Table I: Ovothiol Content of Invertebrate Tissues

species/tissue	ovothiol (nmol/mg of protein)[a] C	B	A	other[b]
S. purpuratus (urchin)				
egg	28.5	<0.2	<0.2	
ovary	19.2	1.6	<0.2	
sperm	0.16	0.11	<0.01	
gut (female)	0.05	0.35	0.06	0.64
C. hastata (scallop)				
ovary	<0.01	4.04	1.03	
testis	<0.01	0.1	0.05	
mantle (female)	<0.01	0.015	0.016	
adductor (female)	<0.01	0.03	0.08	
E. troschelii (starfish)				
ovary	<0.1	<0.1	24.3	
testis	0.2	<0.1	4.1	
gut (female)	0.3	<0.1	5.7	
tube feet (female)	3.2	<0.1	<0.1	0.52

[a] The principal form of ovothiol in the eggs of *S. purpuratus* and ovaries of *C. hastata* and *E. troschelii* (underlined) was identified by ^{1}H NMR as described under Results; other forms were identified by reactivity with iodoacetic acid, coisolation on Dowex 50, and comigration with authentic internal standards on ion-pairing HPLC, as described under Materials and Methods. [b] Compounds that labeled with iodoacetic acid and migrated near but not exactly with known ovothiols.

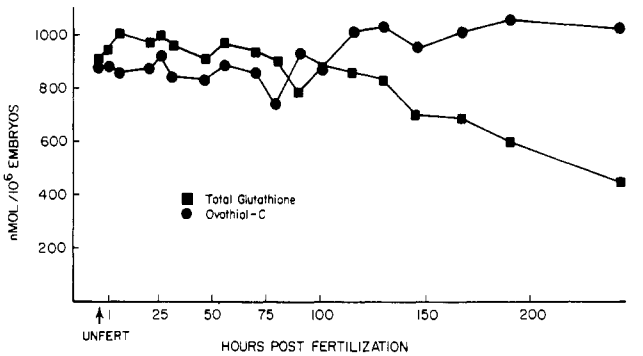

FIGURE 6: Total ovothiol C and glutathione content in early development. Symbols: ●, ovothiol C; ■, glutathione (GSH + GSSG). Embryos of *S. purpuratus* were grown as described under Materials and Methods. Time zero represents unfertilized eggs. Hatching and gastrulation were synchronous within ±10% of elapsed time in >95% of embryos. Embryos were not fed, and viability (swimming) decreased significantly after 168 h (1 week) although the embryos remained intact. A significantly lower value for ovothiol content at 70 h (600 nmol/10^6 embryos) due to error in sample size was normalized to the protein content of adjacent samples. Concentrations are total reduced monomer equivalent for both compounds. Ovothiol C was assayed by S-carboxymethylation and ion-exchange HPLC and glutathione by the glutathione reductase assay as described under Materials and Methods, after reduction of the homogenate by dithiothreitol.

with a cycle of eight on-resonance and eight off-resonance scans. Referring to Figure 5, ring H represents a single aromatic proton, ring N-CH_3 a methyl linked to imidazole nitrogen, S-CH_2 the methylene of the *S*-carboxymethyl group, $C^\alpha H$ the C^α proton, $C^\beta H_2$ the β-methylene group, and N^α-CH_3 the α-aminomethyl group. In NOE studies, only the ring N-CH_3 and S-CH_2 were irradiated. The imidazole methyl gave an NOE to peaks ring H, $C^\alpha H$, and $C^\beta H_2$; no NOE was seen from the *S*-carboxymethyl protons. The position of the imidazole ring methyl group was thus confirmed as N-1 by the observed NOE from the ring methyl group to the α-methine and β-methylene protons. A methyl group at N-3 would not be expected to give these NOEs. The absence of NOE from the sulfur-linked methylene group resulting from S-carboxymethylation again confirmed that the ring methyl group is linked to N-1, not adjacent to sulfur at N-3.

Mercaptohistidine Content of Various Tissues. Table I shows the content of ovothiols A, B, and C in various tissues of *S. purpuratus*, *E. troschelii*, and *C. hastata*. Levels were determined by gradient ion-pairing HPLC of [^{3}H]-*S*-carboxymethyl derivatives and comparison with internal standards of authentic *S*-(carboxymethyl)ovothiols prepared described under Materials and Methods; quantitative analysis was based on the known specific activity of the iodoacetic acid labeling reagent.

Figure 6 shows the persistence of ovothiol C throughout early development in the sea urchin. Total ovothiol remained virtually constant on a per embryo basis; glutathione levels declined somewhat after about 1 week, although this may be related to nutrient deprivation and declining viability of the embryos. Total glutathione was measured after reduction by 1 mM dithiothreitol, which did not affect the assay of internal

GSH standards or GSH alone. The glutathione concentration of unfertilized eggs of *S. purpuratus* as measured by the glutathione reductase assay (Materials and Methods) is approximately 0.9 μmol/10^6 eggs (range 0.78–0.95); the ovothiol content is 1.14 μmol/10^6 eggs (range 0.9–1.2). Together these compounds accounted for essentially all of the trichloroacetic acid soluble sulfhydryl groups present (2.05 μmol/10^6 eggs) as determined by assay with Ellman's reagent (Materials and Methods).

NAD(P)H–O_2 Oxidoreductase Activity of Ovoperoxidase. Ovothiol A disulfide was isolated and reduced to the sulfhydryl form as described above. Under standard assay conditions (Materials and Methods) 35 μM ovothiol A gave 610 nmol/min NADH consumption in the absence of cyanide and 600 nmol/min in the presence of 1 mM cyanide. Replacement of $MnCl_2$ with 1 mM EGTA in the assay buffer and 175 μM ovothiol A gave 38 nmol/min NADH consumed. These results are very similar to those previously observed for ovothiol C (Turner et al., 1986).

In Vivo Derivatization of Ovothiol. Ovothiol C can be derivatized in vivo by incubation of unfertilized eggs in [^{14}C]iodoacetamide, a permeant analogue of the derivatizing reagent used in routine assays. Following derivatization in vivo, the egg functions of fertilization membrane elevation and hardening, respiration, and cleavage were assessed. In these experiments, 5 mL of egg suspension containing 3.4 × 10^5 eggs/mL was incubated 20 min with a given concentration of nonradioactive iodoacetamide in MFSW seawater containing 20 mM Tris, pH 8.0. The eggs were then washed twice, resuspended in the same volume, and used in the appropriate experiment. To determine the remaining underivatized ovothiol, 0.5-mL aliquots were brought to 5 mM with [^{14}C]iodoacetamide, 500 cpm/nmol, and incubated with frequent vortex mixing. After 1 h, samples were precipitated with 10% trichloroacetic acid and vortexed 1 min, and the supernatant after microcentrifugation was applied to a 1-mL Dowex 50 column as described under Materials and Methods. These conditions were shown to give incorporation of label equivalent to the homogenization with dithiothreitol and derivatization with iodoacetic acid described under Materials and Methods. Vortexing in TCA effectively extracted the derivatized ovothiol from whole eggs. The counts incorporated in the second derivatization were used to calculate the amount of ovothiol C not initially derivatized with cold reagent; the percent remaining ovothiol equals (experimental cpm/control cpm) × 100, where the control represents zero cold iodoacetamide in the initial derivatization. Ovothiol C carboxymethylamide was not subsequently isolated by HPLC in these experiments. However, as ovothiol derivatives routinely account for more than 90% of the counts eluted from Dowex 50 (e.g., see Figure 1), the Dowex step can be used alone to estimate label incorporation into ovothiol (higher pH, a greater excess of alkylating agent and longer incubation were required to achieve significant incorporation into GSH).

As shown in Table II, incubation in 1.0 mM iodoacetamide derivatized about 50% and 4.0 mM iodoacetamide >90% of the ovothiol present. High concentrations (>4.0 mM) of reagent had a generally deleterious effect on fertilization and egg morphology. At 4.0 mM iodoacetamide or less, virtually all of the eggs that elevated fertilization membranes also cross-linked or hardened them, as assayed by resistance to DTT solubilization (Foerder & Shapiro, 1977). Thus, derivatization of ovothiol per se does not inhibit the cross-linking reaction. The ovoperoxidase inhibitor aminotriazole prevents fertilization membrane hardening (Foerder & Shapiro, 1977) and is used in one control to demonstrate the effectiveness of the membrane removal procedure. Iodoacetamide at 1.0 mM or greater, corresponding to derivatization of over half of the ovothiol, blocked egg cleavage; similar effects have been seen previously after altering the thiols of eggs (Nath & Rebhun, 1976).

Table II: Fertilization Membrane Elevation and Hardening and Division after Iodoacetamide Derivatization

iodoacetamide (mM)	% underivatized ovothiol	% FM elevation (% DTT insoluble)[a]	% first cleavage[b]
control	100[c]	100 (100)	100
control + 1 mM aminotriazole		53 (0)	
0.5	77	100 (100)	98
1.0	53	100 (100)	72
2.0	31	93 (83)	0
3.0	17	93 (90)	0
4.0	8	48 (39)	0
10.0	0.6	3 (0)[d]	0[d]

[a] 3.4 × 10^5 eggs were fertilized with 1 μL of packed sperm. After 10 min, eggs were scored for fertilization membrane (FM) elevation and DTT was added to 10 mM. At 45 min postinsemination, eggs were vortexed and the fraction of intact membranes counted again. Aminotriazole was added 10 s postinsemination. [b] Division was scored 5 h after sperm addition, and embryos with two cells or more counted as positive. Data are expressed as percent of control eggs with fertilization membrane elevation. [c] Control value was 1.56 × 10^5 cpm bound to Dowex 50. [d] Extensive egg lysis and aggregation.

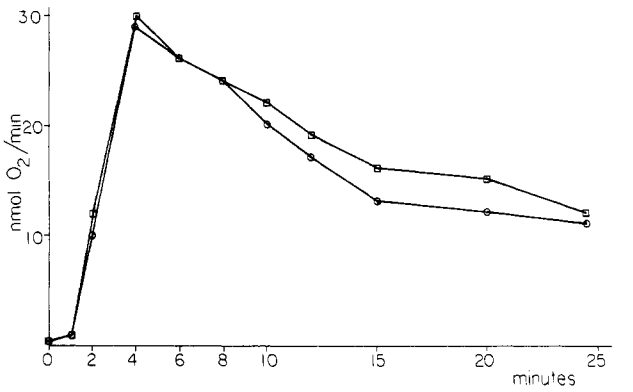

FIGURE 7: Respiration after fertilization in eggs of *S. purpuratus*. Respiration was measured in a Clark O_2 electrode as previously described (Turner et al., 1985). Rates represent 1.4 × 10^5 eggs in a final volume of 5 mL of Millipore-filtered seawater, with (■) and without (●) 10 mM aminotriazole. Fertilization was with 5 × 10^7 sperm diluted to a 1% suspension prior to addition to the electrode. Parthenogenic activation with 80 μg of ionophore A23187 in 20 μL of dimethyl sulfoxide gave similar results (data not shown).

Egg Respiration following Fertilization. Because the ovothiol-dependent NAD(P)H–O_2 oxidoreductase activity of ovoperoxidase has been suggested as a candidate for the enzyme responsible for the respiratory burst following fertilization (Turner et al., 1986), we tested egg respiration in the presence of aminotriazole, a potent inhibitor of the ovoperoxidase oxidase activity. As shown in Figure 7, aminotriazole had little effect on the respiratory burst following fertilization.

Egg respiration following fertilization was also measured in iodoacetamide-treated eggs. Eggs treated under the conditions described above with 3.0 mM iodoacetamide or less displayed respiration essentially identical with that shown in Figure 7 for untreated eggs. Higher iodoacetamide concentrations produced eggs too fragile to be effectively assayed in the oxygen electrode.

DISCUSSION

The method of isolation and detection of 4-mercaptohistidines reported here, using S-carboxymethylation and

ion-pairing HPLC, represents a significant improvement over isolation of the native material by ion-exchange HPLC as previously described (Turner et al., 1986). Because the *S*-carboxymethyl derivatives of the 4-mercaptohistidines are not redox labile, the problem of separation of multiple oxidation states (dimer vs. monomer) does not complicate the isolation procedure. Quantities in the range of 0.1–1.0 nmol are detectable by UV absorbance at 210 nm, and quantitation is facilitated by incorporation of a label of known specific activity.

This technique has allowed the isolation of two new 4-mercaptohistidine compounds: from the starfish *E. troschelii*, 1-methyl-4-mercaptohistidine or ovothiol A, and from the bay scallop *C. hastata*, 1-methyl-N^{α},N^{α}-methyl-4-mercaptohistidine or ovothiol B. These differ from the 4-mercaptohistidine previously reported by our group from *S. purpuratus*, 1-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine or ovothiol C, only in the extent of methylation of the α-amino nitrogen. In the case of ovothiol A (this paper) and the case of ovothiol C (Turner et al., 1986), the identities of the compounds, particularly with regard to the position of the ring methyl group, have been clearly confirmed by comparison with authentic compounds. The identity of the commercial standards was in turn confirmed by ^{1}H NMR and NOE (Turner et al. 1986). In ovothiol B such a direct comparison was not made, but NOE results are consistent only with methylation at N-1 of the imidazole ring in this compound as well. Two related mercaptohistidine compounds from echinoderms have been previously reported by Palumbo et al. (1984), which in the system of nomenclature used in this paper would be designated 3-methyl-4-mercaptohistidine and 3-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine, respectively. We have compared a sample of the latter compound provided by Dr. Palumbo to ovothiol C from *S. purpuratus*, using ^{1}H NMR at 500 MHz (Materials and Methods). In binary mixtures of the two compounds single peaks appear in all locations (data not shown). Because this technique has clearly resolved positional isomers of the methylhistidines before [Turner et al. (1986) and this paper], it demonstrates that the compounds are identical and resolves the question of the imidazole ring locus of methylation in these thiolhistidines, showing it to be at the 1-position. Similarly, the monomethyl compound previously reported is probably identical with ovothiol A. The incorrect assignment of the ring methyl group apparently arose from confusion regarding the nomenclature of substituted histidines (see Appendix). A 4-mercaptohistidine with a single α-aminomethyl group such as ovothiol B has not been previously reported.

Pathirana and Andersen (1986) have recently described imbricatine, a benzyltetrahydroisoquinoline alkaloid from the asteroid *Dermasterias imbricata* that contains a mercaptohistidine moiety linked via a thioether bond to an isoquinoline nucleus. The mercaptohistidine would be designated 1-methyl-4-mercaptohistidine (ovothiol A) by our nomenclature, the form of ovothiol we have found to predominate in asteroids. These results offer further confirmation of the N-1 position as the site of ring methylation in these compounds.

The ovothiol C content of *S. purpuratus* eggs was previously estimated as 1.8 pmol/egg, on the basis of preparative isolation of the compound and an erroneous value of 94 mg of protein/10^6 eggs (Turner et al., 1986). A better value for egg protein content in this species is 40 mg of protein/10^6 eggs (Guidice, 1973), which agrees with our experimental determinations by the method of Lowry (1951). Thus 28.5 nmol of ovothiol C/mg of protein corresponds to 1.14 pmol/egg. With an egg diameter of 80 μm, this would give 4.3 mM average concentration in the interior of the egg. As previously noted (Turner et al., 1986), the concentration of ovothiol in the egg is greater than that of NADH or ATP and approximately twice the amount of H_2O_2 produced per egg following fertilization (Foerder et al., 1978). The 4-mercaptohistidine content reported here is comparable to total mercaptohistidine levels ranging from 8.5 to 22 nmol/mg of protein found in five asteroid and echinoid species by Palumbo et al. (1984).

The glutathione and total sulfhydryl content of the developing sea urchin embryo and the oxidation state of these sulfhydryl compounds have been subjects of considerable previous research. Rapkine (1931) described major fluctuations following fertilization in the non-protein sulfhydryl content of embryos of the sea urchin *Paracentrotus lividus*, including a drop halfway through the cell cycle and a rise preceding first division. He demonstrated that $HgCl_2$ blocked division and that this effect was partially reversed by exogenous –SH-containing compounds. The importance of free sulfhydryl groups has been subsequently confirmed by the inhibitory effect of diamide, which oxidizes glutathione in vivo, on cell division in embryos (Nath & Rebhun, 1976). Neufeld and Mazia (1957) demonstrated that non-protein sulfhydryls did not appear to fluctuate with the cell cycle, and Sakai and Dan (1959) showed that the cyclic changes in Rapkine's report may have been due to fluctuations in protein sulfhydryls that remained soluble under Rapkine's original conditions. All of these early studies suffered from lack of specificity in GSH measurement, using the nitroprusside reaction or iodimetry for quantitation of non-protein –SH. In *Pseudocentrotus depressus* and *Clypeaster japonicus* Sakai and Dan found 2.38 μmol/10^6 eggs and 3.32 μmol/10^6 eggs of non-protein sulfhydryl ("GSH"), respectively. By use of published figures for the diameter of the larger eggs of the Japanese urchins (Czihak, 1975, p 28), this concentration yields values for intracellular non-protein –SH concentration of 5.3 and 4.8 mM, which lie between our values for GSH (3.3 mM) and total TCA-soluble –SH (7.6 mM) in *S. purpuratus*.

Fahey et al. (1976) applied modern enzymic glutathione assays to these questions and confirmed in *L. pictus* and *S. purpuratus* that glutathione exists almost entirely in the reduced state before and after fertilization, without cyclic fluctuation. The GSH content of unfertilized *S. purpuratus* eggs obtained in their study was 14.0 μmol/g dry weight, equivalent to 0.89 μmol/10^6 embryos by using a hydration factor of 4 and published data for the nitrogen and protein content of these eggs (Czihak, 1975, p 610); this value agrees closely with our results. Fahey found that the total acid-soluble thiol levels exceeded the GSH content by as much as a factor of 3 in *Lytechinus* but by only about 20% in *S. purpuratus*; the excess thiol was not analyzed further.

Although the 4-mercaptohistidine levels determined by Palumbo et al. (1984) correspond closely to those in this study, the authors report GSH levels 60-fold lower than mercaptohistidine in *Paracentrotus lividus* eggs as determined by a several-step extraction procedure followed by amino acid analysis. This is far below any other published figure for egg GSH content. In general, a specific enzymic assay employing glutathione reductase (Material and Methods) is probably the best way to assay glutathione in eggs, as ovothiol C showed no reactivity in the assay and did not interfere with the assay of glutathione standards when added in equimolar amounts.

No attempt was made to determine the redox state of ovothiol or glutathione in this study. The total amounts of both compounds present in the unfertilized egg persist for the first week of development in fasted *S. purpuratus* embryos

without significant change (Figure 6). Glutathione content declines after 1 week, but this may be due to exhaustion of energy reserves of the unfed embryos and declining viability.

The specific tissue distribution of the ovothiols is striking. In all three species studied, ovothiol was far more abundant in the egg or female gonad than in any other tissue; it was low to absent in sperm and testis. The identity of the compounds in nonovarian tissues must be regarded as tentative, as these compounds were identified only by comigration with internal ovothiol standards in HPLC, but in every case it is clear that ovothiol is present in these tissues at low levels, if at all. It is also clear that although high average levels of ovothiol persist in the first 2 weeks of embryonic development in *S. purpuratus*, at some developmental stage the decision is made to cease accumulation of the compound in somatic tissues.

Equally remarkable is the species specificity of these compounds; at least 80% of the total mercaptohistidine accumulated in the egg or ovary of a given species was the most abundant methylation form, i.e., 1-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine in *S. purpuratus* (ovothiol C), the N^{α}-monomethylated form in *C. hastata* (ovothiol B), and the free α-amino form (ovothiol A) in *E. troschelii*. Similarly, Palumbo et al. (1984) observed that at least 80% of the 4-thiolhistidine accumulated was the principal methylation form in the six echinoderm species they examined. It is not known if these species differences represent specific synthesis, preferential accumulation, or differences in dietary intake.

The biological role of 4-mercaptohistidines is still unclear, but the remarkably and specifically high concentration in eggs suggests some central role in egg metabolism or early development. Among the most striking metabolic events following fertilization are the respiratory burst of cyanide-insensitive O_2 consumption and H_2O_2 production with concomitant H_2O_2-dependent dityrosyl cross-linking of the extracellular protein coat or fertilization membrane of the egg by ovoperoxidase (Foerder & Shapiro, 1977; Foerder et al., 1978). The previously demonstrated ability of ovothiol C to confer cyanide-insensitive NAD(P)H–O_2 oxidoreductase activity on the ovoperoxidase has been confirmed in this study for ovothiol A as well but is not a property of other known biological thiols (Turner et al., 1986). Initially, this suggested a role for ovothiol in this burst of oxygen consumption, with ovoperoxidase participating in production as well as consumption of hydrogen peroxide (Turner et al., 1985, 1986). Data presented here, however, make a role for ovothiol and the ovothiol-dependent oxidoreductase activity of ovoperoxidase in the respiratory burst much less likely. First, derivatization of >90% of the ovothiol with iodoacetamide, rendering it redox inactive, did not interfere with fertilization membrane cross-linking (Table II) or the respiratory burst (data not shown). Second, egg respiration after fertilization was unchanged in the presence of 10 mM aminotriazole (Figure 7), a potent inhibitor of the peroxidase (Deits et al., 1977) and oxidase (Turner et al., 1986) activities of ovoperoxidase. Finally, during the time period of the respiratory burst, the ovoperoxidase is extracellular, and we have no evidence that ovothiol is released from the egg to allow interaction with the enzyme at the relevant time.

The significant nonenzymic rate of reaction between reduced ovothiol C and H_2O_2 (Turner et al., 1986) suggests the alternate hypothesis that ovothiol may function not in the production of H_2O_2 but in its detoxification. In using H_2O_2 as an oxidative metabolite for cross-linking its extracellular coat, the egg risks potential genetic and cytoplasmic damage at the beginning of its developmental program. Whether ovothiol acts as a significant component of the protective mechanism of the egg, relative to other possible systems of activated oxygen detoxification (catalase, superoxide dismutase, glutathione peroxidase), remains to be seen.

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Appendix

Nomenclature of Substituted Mercaptohistidines. The studies reported here and previously (Turner et al., 1986), as well as complete synthesis (P. Hopkins, personal communication), have unambiguously determined the structure of the ovothiols from marine invertebrates to correspond to the diagram shown in Figure 4. However, conflicting systems of nomenclature have confused the identity of these compounds.

Two systems of nomenclature for the imidazole ring of substituted histidines have been used historically (IUPAC, 1975), with biochemists generally designating the side chain as C-5 and the adjacent nitrogen as N-1 (Figure 4) and organic chemists designating the side chain as C-4 and the adjacent nitrogen as N-3. A third system has been suggested by IUPAC (1975) in which the nitrogen proximal to the side chain is designated π and that distal to the side chain τ, with the organic chemistry notation for the other positions (i.e., side chain at C-4), but this system has not been widely used. This confusion extends even to commercially supplied methylhistidines; for instance, 1-methylhistidine and 3-methylhistidine supplied by Sigma Chemical Co. are designated according to the system of nomenclature used in Figure 4 (corresponding to π-methyl- and τ-methylhistidine, respectively), while "1-methyl-L-histidine" supplied by Aldrich Co. is actually τ-methylhistidine and would be designated 3-methylhistidine by the system of nomenclature used in Figure 4. This problem in nomenclature apparently led to error in the assignment of the position of the ring methyl group of these mercaptohistidines to the τ position by Palumbo et al. (1982, 1984). They described Raney nickel desulfuration of a mercaptohistidine from starfish as "affording 1-methyl-L-histidine ... identified by comparison with an authentic sample" (Palumbo et al., 1982), which they apparently interpreted using the organic chemistry nomenclature and thus assigned the methyl group in the starfish compound to the τ-position. As noted in the text, we have shown that ovothiol C identified in our laboratory is identical with the N^{α}-dimethyl compound supplied by Dr. Palumbo and that the 1-methyl (i.e., π-methyl) position is the correct assignment (Figure 4).

We have chosen to use consistently the system designating the π-nitrogen as N-1 and side chain as C-5 because of the longstanding biochemical usage of this convention.

Registry No. GSH, 70-18-8; NAD(P)H–O_2 oxidoreductase, 9032-22-8; ovothiol B, 108418-14-0; ovothiol A, 108418-13-9; ovothiol C, 105496-34-2; iodoacetic acid, 64-69-7; *S*-(carboxymethyl)ovothiol A, 108418-15-1; *S*-(carboxymethyl)ovothiol B, 108418-16-2; *S*-(carboxymethyl)ovothiol C, 108418-17-3; ovothiol A disulfide, 73491-33-5; des-*S*-ovothiol A, 368-16-1; ovoperoxidase, 9003-99-0.

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Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing *dl*-Methyl Anteisobranched Fatty Acids. 1. Differential Scanning Calorimetric and ^{31}P NMR Spectroscopic Studies†

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ABSTRACT: The thermotropic phase behavior of aqueous dispersions of nine *dl*-methyl branched anteisoacylphosphatidylcholines was studied by differential scanning calorimetry and ^{31}P nuclear magnetic resonance spectroscopy. The calorimetric studies demonstrate that these compounds all exhibit a complex phase behavior, consisting of at least two minor, low-enthalpy, gel-state transitions which occur at temperatures just prior to the onset of the gel/liquid-crystalline phase transition. In addition, at still lower temperatures, anteisobranched phosphatidylcholines containing fatty acyl chains with an odd number of carbon atoms show a major, higher enthalpy, gel-state transition, which was assigned to a conversion from a condensed to a more loosely packed gel phase. No such transition was observed for the even-numbered compounds in aqueous dispersion, but when dispersed in aqueous ethylene glycol, a major gel-state transition is clearly discernible for two of the even-numbered phospholipids. The major gel-state transition exhibits heating and cooling hysteresis and is fairly sensitive to the composition of the bulk aqueous phase. ^{31}P NMR spectroscopic studies indicate that the major gel-state transition is accompanied by a considerable change in the mobility of the phosphate head group and that, at temperatures just prior to the onset of the gel/liquid-crystalline phase transition, the mobility of the phosphate head group is comparable to that normally exhibited by the liquid-crystalline state of most other phospholipids. The temperatures at which the gel/liquid-crystalline phase transition occurs and the enthalpy change associated with this process are considerably lower than those of the saturated *n*-acyl-PC's of comparable acyl chain length. The calorimetric and ^{31}P nuclear magnetic resonance data thus indicate that, although the methyl anteisobranched phosphatidylcholines can form a fairly condensed gel state at sufficiently low temperatures, these phospholipids normally adopt a more loosely packed gel state, and possibly a more ordered liquid-crystalline state, than do phosphatidylcholines containing linear saturated fatty acids of comparable chain length.

Fatty acids containing a single methyl branch near the methyl terminus of the hydrocarbon chain are abundant and wide-spread constituents of the membrane lipids of a number of genera of eubacteria. In those microorganisms the methyl iso- and anteisobranched fatty acids usually account for 65–95% of the acyl chains in the membrane lipids, and they are believed to be the functional equivalents of the linear saturated and unsaturated fatty acids, respectively, of eucaryotic cell membranes [for a review and discussion, see Kaneda (1977), Kannenberg et al. (1983), and Lewis and McElhaney (1985a)]. In addition to being abundant and widely distributed in a variety of microorganisms, these branched-chain fatty acids can also support the growth of several procaryotic microorganisms in which they do not

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