Molecular Variants of *Golfingia gouldii* Hemerythrin. The Primary Structure of the Variants Arising from Five Amino Acid Interchanges*

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ABSTRACT: Monomeric hemerythrin from the coelom of the sipunculid *Golfingia gouldii* has been resolved into two components by disc electrophoresis and DEAE-cellulose chromatography. These hemerythrins differ in amino acid composition. The major species which comprises 80–85% of the hemerythrin in pooled blood and 50–100% in individual animals has the amino acid composition and sequence reported previously for *G. gouldii* hemerythrin (Klippenstein, G. L., Holleman, J. W., and Klotz, I. M., *Biochemistry 7*, 3868 (1968)). This major species actually consists of four variant proteins. The minor component differs in its content of histidine, aspartic acid, glutamic acid, threonine, serine, glycine, and alanine. These differences have been characterized as amino acid interchanges involving substitution of glutamic acid for glutamine at residue 63, aspartic acid for glutamic acid at residue 78, glycine for threonine at residue 79, asparagine for histidine at residue 82, and alanine for serine at residue 96. In spite of these substitutions, the ultraviolet and visible spectra of both hemerythrins are virtually identical indicating that the iron-binding site is unchanged.

The structure of hemerythrin, the nonheme iron respiratory protein of sipunculids, priapulids, and some brachiopods and annelids is of considerable interest in connection with studies of the chemistry of reversible oxygen binding. The most extensively characterized of the hemerythrins, that of the sipunculid *Golfingia gouldii*, is a 108,000 molecular weight octamer arising from the association of 13,500 molecular weight subunits (Klotz and Keresztes-Nagy, 1962, 1963). The identical subunits consist of 113 amino acid residues, the sequence of which is known (Klippenstein *et al.*, 1968). Each monomer contains two iron atoms and can combine with one molecule of oxygen (Klotz and Klotz, 1955; Klotz *et al.*, 1957). The iron atoms are bound directly to the protein molecule, presumably through specific amino acid side chains. Histidine (Fan and York, 1969) and tyrosine (Rill and Klotz, 1970; York and Fan, 1971) residues have been implicated as iron ligands.

It has been known for some time that there are differences in electrophoretic behavior and in peptide maps of hemerythrin from individual animals of the species *G. gouldii*. Manwell (1963) found individuals with electrophoretically "fast" and "slow" classes of hemerythrin and demonstrated corresponding differences in peptide maps. Groskopf *et al.* (1963) also observed two types of peptide maps among individual animals. Amino acid sequence analysis on pooled preparations of hemerythrin (Klippenstein *et al.*, 1968) subsequently revealed two sites of amino acid interchange in the polypeptide chain, the substitution of a threonine for a glycine at residue 79 and of a serine for an alanine at residue 96. These results, however, did not account for the electrophoretic variation observed by Manwell since both interchanges involve only neutral amino acids. Therefore, an additional site or sites of amino acid interchange must occur resulting in the observed change in the net charge on the protein molecule.

The present study has been undertaken to identify the site or sites of amino acid interchange in hemerythrin and to examine the effect of such variations on the properties of the protein molecule. Hemerythrin both from individual animals and from pooled blood was analyzed. Since octameric hemerythrin would be expected to consist of hybrids of any variant subunits (Keresztes-Nagy *et al.*, 1965), the monomeric protein was examined. The results of these studies show that the differences between individual animals are caused by the occurrence of varying quantities of two major hemerythrin components. These two proteins differ at five sites in the amino acid sequence.

Experimental Section

Preparation of Hemerythrin. Sipunculid worms of the species *Golfingia gouldii* were obtained from Marine Biological Laboratory, Woods Hole, Mass. The coelomic fluid was either collected separately from individual animals or was pooled in batches from approximately 100 worms. Hemerythrin was purified by the method of Klotz *et al.* (1957), with the minor modifications described by Subramanian *et al.* (1968). *Dendrostomum pyroides* worms were obtained from Pacific Bio-Marine Supply Co., Venice, Calif. Hemerythrin from the coelomic fluid of this species was prepared in essentially the same way as that of *G. gouldii* except that the crystallization step was omitted.

The oxyhemerythrin thus obtained was converted directly to metazidehemerythrin by dialysis against 0.1 M NaN_3 or was converted to metaquohemerythrin and then to the azide form. To obtain metaquohemerythrin, crystals of oxyhemerythrin were dissolved in 0.1 M Tris-acetate (pH 8.0) and then dialyzed against this buffer. Potassium ferricyanide, 2.2 moles/mole of hemerythrin monomer, was added to remove the oxygen and reaction was allowed to proceed overnight at 5°. Finally the mixture was passed through a column

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of Sephadex G-25, (Pharmacia Fine Chemicals) which had been equilibrated in the 0.1 M Tris-acetate buffer.

Metazidehemerythrin monomer can be prepared by reaction of the single cysteine sulfhydryl in the protein with mercurials (Keresztes-Nagy and Klotz, 1963). A small molar excess of sodium *p*-hydroxymercuribenzoate (Sigma) as a 5×10^{-3} M solution in 0.1 M Tris-chloride (pH 8.6) was added to metazidehemerythrin in the same Tris buffer. Reaction was allowed to proceed for 30 min at 5°. This solution of hemerythrin monomer was either used directly or was dialyzed against appropriate buffers before use.

Disc Electrophoresis. The method of Davis (1964) was used for disc electrophoresis of hemerythrin octamer and monomer. The protein sample in a 10–20% sucrose solution was layered directly onto the surface of the stacking gel and electrode buffer was then layered onto the sample. Electrophoresis was done at 5° at a constant current of 3.5 mA/gel. Before staining, the position of the tracking dye was marked by the insertion of a piece of fine wire.

DEAE-cellulose Chromatography. DEAE-cellulose (Whatman DE52, Reeve Angel) was washed with acid, base, and water and then equilibrated with 0.01 M Tris-chloride buffer (pH 8.0). For preparative separations, columns of 2.5 × 55 cm were used. All columns were run at 5°. In a typical experiment 50 μmoles of metazidehemerythrin monomer at a concentration of 1.9×10^{-3} M was dialyzed against 0.01 M Tris buffer (pH 8.0) and then applied to the column. The column was eluted with a 2-l. linear salt gradient consisting of 1 l. of 0.01 M Tris (pH 8.0) and 1 l. of 0.1 M NaCl in 0.01 M Tris (pH 8.0). A flow rate of 60 ml/hr was maintained and 8-ml fractions were collected. The effluent was monitored by the absorption of the fractions at 445 and 280 nm. DEAE-cellulose chromatography of 2 to 3 μmoles of hemerythrin from individual animals was done on 0.9 × 25 cm columns, using a 500-ml linear NaCl gradient, a flow rate of 12 ml/hr, and 2.8-ml fractions.

Enzymatic Digestion and Peptide Mapping. Monomeric hemerythrin samples from individual animals or fractions from DEAE-cellulose columns were denatured with guanidinium chloride, digested with chymotrypsin or with a combination of trypsin and chymotrypsin, and then subjected to paper electrophoresis–chromatography. For samples from individual animals, 1 μmole (13.5 mg) of protein was dissolved in 1.75 ml of 6 M guanidinium chloride solution, allowed to stand overnight at 5°, and then dialyzed against distilled water. The resulting suspension was diluted to 5 ml and made 0.1 M in NH_4HCO_3 and pH 8.0 by addition of 1.0 M NH_4HCO_3 (pH 8.0). Of this protein suspension 1.5 ml was taken for chymotryptic digestion and 3.5 ml for the combination tryptic–chymotryptic digestion. For the chymotryptic digestion, 0.04 mg (1% by weight) of α-chymotrypsin (Worthington) was added as a 1-mg/ml solution of the enzyme and digestion was allowed to proceed at 37° for 3 hr. An additional 1% by weight of the enzyme was then added and the reaction continued for another 3 hr, after which the sample was lyophilized. The combined tryptic–chymotryptic digest was initiated by the addition of 1% by weight (0.1 mg) trypsin (Worthington) which had been treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Schoellman and Shaw, 1963). A second 1% aliquot was added after digestion at 37° for 3 hr. At 6 hr, 1% by weight (0.1 mg) of α-chymotrypsin was added and an additional 0.05 mg of this enzyme was added after 9 hr had elapsed. The digestion was terminated at 12 hr by lyophilization.

For the chymotryptic digestion of DEAE-cellulose fractions of pooled hemerythrin, the procedure described above was scaled up to accomodate 5–10 μmoles of protein. In some cases milder (1% enzyme by weight, 4 hr) or more extensive (3% enzyme, 20 hr) digestion conditions were used.

Electrophoresis–chromatography (Ingram, 1958) was carried out under the following conditions: electrophoresis, 55 V/cm, 75 min, 5–10°, pH 6.4 pyridine–acetic acid–water buffer (133:4.6:1860 v/v) (Michl, 1951); chromatography, 16 hr, 1-butanol–acetic acid–water (40:6:15 v/v) for the chymotryptic digests and pyridine–water (80:20 v/v) for combined tryptic–chymotryptic digests. The peptides were visualized with ninhydrin reagent (0.1% in 1-butanol–acetone, 7:3 v/v). Tryptophan (Smith, 1953a), arginine (Smith, 1953b), methionine (Toennies and Kolb, 1951), histidine, and tyrosine (Smith, 1960) were visualized by standard techniques.

Purification of Peptides. Preparative peptide mapping was used to purify small quantities of peptides. Usually, 0.5–1.0 μmole of the enzyme digest or impure peptide fraction was applied to each sheet, and electrophoresis and then chromatography was carried out as described above. Peptides were located with a dilute (0.01%) ninhydrin reagent. Somewhat larger quantities (1–10 μmoles) of material were purified either by paper electrophoresis or paper chromatography alone or by a combination of these techniques in separate steps with intervening elution and lyophilization of the desired fractions. Conditions were generally as described above. Some peptides which were neutral in electrophoresis with the pH 6.4 buffer were purified by electrophoresis in 0.025 M ammonium bicarbonate buffer (pH 8.0) or in pH 5.3 pyridine–acetic acid–water (20:8:2972 v/v).

Thin-layer chromatography was also used for the final purification of some peptides. Cellulose layers (Whatman CC 41) 250 μ thick were used with pyridine–1-butanol–acetic acid–water (60:40:12:48 v/v) as the developing solvent.

Gel filtration on a column (1.5 × 92 cm) of Sephadex G-15 was used as a purification step with some peptides. Samples of 0.5–1.0 ml were applied, the column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 7.5 ml/hr and fractions of 1.0 ml were collected. The effluent was monitored by the absorbance of the fractions at 220 nm.

Amino Acid Analysis. Amino acid compositions were determined on a Beckman 120C amino acid analyzer according to Spackman *et al.* (1958). Peptides were hydrolyzed for 24 hr at 108° in triply glass-distilled 5.7 M HCl to which had been added 0.1 ml of 0.5 M hydrazine to prevent destruction of tyrosine (Sanger and Thompson, 1963). Protein samples were hydrolyzed for varying amounts of time and the quantities of threonine and serine, which are progressively destroyed, extrapolated to zero time.

Sequence Studies. The carboxyl-terminal sequences of peptides were determined by carboxypeptidase A digestion and amino terminal residues were determined by digestion with leucine aminopeptidase or by the dansyl chloride reaction. Carboxypeptidase A digestion was performed as described by Groskopf *et al.* (1966a). Amino acids liberated at various times from 2 to 22 hr were detected by automatic amino acid analysis. Leucine aminopeptidase digestions were done as described by Margoliash and Smith (1962). Digestion times from 2 to 8 hr were used and amino acids released were determined on the amino acid analyzer. The dansyl chloride procedure of Gray (1967) was used. Dansylamino acids were identified by thin-layer chromatography on silica gel plates (Analtech, Inc.) with benzene–pyridine–acetic acid (16:4:1 v/v) (Deyl and Rosmus, 1965) as solvent.

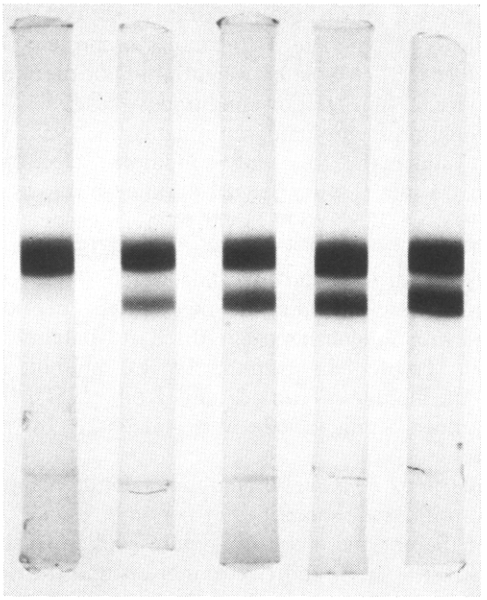

FIGURE 1: Disc electrophoresis of hemerythrin monomer from five individual animals of the species *G. gouldii*. Monomer was prepared from octameric metazidehemerythrin by addition of *p*-hydroxymercuribenzoate.

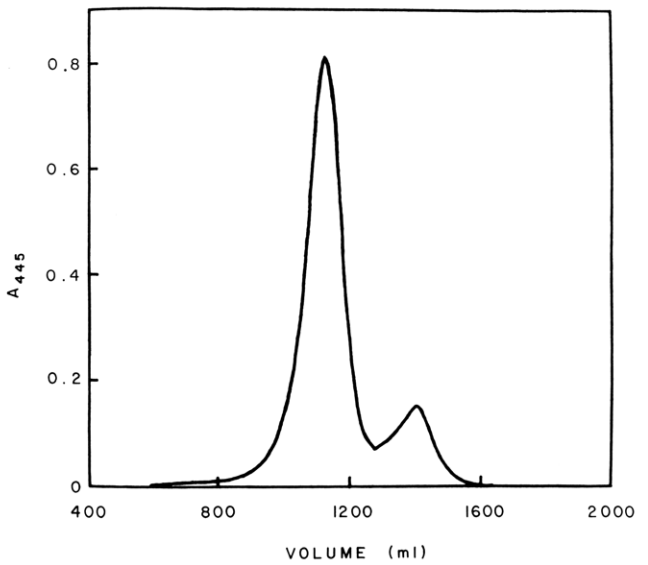

FIGURE 3: DEAE-cellulose chromatography of a pooled preparation of metazidehemerythrin monomer. A 50-μmole sample of hemerythrin subunit was applied to a 2.5 $\times$ 55 cm column and eluted with a 2-l. linear sodium chloride gradient. Details of procedure are given in the text.

The sequence of one peptide, C(76–80), was confirmed by dilute acid hydrolysis (Schultz *et al.*, 1962). The peptide was hydrolyzed for 7 hr, 40 min at 108° in 0.03 M HCl, and the hydrolysate was then lyophilized and analyzed for free amino acids.

Spectra. Ultraviolet and visible spectra of the hemerythrin variants in the monomeric metazide form were obtained using either a Cary 15 or a Hitachi Perkin-Elmer 124 spectrophotometer.

Nomenclature. The two hemerythrin components are designated hemerythrin A (Hr A) and hemerythrin B (Hr B) based on their order of elution from DEAE-cellulose columns. Peptides are designated C for chymotryptic digest, T for tryptic digest, and this letter is followed by the numbers, in parentheses, of the amino terminal and carboxyl-terminal residues of the peptide in the total amino acid sequence of hemerythrin.

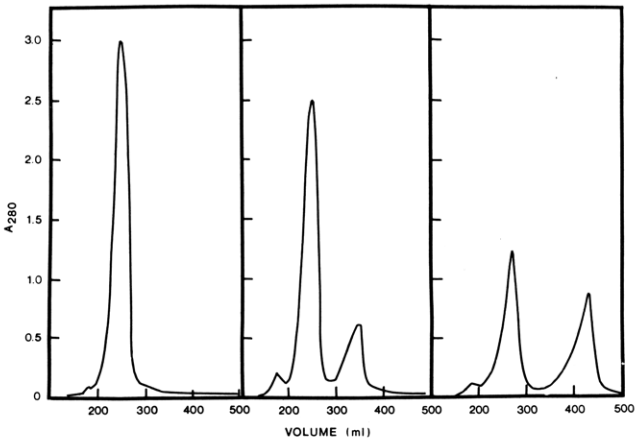

FIGURE 2: DEAE-cellulose chromatography of metazidehemerythrin monomer from three individual animals of the species *G. gouldii*. Samples of 2.8–3.2 μmoles of hemerythrin subunit were applied to 0.9 $\times$ 25 cm columns, and eluted with 500-ml linear sodium chloride gradients. Details of procedure are given in the text.

Results

Hemerythrin Variants in Individual Animals. Coelomic hemerythrin monomer from each of 101 individual animals was examined by disc electrophoresis. The two fundamental patterns which emerged are shown in Figure 1. While 37 animals contained a single electrophoretic species (Hr A), 64 others contained, in addition, a second, faster-moving band (Hr B). In the latter 64, the relative quantities of the two hemerythrins varied considerably. This can be seen by

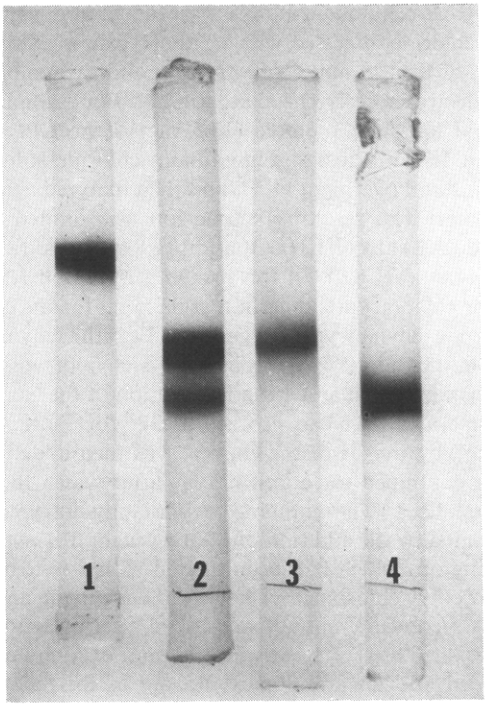

FIGURE 4: Disc electrophoresis of metazidehemerythrin octamer (gel 1), monomer (gel 2), DEAE-cellulose fraction A (gel 3), and DEAE-cellulose fraction B (gel 4).

TABLE I: Distribution of Threonine–Glycine and Serine–Alanine Interchanges in Hemerythrins from Individual Animals.

No. of Animals	Electrophoretic Type	Amino Acid at Residue 79	Amino Acid at Residue 96
8	Hr A	Thr or Gly	Ser or Ala
6	Hr A	Thr or Gly	Ser
1	Hr A	Thr or Gly	Ala
1	Hr A	Thr	Ser
2	Hr A	Gly	Ser
1	Hr A	Thr or Gly	Ser
	Hr B	Gly	Ala
1	Hr A	Thr	Ser or Ala
	Hr B	Gly	Ala
2	Hr A	Thr	Ala
	Hr B	Gly	Ala

disc electrophoresis (Figure 1), or DEAE-cellulose chromatography (Figure 2). No animals, however, were found which contained only the faster moving component (Hr B) nor did the quantity of this protein exceed 50% of the total hemerythrin in any animal.

Pooled preparations of hemerythrin monomer invariably exhibited both electrophoretic bands. The two components were separated on DEAE-cellulose columns (Figure 3) and the purity of the resulting two fractions assessed by electrophoresis (Figure 4). Hr B comprised approximately 15–20% of the total hemerythrin in these pooled samples, (average of 17% for five runs) while Hr A accounted for the other 80–85%.

DEAE-cellulose chromatography of pooled hemerythrin from five batches of worms differing in average weight from 0.2 g to over 1.3 g revealed the presence in all five of both Hr A and Hr B. However, there was no correlation between size (and presumably age) of the animals and the relative quantity of these hemerythrin variants.

The previously reported amino acid interchanges at residues 79 (threonine/glycine) and 96 (serine/alanine) involve only uncharged amino acid side chains and thus would not give rise to electrophoretic differences. To determine, however, whether the observed electrophoretic variation was related in some way to these interchanges, peptide mapping experiments were done on hemerythrin from individual animals. The interchange at residue 79 was detected by a combined tryptic–chymotryptic digest followed by paper electrophoresis–chromatography. Peptide TC (76–80) (Glu-His-Glu-$\overset{\text{Thr}}{\underset{\text{Gly}}{}}$-Phe) was resolved into the threonine and glycine components by electrophoresis followed by chromatography in the pyridine–water system. The interchange at residue 96 was detected by resolution of the serine and alanine components of chymotryptic peptide C(94–97) (Ala-Lys-$\overset{\text{Ser}}{\underset{\text{Ala}}{}}$-Trp) (peptides 4 and 5; see below) in the standard peptide mapping system using 1-butanol–acetic acid–water as chromatography solvent (Subramanian *et al.*, 1968). Hemerythrin from 18 animals which had contained only Hr A and both DEAE-cellulose fractions (Hr A and Hr B) from 4 other animals were examined. The results of these experiments are listed in Table I.

TABLE II: Amino Acid Composition of the Variants of *Golfingia gouldii* Hemerythrin.

Amino Acid	Residues/Mole of Monomer		
	Pooled Hemerythrin[a]	Hemerythrin A[b]	Hemerythrin B[c]
Lysine	11.0	11.0	11.0
Histidine	6.7	6.8	6.1
Arginine	3.2	2.9	2.9
Aspartic acid	16.9	17.0	19.1
Threonine	4.6	4.4	4.1
Serine	3.6	3.8	3.3
Glutamic acid	9.9	10.0	9.0
Proline	4.2	4.1	4.0
Glycine	6.4	6.6	7.0
Alanine	5.5	5.2	5.8
$^1/_2$-Cystine	1.1	n.d.	n.d.
Valine	3.9	3.9	4.0
Methionine	1.1	0.84	0.83
Isoleucine	8.7	8.2	8.9
Leucine	7.7	8.0	8.4
Tyrosine	5.0	4.9	4.8
Phenylalanine	8.8	9.2	8.8

[a] From Groskopf *et al.* (1966a). [b] Average of six analyses. [c] Average of four analyses.

No clear pattern of threonine and glycine at position 79 or of serine and alanine at residue 96 emerges in Hr A. Of the 22 individuals from which Hr A was analyzed, only five contained a single amino acid at both positions 79 and 96. Three of the four possible combinations of amino acids at 79 and 96 were represented in these five samples. The existence of the fourth combination (79 Gly; 96 Ala) can be deduced from results in an animal containing Gly and Thr at residue 79 but only alanine at 96. Thus, all possible combinations of variants at residues 79 and 96 occur in the major electrophoretic hemerythrin species, Hr A.

In contrast, Hr B contained only glycine at residue 79 and alanine at residue 96 in the four samples examined. This result was verified by peptide mapping and amino acid analysis of Hr B from pooled preparations (see below). The amino acid interchanges at residues 79 and 96 seem, therefore, to be related to the interchange(s) responsible for the observed electrophoretic variation.

Identification of Sites of Amino Acid Interchange. To identify the amino acid interchanges responsible for the electrophoretic heterogeneity observed, the amino acid compositions and chymotryptic peptide maps of Hr A and Hr B were compared.

Amino acid analysis of purified samples of Hr A and Hr B gave the results shown in Table II. The compositions of these proteins differ in the quantity of histidine, aspartic acid, glutamic acid, threonine, serine, glycine, and alanine. In Hr B, the numbers of residues of each of the latter four amino acids are very nearly integral and are consistent with the presence of only glycine at residue 79 and only alanine at residue 96. The quantities of histidine and glutamic acid are each one residue less in Hr B than in Hr A, and the quantity of aspartic acid is two residues more in Hr B than in Hr A.

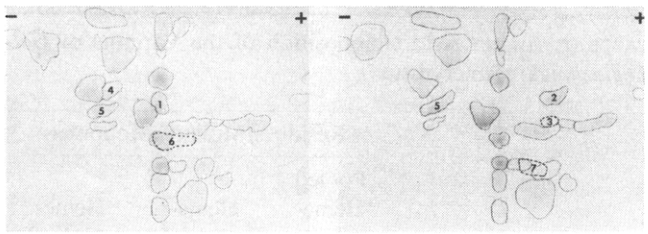

FIGURE 5: Peptide maps of chymotryptic digests of hemerythrin A (map on left) and hemerythrin B (map on right). Electrophoresis, pH 6 4, pyridine–acetate buffer, 75 min, 55 V/cm. Chromatography, 1-butanol–acetic acid–water (40:6:15 v/v), 16 hr. Peptide spots were revealed with ninhydrin. Dashed lines outline peptides which gave no apparent ninhydrin color and were located by specific staining reactions.

This suggests that in Hr B, aspartic acid of asparagine replaces one residue of histidine and one residue of glutamic acid or glutamine.

Representative peptide maps of the chymotryptic digests of Hr A and Hr B are shown in Figure 5. Three differences between the two maps are observed. The most obvious difference is the presence in Hr A of a neutral peptide (1) which is not found in Hr B and of two acidic peptides (2 and 3) in Hr B which are not found in Hr A. These three peptides all contain tyrosine (Pauly reaction) and peptides 1 and 3 contain methionine (Toennies-Kolb reaction). In some maps of Hr A, another peptide which contains tyrosine but not methionine appears just above peptide 1 (peptide 1′). A second difference involves the presence in Hr A of two basic, tryptophan-containing (Ehrlich reaction) peptides (4 and 5) and the absence of one of these (4) and the intensification of the second (5) in Hr B. A third difference in the peptide maps is the presence of a histidine- and tryptophan-containing peptide (6) in the neutral region of the Hr A map which in Hr B (peptide 7) moves somewhat faster in chromatography, is slightly more acidic in electrophoresis, and no longer gives a test for histidine with the Pauly reagent. Neither peptide 6 or 7 appears to give a strong color with ninhydrin but the spots can be located in the peptide map by their reaction with Ehrlich reagent and by the reaction of peptide 6 with Pauly reagent.

Peptides 1, 1′, 2, and 3 (*C(62–67)*, *C(63–67)*, *C(56–67)*). Peptides 1, 1′, 2, and 3 were purified by preparative peptide mapping. The amino acid compositions were: peptide 1: Ser, 0.93 (1); Glu, 2.09 (2); Ala, 1.03 (1); Met 0.77 (1); Tyr, 0.95 (1); peptide 1′: Ser, 1.09 (1); Glu, 1.82 (2); Ala, 1.11 (1); Tyr, 0.98 (1); peptide 2: Ser, 0.93 (1); Glu, 1.86 (2); Ala, 1.15 (1); Tyr, 1.05 (1); peptide 3: Asp, 1.33 (1); Ser, 0.95 (1); Glu, 4.05 (4); Ala, 1.07 (1), Val, 0.91 (1); Met, 1.04 (1); Leu, 1.82 (2); Tyr, 0.85 (1). Compositions identify peptide 1 as C(62–67), 1′ and 2 as C(63–67), and 3 as C(56–67) and are consistent with the sequence of hemerythrin in this region. In spite of the electrophoretic difference between C(62–67) and C(63–67) from Hr A and C(63–67) from Hr B, no difference in the amino acid sequences is revealed by the amino acid compositions. This result suggests that in C(63–67) from Hr B, one of the glutamine residues has been replaced by glutamic acid. This possibility was confirmed by sequence analysis. The electrophoretic mobility of C(62–67) and C(63–67) from Hr A indicates that both residues 63 and 66 are glutamine. Peptide C(63–67) from Hr B, however, gave N-terminal glutamic acid (residue 63) upon digestion with leucine aminopeptidase. Residue 66, on the other hand, was found to be glutamine by carboxypeptidase A digestion. Thus, the presence of a glutamic acid in place of a glutamine at residue 63 has been identified in Hr B and explains the difference in electrophoretic mobility of C(62–67) and C(63–67 from Hr A and C(63–67) from Hr B.

Peptides 4 and 5 (*C(94–97)*). Peptides 4 and 5 were purified by preparative peptide mapping. Their compositions were: peptide 4: Lys, 1.20 (1); Ser, 0.78 (1); Ala, 1.02 (1); Trp (1); peptide 5: Lys, 1.00 (1); Ala, 2.00 (2); Trp (1). The sequences of these peptides (4, Ala-Lys-Ser-Trp; 5, Ala-Lys-Ala-Trp) correspond to residues 94–97 in the protein chain and have serine and alanine, respectively, at position 96. As had been observed in the hemerythrin from individual animals, in these pooled preparations, Hr B contained only peptide 5 (alanine at residue 96) while Hr A contained both peptides 4 and 5.

Peptides 6 and 7 (*C(81–84)*, *C(81–87)*). Peptide 6 from Hr A contained both tryptophan and histidine and therefore was suspected to be C(81–87) (Ile-His-Ala-Leu-Asp-Asn-Trp), the only chymotryptic peptide in hemerythrin containing both of these amino acids. Peptide 7 from Hr B contained tryptophan but no histidine and therefore was thought to be C(81–87) with histidine 82 having been replaced by some other amino acid. Since peptide C(81–87) had been previously recovered as two fragments, C(81–84) and C(85–87) due to a chymotryptic cleavage at leucine-84 (Subramanian *et al.*, 1968), it seemed feasible to obtain the smaller fragment C(81–84), which would still contain an interchange at residue 82 from both Hr A and Hr B.

Hr A was digested extensively (20 hr, 3% chymotrypsin by weight) and was fractionated by paper electrophoresis (pH 6.4), paper chromatography, and a second electrophoretic separation (pH 5.3). The peptide obtained in this way had the composition, His, 0.98 (1); Ala, 1.05 (1); Ile, 0.94 (1); Leu, 1.04 (1), which confirmed its identity as C(81–84), Ile-His-Ala-Leu.

Two peptides were prepared from Hr B. Peptide C(81–84) was purified after extensive chymotryptic digestion (20 hr, 3% by weight) by gel filtration on G-15 Sephadex, followed by preparative peptide mapping of the appropriate Sephadex fraction. The composition of the purified peptide, Asp, 1.02 (1); Ala, 0.95 (1); Ile, 1.01 (1); Leu, 1.03 (1), suggested the sequence Ile-Asx-Ala-Leu. Since the peptide was neutral in electrophoresis, the Asx residue is asparagine. Peptide C(81–87) was purified after a limited chymotryptic digestion (4 hr, 1% enzyme by weight) by gel filtration on G-15 Sephadex followed by DEAE-Sephadex chromatography. The ion-exchange column, 0.9 × 95 cm in size, was equilibrated with 0.1 M ammonium acetate, pH 5.0, and after the sample had been applied was eluted with 1.0 M ammonium acetate buffer, pH 5.0. The purified peptide gave the composition, Asp, 2.96 (3); Ala, 0.98 (1); Ile, 1.01 (1); Leu, 1.05 (1); Trp (1), corresponding to the sequence Ile-Asn-Ala-Leu-Asp-Asn-Trp. Finally, the presence of asparagine at residue 82 in Hr B was firmly established by leucine aminopeptidase digestion of this peptide.

Aspartic Acid–Glutamic Acid Interchange. The differences between the amino acid compositions of Hr A and Hr B suggested the replacement of a glutamic acid (or glutamine) in Hr A by an aspartic acid (or asparagine) in Hr B. Such an interchange was not detected in the peptide maps of the two variants. Therefore, the Hr B molecule was fragmented and each peptide which would be expected to contain glutamic acid, was examined for the substitution of aspartic acid.

Hr B was treated with iodoacetamide to protect the cysteine,

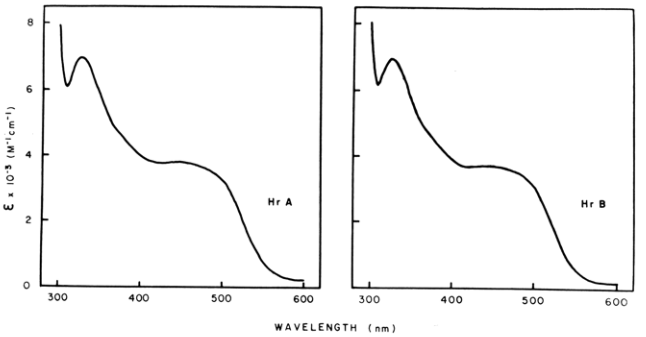

FIGURE 6: Absorption spectra of the metazide form of hemerythrin A and hemerythrin B. Extinction coefficients are given in terms of moles of hemerythrin monomer.

succinylated, and digested with trypsin, and the digest was fractionated on a column of G-50 Sephadex. Three peptides are produced by this method, two of which contain glutamic acid. Peptide sHr-TT-3 (residues 16–48) contains 2 Glu and 8 Asx residues and sHr-TT-2 (residues 50–113) contains 8 Glx and 7 Asx residues (Klippenstein *et al.*, 1968). While peptide sHr-TT-3 from Hr B gave the same composition as in the previous report, sHr-TT-2 contained 6.97 (7) Glx residues and 8.99 (9) Asx residues. One of these additional Asx residues is accounted for by the asparagine–histidine substitution at residue 82. The other Asx represents an Asx–Glx interchange in the C-terminal half of the molecule.

Four of the Glx residues in this region of the molecule are found in peptide 3 (C(56–67)) described above. It is obvious from the amino acid composition of C(56–67) from Hr B that the expected Asx–Glx interchange is not in this region. The remaining Glx residues in the C-terminal half of the molecule are found between residues 68 and 80. A peptide encompassing this entire region was isolated from a chymotryptic digest of Hr B. The digest was subjected to paper electrophoresis and the neutral region was then fractionated by paper chromatography. The fraction moving only 1.5 cm from the origin was isolated. The composition of this peptide, Lys, 1.91 (2); His, 1.89 (2); Asp, 2.11 (2); Glu, 2.53 (3); Gly, 1.10 (1); Tyr, 0.74 (1); Phe, 1.71 (2); identified it as C(68–80), the sequence for which is Gln-Phe-Tyr-Asp-Glu-His-Lys-Lys-Glu-His-Glu-$\frac{\text{Gly}}{\text{Thr}}$-Phe in the major hemerythrin component. The low, nonintegral values for Glu, Tyr, and Phe indicate that the fraction contains a small amount of C(71–80) and thus is missing a small amount of the tripeptide Gln-Tyr-Phe. As anticipated, the Asx–Glx interchange does occur in this region since the aspartic acid content is one residue high, the glutamic acid content low. To determine whether the Asx–Glx interchange occurs at residue 68, 72, 76, or 78, the peptide was further fragmented by tryptic digestion. The products of this digestion were separated by paper electrophoresis. The most acidic of these peptides, CT(76–80) had the composition: His, 0.94 (1); Asp, 1.03 (1); Glu, 1.13 (1); Gly, 0.97 (1); Phe, 0.93 (1), a clear demonstration that the interchange of an aspartic acid for a glutamic acid residue was at residue 76 or 78. The exact position was determined by three methods, leucine aminopeptidase digestion, dansylation, and dilute acid hydrolysis.

Digestion of CT(76–80) from Hr B with leucine aminopeptidase liberated only glutamic acid, positively identifying residue 76 and implicating residue 78 as the site of the interchange. Dansylglutamic acid was obtained using the dansyl chloride procedure. If the interchange were at residue 78, only the aspartic acid there should be rapidly cleaved in dilute acid hydrolysis. If, on the other hand, it were located at residue 77 or 79, glutamic acid or phenylalanine should be liberated in equimolar quantities with aspartic acid. In 7 hr, 40 min of hydrolysis, only aspartic acid was released. This confirms the location of the aspartic acid–glutamic acid interchange at residue 78.

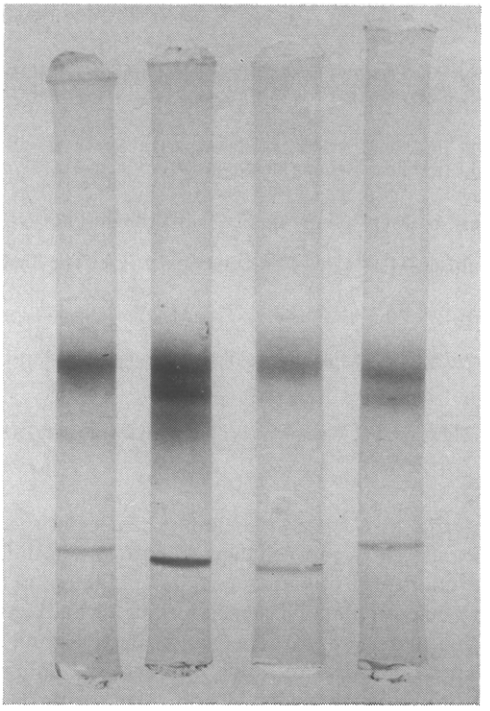

FIGURE 7: Disc electrophoresis of hemerythrin monomer from selected individual animals of the species *Dendrostomum pyroides*. Monomer was prepared from octameric metazidehemerythrin by addition of *p*-hydroxymercuribenzoate.

This peptide contained an integral residue ratio of glycine, confirming the results from amino acid analysis and peptide maps of hemerythrin from individual animals that whereas both threonine and glycine occurs at position 79 in Hr A only glycine occurs at residue 79 in Hr B.

Spectral Properties of Hemerythrin Variants. The absorption spectra of Hr A and Hr B in the monomeric metazide form are shown in Figure 6. The spectra are virtually identical with one another, both corresponding in shape and extinction coefficients to the spectrum reported previously (Keresztes-Nagy and Klotz, 1965).

Hemerythrin Variants in Dendrostomum Pyroides. Hemerythrin from the coelom of 46 individuals of the sipunculid *Dendrostomum pyroides* was examined by disc electrophoresis. As with *Golfingia* hemerythrin the monomeric protein in the metazide form was used. There are at least three monomeric components of this hemerythrin. Furthermore, there are differences in the proportions of these hemerythrin species in different individual animals (Figure 7). The electrophoretic differences between monomers are more subtle than in *G. gouldii*, possibly explaining the fact that octameric hemerythrin from individuals of *D. pyroides* are virtually indistinguishable on starch–gel electrophoresis (Ferrell and Kitto, 1970).

Discussion

This investigation confirms the existence of two electro-

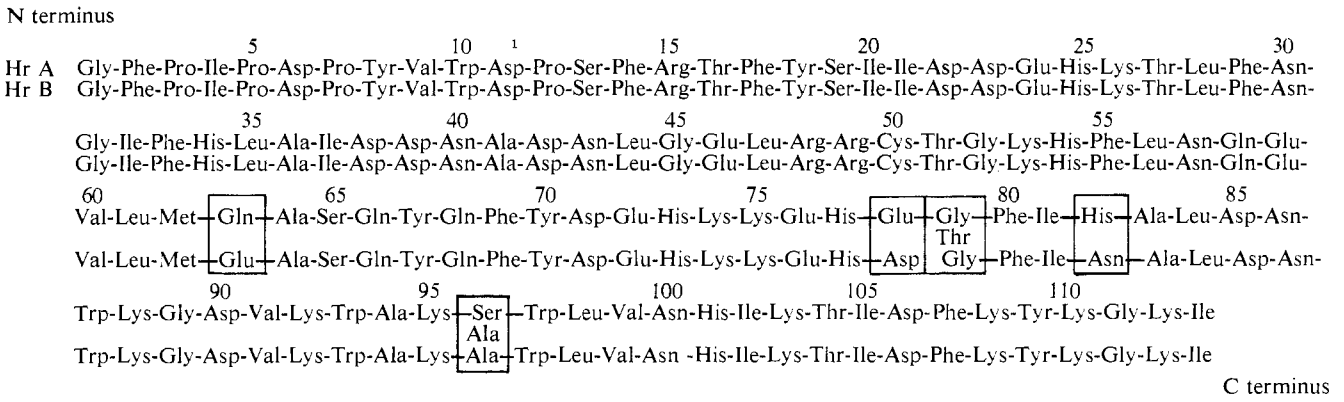

FIGURE 8: The amino acid sequences of hemerythrin A and hemerythrin B. Enclosures identify sites of amino acid interchange.

phoretic forms of *G. gouldii* coelomic hemerythrin and describes the differences between them (Figure 8). The differences are substitution of a glutamic acid for glutamine at residue 63, aspartic acid for glutamic acid at residue 78, and asparagine for histidine at residue 82. The major electrophoretic component, Hr A consists of four variants containing either threonine or glycine at residue 79 and either serine or alanine at residue 96. Hr B contains only glycine at residue 79 and alanine at residue 96. Thus, Hr B differs from one Hr A variant by three residues, with two of the Hr A variants by four residues and with the fourth Hr A variant by five residues.

Manwell (1963), using octameric hemerythrin, found that individual animals contain either electrophoretically "fast," "slow," or "intermediate" hemerythrins on starch–gel electrophoresis. Using monomeric hemerythrin, we have found some animals which contain only a slower moving monomeric species (Hr A) and many which contain both a faster moving (Hr B) and the slower moving species in varying proportions. None have been found, however, which contain only the fast (Hr B) monomer. Since octameric hemerythrin probably consists of hybrids of any variant subunits present (Keresztes-Nagy *et al.*, 1965), Manwell (1963) may well have been unable to distinguish between, for example, the hybrid octamers produced by a 1:1 mixture of the two monomeric species Hr A and Hr B and a pure octamer made up entirely of Hr B. It seems likely, therefore, that while the slow band reported in in this early work represents a homogeneous species consisting entirely of Hr A, both the intermediate and fast bands represent hybrids of Hr A and Hr B, the two bands differing in the proportion of the two variant monomers. This contention is supported by the width of the bands on starch–gel electrophoresis. The intermediate and fast octameric bands were of equal width whereas the slow band was only one-half the width of the other two (Manwell, 1963).

It has been reported that hemerythrin from individual animals of the sipunculids *Dendrostomum zostericolom* (Manwell, 1963) and *Dendrostomum pyroides* (Ferrell and Kitto, 1970, 1971a) do not exhibit electrophoretic heterogeneity. Both of these studies were carried out on the octameric protein. Furthermore, Ferrell and Kitto (1971b) have reported a partial sequence of *D. pyroides* hemerythrin and have found no variant residues. Nevertheless, using monomeric *Dendrostomum pyroides* hemerythrin, we have found three hemerythrin components which vary in relative quantity in different individual animals. We have also found differences in the hemerythrins of different individuals of the species *Phascolosoma agassizii* (G. L. Klippenstein and F. Liberatore, 1971, unpublished data). It is apparent that multiple hemerythrins are found in other sipunculids and that this phenomenon is not limited to *G. gouldii*.

Groskopf *et al.* (1963) reported observing differences in chymotryptic peptide maps of *Golfingia* hemerythrin from individual animals. They found that an acidic, tyrosine-containing peptide and a basic, tryptophan-containing peptide were both present in one class of worms, and both absent in another. These peptides correspond to peptides 2 (C(63–67)) and 5 (C(94–97)) reported here. Both peptides occur in Hr B and it thus appears that animals in which both were present contained Hr B, while animals in which both were absent contained only Hr A. It is not clear, however, why some animals were not found by Groskopf *et al.* whose hemerythrin had the basic trytophan-containing peptide C(94–97) but not the acidic peptide C(63–67), since a number of such animals have been found in this present work.

At residue 63, a glutamine is found in Hr A, a glutamic acid in Hr B. The assumption that a true amino acid interchange has occurred can be questioned, since cyclization or deamidation of the glutamine would give the same experimental behavior. However, cyclization to form a pyrrolidonecarboxylic acid residue is ruled out because peptide C(63–67) from Hr B reacts with ninhydrin and is degraded by leucine aminopeptidase. Deamidation of the glutamine is unlikely since (1) all hemerythrin samples were treated in an identical manner and prior to disc electrophoresis were never subjected to conditions of pH or temperature which would favor deamidation, and (2) the interchange at residue 63 is accompanied by interchanges at residues 78 and 82, neither of which could be the result of deamidation.

The primary structure reported previously for hemerythrin (Klippenstein *et al.*, 1968) corresponds to the structure of the major hemerythrin component Hr A. This component consists of four homogeneous variants, the differences between them determined by the amino acid residue found at position 79 (threonine or glycine) and at position 96 (serine or alanine). The residue ratios for most of the remaining amino acids in the protein are nearly integral. However, two residues, phenylalanine and isoleucine, deviate slightly from the value expected and thus make it impossible to rule out other amino acid interchanges. In addition, histidine is still slightly less

[1] Ferrell and Kitto (1971a,b) using an automatic protein sequenator have obtained results which suggest that residues 10 and 11 have the sequence Trp-Asp rather than Asp-Trp as was previously reported (Groskopf *et al.*, 1966b). W. R. Groskopf (personal communication, 1971) has indicated that the sequence Trp-Asp is not inconsistent with his data.

than 7 residues and the possibility remains that it may be replaced by asparagine at residue 82 in a small fraction of the Hr A molecules.

The absorption spectra of the metazide form of Hr A and Hr B are essentially the same indicating that despite the structural differences between them, both have an intact iron-binding site. This leads to the important conclusion that while histidine is involved in iron binding in hemerythrin (Fan and York, 1969) histidine-82 can clearly be eliminated as an iron ligand. Manwell (1963) measured the oxygen affinities of the fast, slow, and intermediate hemerythrin species. He observed that the oxygen affinity of the fast hemerythrin is 37% greater than the slow. This considerably increased oxygen affinity, due presumably to the presence of Hr B in the fast hemerythrin fraction indicates that the amino acid interchanges described in this paper have an important though probably indirect effect on the oxygen-binding site in hemerythrin. Whether this effect involves interaction with the active center or the stabilization of a protein conformation more favorable to oxygen binding is a matter of speculation.

A number of examples of modification of oxygen affinity have been found in the mutant human hemoglobins. Substitutions of residues in contact with the heme group, of nonpolar residues in the interior of the molecule with polar groups, and of hydrogen-bonding with nonhydrogen-bonding residues have particularly profound effects on the biological properties of hemoglobin (Perutz and Lehmann, 1968). In hemerythrin, a new negative charge introduced at residue 63 would have the potential to interact electrostatically with the iron in the active center, its protein ligands, or the peroxo or hydroperoxo (Garbett *et al.*, 1969) group bound to iron in the oxy form. Any of these interactions might favor the oxy form and increase the measured oxygen affinity. In this connection, it has been observed that additional negative charges bound *via* external anions like perchlorate do indeed increase the oxygen affinity of hemerythrin (DePhillips, 1971). The introduction of a negative charge at residue 63 in a relatively nonpolar, possibly interior region of the molecule could also result in a conformational change tending to stabilize the structure of oxyhemerythrin. A change in the geometry or strength of hydrogen bonds formed by any of the five interchanged amino acids could also result in such a conformational transition. Finally, Garbett *et al.* (1971) have postulated a mechanism for the oxygenation of hemerythrin in which two potentially basic amino acid side chains participate and a third protein group coordinates to the peroxo group. An amino acid interchange involving any of these three groups would be expected to modify the ability of the protein to bind oxygen.

The fact that no animals which contained only Hr B were found suggests that this hemerythrin variant when it occurs alone may be lethal to the animal. Most animals contained at least two hemerythrins and some contained three or possibly more hemerythrin variants. On the other hand, 3 of 22 animals examined were found to contain only one hemerythrin and among these, two of the four observed forms of Hr A were represented. The observation that three hemerythrins occur in some individuals of this diploid animal indicates that a minimum of two cistrons specifies the structure of the five hemerythrin variants. The data in Table I are consistent with the idea that alleles of one cistron specify the structures of three forms of Hr A and the second cistron specifies the structure of Hr B. The cistron to which Hr A (79 Gly; 96 Ala) should be assigned cannot be deduced with the data available. Further interpretation of these results on the genetic level must await the accomplishment of appropriate genetic crosses.

Acknowledgment

I thank Dr. D. MacDonald Green and Dr. James A. Stewart for helpful discussions and Miss Suzanne Boydston for excellent technical assistance.

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Conversion of Retinyl Methyl Ether into Retinol in the Rat *in Vitro**

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ABSTRACT: In rats retinyl methyl ether (RME) is converted into retinol by everted intestinal sacs, liver slices and liver homogenates. The RME cleavage enzyme of liver is localized in the microsomal fraction, and can be solubilized and stabilized by the preparation of an acetone powder. Mg^{2+} and EDTA have an additive stimulatory effect on the fresh microsomal enzyme but not on the acetone powder preparation. The K_m for RME was found to be 4×10^{-4} M from kinetic studies. Tetrahydropteridine is a required cofactor, also with a K_m of 4×10^{-4} M. The pteridine analog, tetrahydroquinazoline, inhibits the reaction by competing with tetrahydropteridine, and has a K_i of 4.25×10^{-4} M. Molecular oxygen is also required, and NADPH enhances the enzyme activity, presumably by reducing dihydropteridine. Thus, the microsomal enzyme which catalyzes the cleavage of RME to retinol appears to be a typical pteridine requiring monooxygenase.

Previous studies in this laboratory (Narindrasorasak *et al.*, 1971) and elsewhere (Thompson and Pitt, 1963) have shown that retinyl methyl ether (RME),[1] which has good growth-promoting activity (Isler *et al.*, 1949), is efficiently converted into retinol when fed to rats. Analysis of the kinetics of uptake, storage, and cleavage indicated that the intestine and liver were probably the major conversion sites (Narindrasorasak *et al.*, 1971). In this paper, we wish to report the oxidation of RME into retinol by liver slices, everted intestinal sacs, and a microsomal enzyme of rat liver. The enzyme has been characterized as a typical pteridine requiring monooxygenase.

Materials and Methods

Preparation of [15-^{3}H]RME. [$^3H_4$]LiAl (5 mCi, specific activity 1.94 mCi/mg) was dissolved in dry diethyl ether and cooled to −15°. *all-trans*-Retinal (5 mCi) in dry diethyl ether was added to the [$^3H_4$]LiAl solution drop by drop until the yellow color of retinal disappeared, which indicated the end point for the reduction of retinal to retinol. One drop of a saturated solution of nonradioactive $LiAlH_4$ in diethyl ether was then added to reduce the last traces of retinal. The reaction was stopped by the addition of distilled water. The ether layer was washed, dried over anhydrous Na_2SO_4, reduced in volume *in vacuo*, and finally dissolved in petroleum ether (bp 40–60°). The product had the characteristic fluorescence and absorption spectrum (λ_{max} at 325 nm) of retinol. The yield of [15-^{3}H]retinol was 22.7%. [15-^{3}H]Retinol was then mixed with 100 mg of nonradioactive retinol and methylated with dimethyl sulfate by the method described by Hanze *et al.* (1948). The yield of [15-^{3}H]RME[1] from labeled retinol was 12.5%.

Preparation of the Enzyme from Rat Liver. METHOD A. A normal male rat, fasted for 24 hr, was killed by a blow on the head. After perfusing with 0.16 M Krebs–Ringer phosphate buffer (pH 7.4), the liver was removed and homogenized with two volumes of 0.05 M potassium phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer together with four volumes of ice-cold 0.25 M sucrose. Cell debris and nuclei were removed by centrifugation at 400*g* for 5 min in a Sorvall RC-2 refrigerated centrifuge. The mitochondrial fraction was sedimented by centrifugation at 10,000*g* for 10 min, and the microsomes were then collected by centrifuging at 100,000*g* for 60 min in a Beckman Model L2-65 ultracentrifuge. The mitochondrial and microsomal fractions were resuspended in a volume of fresh 0.25 M sucrose equivalent to one-fifth the volume of the original homogenate. The crude homogenate and the mitochondrial, microsomal, and final supernatant fractions were then tested for enzyme activity.

METHOD B. The microsomal fraction obtained as described in method A was further homogenized with five volumes of cold acetone (−15°) for 2 min in a Waring blender and then quickly filtered through a sintered-glass funnel with suction. The residue was thoroughly washed with cold acetone, dried, and stored in a desiccator at −15°. Although the acetone powder was no more active than the original microsomal enzyme, it could be stored at −15° for 2 weeks without appreciable loss of activity. The acetone powder enzyme was used in the assay for cofactor requirements.

Measurement of Enzyme Activity. [15-^{3}H]RME (5 μg; specific activity 4×10^5 cpm/mg) was solubilized in 0.5 ml of propylene glycol and incubated in a 50-ml Raysorb flask with various subcellular fractions in a final volume of 10 ml of

* From the Biochemistry Department, Faculty of Science, Mahidol University, Bangkok, Thailand. *Received June 7, 1971.* This study was supported by grants from National Institutes of Health (5 ROI-AM 11367), the Rockefeller Foundation (GA-BMS-7006) and the Faculty of Graduate Studies, Mahidol University, Bangkok. Part of this work was conducted by S. N. in partial fulfillment of the requirements of a M.Sc. degree.

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[1] Abbreviation used is: RME, retinyl methyl ether.