Incorporation of *S*-(9-Hydroxy-9,10-Dihydro-10-Phenanthryl)-L-Cysteine into Rabbit Hemoglobin (36103)

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Previous work from our laboratory has shown that arylcysteine conjugates, such as *S*-(9-hydroxy-9,10-dihydro-10-phenanthyrl)-L-cysteine (1), can be incorporated into ribosomal protein by a rat liver biosynthetic system (2). Also, when the compound is incubated with resting *Escherichia coli,* induced β-galactosidase contains the amino acid analogue in peptide combination (3).

It was desirable to extend these findings to a mammalian protein. Hemoglobin synthesis by rabbit reticulocytes offered a convenient system. When the phenanthrylcysteine conjugate was incubated with a lysate of rabbit reticulocytes, newly synthesized hemoglobin contained the amino acid analogue in its primary structure.

Materials and Methods. *S*-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (DHP-cysteine) was synthesized from 9,10-phenanthrene epoxide and labeled L-cysteine by the method previously described (2). Cysteine-^{35}S was obtained from Schwarz/Mann and cystine-^{14}C from Amersham/Searle.

Reticulocytes were obtained from New Zealand white rabbits made anemic by six daily injections of 6.4 mg of neutralized phenylhydrazine per kilo. The hematocrit of the blood used was 16–20% and the reticulocyte count was more than 80%. Blood was taken by cardiac puncture on the seventh day. Lysates were prepared by the method of Adamson *et al.* and were incubated promptly with their mixture of amino acids, hemin, creatine phosphate, creatine phosphate kinase, ATP and GTP (4). The incubation mixture consisted of 0.8 ml of lysate and 0.2 ml of amino acid mixture. The concentration of DHP-cysteine in the incubation mixture was 0.6×10^{-3} *M*. In some experiments chloramphenicol, 5×10^{-3}*M*, was added. The samples were placed in 25 ml beakers and incubated for one hour at 25° in a Dubnoff shaker under air. The pooled contents of the beakers were centrifuged for 90 min at 105,000*g* to remove ribosomes. The hemoglobin was purified by gel filtration on Sephadex G-100 (5). Globin was prepared from hemoglobin by the acid acetone method (6). Alpha and beta chains were separated by the column chromatography method of Dintzis (7). Protein concentrations were estimated spectrophotometrically or by a turbidimetric method (8). For radioactivity measurements, samples of protein solutions were plated on paper discs by the method of Bollum (9) and counted in a Nuclear Chicago Mark II liquid scintillation counter.

Globin and the separated chains were digested with trypsin according to the procedure of Tavill *et al.* (5). Peptides were separated by high voltage paper electrophoresis followed by chromatography according to the methods used by Dintzis (7). Peptide spots were visualized by light ninhydrin staining, cut out, and then eluted by repeated centrifugation of the moistened paper. The peptides are numbered on the chromatogram according to the scheme of Naughton and Dintzis (10). For isolation of DHP-cysteine from globin, a 10 mg sample was digested by trypsin and then by Pronase. The digest was lyophilized and extracted by dry methanol. The methanol extract was applied on filter paper and developed with *n*-butanol, acetic acid, water (2:1:1). The chromatogram was stained with ninhydrin and DHP-cysteine

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was indentified by R_f value and radioactivity.

Results and Discussion. Newly-synthesized hemoglobin was found to contain DHP-cysteine in all experiments excepting those utilizing chloroamphenicol. The radioactivity marker survived extensive purification of the hemoglobin, its conversion to globin, separation of the alpha and beta chains and fingerprinting the tryptic peptides. The radioactive compound isolated from the Pronase digest of globin was identified as DHP-cysteine-^{35}S by paper chromatography. Table I shows the incorporation of the analogue was greater into the beta chain than into the alpha. The influence of chloramphenicol on the incorporation is shown in Table II. Figure 1 shows the distribution of radioactivity in relation to the hemoglobin emerging from the CM-cellulose column used in the separation.

TABLE II. The Effect of Chloramphenicol on the Incorporation of DHP-Cysteine-^{35}S into Hemoglobin.

Molar concentration of chloramphenicol[a]	Specific radioactivity of hemoglobin (dpm/mg)	% of inhibition
None	590	0
0.6×10^{-3}	331	56
5.0×10^{-3}	24	96

[a] Procedure described in this paper.

Fingerprints from 1 mg of protein showed a number of radioactive peptides but only one which was identified as peptide No. V in

TABLE I. Specific Radioactivity of Hemoglobin and Components.

Experiment number[a]	Hemoglobin (dpm/mg)[b]	Globin (dpm/mg)	α-Chain (dpm/mg)	β-Chain (dpm/mg)
1	80	84	—	—
2	58	61	—	—
3	590	629	167	726
4	618	626	188	729

[a] Procedure described in this paper.

[b] In Experiments No. 1 and 2, DHP-cysteine-^{14}C, 3.7 μCi/mmole was used; in Experiments No. 3 and 4, DNP-cysteine-^{35}S, 27 μCi/mmole.

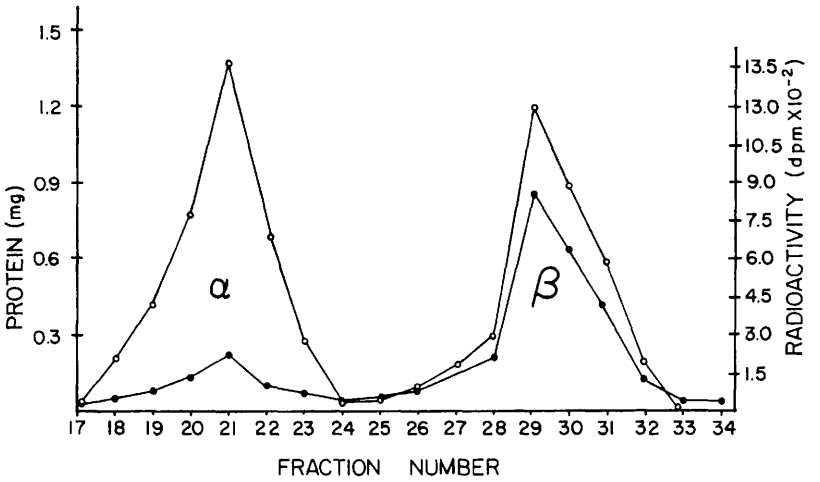

FIG. 1. Separation of alpha and beta chains of labeled globin on a CM-celluose column. Fifty milligrams of protein was applied on a 1 × 20 cm column. Fractions (5 ml) were collected over 30 min intervals. Protein concentration was estimated by measuring the turbidity developed on the addition of trichloroacetic acid. Radioactivity (-●-) and protein values (-O-) were determined on 1 ml aliquots.

the beta chain (βTpV) showed exceptional radioactivity, about 180 dpm per spot (Table III). This peptide is one of two containing

TABLE III. Distribution of ^{35}S Radioactivity Between Tryptic Peptides on "Fingerprint" of Hemoglobin Chains.[a]

α-Chain		β-Chain	
Spot number	dpm/spot	Spot number	dpm/spot
5	29	1	39
6	12	2	180
10	0	3	51
11	0	4	39
14	19	7	28
15	0	8	8
16	—	9	49
19	—	12	46
20	14	13	0
21	27	17	56
22	22	18	0
25	0	23	34
26	12	24	34
28	46	27	34
31	—	29	28
		30	34

[a] One milligram of tryptic digested protein was applied on the chromatogram.

three phenylalanine residues (11). This observation is perhaps significant in view of previous work from this laboratory (12) which suggested that DHP-cysteine is activated and transferred by the synthetase systems operating on phenylalanine, histidine, and glutamic acid.

The amount of analogue incorporated into newly-synthesized hemoglobin is difficult to estimate. The lysates contained approximately 1 μmole/ml of previously synthesized hemoglobin and the amount of new hemoglobin synthesized was estimated at 4 pmoles from leucine incorporation experiments. On this basis as many as 20 residues of DHP-cysteine were incorporated into a hemoglobin molecule.

The incorporation of amino acid analogues into microbial systems is well-established (13). Kruh and Rosa (14) found fluorophenylalanine to be incorporated into hemoglobin. Hemoglobins containing DHP-cysteine are obviously abnormal but insufficient quantities are available to test their physiological properties.

Summary. The incorporation of hydroxydihydrophenanthrylcysteine into hemoglobin has been demonstrated with a lysate of rabbit reticulocytes. The uptake of the amino acid analogue was greater in the beta chain than in the alpha. Incorporation was inhibited by chloramphenicol.[2]

[2] Some preliminary experiments were conducted by Mrs. Barbara Waller with Dr. E. T. Bucovaz.

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