IN VITRO BIOSYNTHESIS OF CHICK HEMOGLOBINS

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It has been reported earlier that the nucleated chick erythrocytes possess two hemoglobins designated as 1 and 2 depending on their paper electrophoretic mobilities in barbital buffer, pH 8.6 (Saha, 1956). Studies made with glycine-2-C^{14}, leucine-U-C^{14}, histidine-(2-ring)-C^{14}, valine-1-C^{14}, and valine-4-C^{14} showed that there is a difference in the rate of synthesis of these two hemoglobins. In vivo studies made with chicks of different age groups showed a higher incorporation of radioactive amino acids in hemoglobin 2, and in vitro studies made with intact chick erythrocytes showed a higher incorporation of labeled amino acids in hemoglobin 1 (Saha and Ghosh, 1959). It appears that the state of internal milieu plays a significant role during the synthesis of hemoglobin forms. Attempts were made during the present investigation to evaluate the effect of the composition of the incubation medium on the biosynthesis of chick hemoglobins 1 and 2. As definite knowledge about the composition of the physiological milieu where the biosynthesis takes place is not available, use has been made of synthetic medium prepared on the basis of the amino acid composition of chick hemoglobins 1 and 2 and of human adult hemoglobin, designated as CH 1, CH 2, and HAH. Attempts were thus made to study the course of protein synthesis in intact chick erythrocytes suspended in media of varying amino acid composition.

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The synthetic media CH 1 and CH 2 were prepared by following the data of Helm and Huisman (1958) on the amino acid composition of chick hemoglobins 1 and 2 respectively. Each ml of the medium (CH 1 or CH 2) contained the same amount of a particular amino acid in micrograms as reported by Helm and Huisman (1958). Furthermore, each medium (CH 1 and CH 2) contained 12.5 μg/ml of both l-cysteine and l-methionine and 15 μg/ml of l-tryptophan. The medium HAH was prepared according to Borsook (1958). The pH of the medium was adjusted to pH 7.4 by the addition of N NaOH with a pH-meter using a glass electrode. Five microcuries of alanine-1-C^{14}, leucine-U-C^{14}, and glycine-2-C^{14} were used for an experiment. Besides these amino acids, the medium contained α-ketoglutaric acid, 20 mg/ml; glucose, 1.0 mg/ml; $MgCl_2$, 2.3 mg/ml; $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 5 μg/ml; $CoCl_2$, 1 μg/ml; pyridoxal-5-phosphate, 17 μg/ml; and Na-penicillin G and streptomycin sulfate, each 0.1 mg/ml.

Adult male white leghorn chicks, approximately six months' old and weighing about 2-3 kg, were injected subcutaneously with a mixture of acetylphenylhydrazine (20 mg) and phenylhydrazine hydrochloride (20 mg) the first week and subsequently with acetylphenylhydrazine (20 mg) once every week during the experimental investigation. Heparinized blood (10-15 ml) was withdrawn from the wing vein of each bird every week 48 hours after the acetylphenylhydrazine injection. When the incubation was carried out in the synthetic medium alone, red blood corpuscles were washed three times with four times their volume of isotonic saline and spun down at 2500 rpm for 15 minutes in a refrigerated centrifuge before the transfer to the incubation vessel. When the incubation was performed in the medium containing an equal mixture of normal chicken plasma and synthetic medium, the erythrocytes were spun down at 2500 rpm for 15 minutes, plasma was removed carefully from the top, and the cells were pipetted directly to the medium. A 3-ml volume of packed erythrocytes was suspended in 6 ml of medium in a 50-ml erlenmeyer flask, and

incubation was carried out in air at 38°C for five hours in a Dubnoff-type metabolic shaker with a rate of 100 cycles per minute. After the incubation, the erythrocytes were washed six times with ten times their volume of cold isotonic saline and hemolyzed with water and toluene in cold. The clear hemoglobin solution was obtained by centrifuging in cold the hemolyzed mixture at 1006 g for 30 minutes and then at 10,000 g for 30 minutes (Saha, 1956).

Paper electrophoretic fractionation of the cell-free hemolyzates was carried out in barbital buffer, pH 8.6, μ - 0.05, for 20-22 hours at 230 volts at 2-3°C in an LKB horizontal paper electrophoresis apparatus. After the completion of an electrophoretic run, the peak areas were cut out and eluted by descending chromatography with 0.05 M Na-phosphate buffer, pH 6.8, in a water-saturated, all glass chromatographic chamber at 20°C. The hemoglobin concentrations of both the effluents were determined spectrophotometrically as HbCO at 540 mμ and then brought to the same concentration with 0.05 M Na-phosphate buffer. The radioactivity was measured with a windowless gas flow counter. The results were calculated as the specific activity per mg of hemoglobin corrected to 1.0 micromole of the particular amino acid present in each component, the value of the amino acid content used in this calculation being based on the data reported by Helm and Huisman (1958).

The results presented in Table 1 show the over-all effect of the amino acid composition of the medium on the biosynthesis of cognate proteins. This effect has been found to be more pronounced with glycine-2-C^{14}. The nucleated chick erythrocytes seem to be capable of synthesizing both hemoglobin forms under the different experimental conditions of amino acid composition, although the rates of synthesis vary. It appears that the change in the composition of milieu induces an alteration in the rate of amino acid incorporation in the chick hemoglobins 1 and 2. When the chick erythrocytes were incubated in a medium CH 2 representing the amino acid composition of chick hemoglobin 2, the rate of labeled amino

acid incorporation in hemoglobin 1 is suppressed as compared with that in the other medium. Thus it implies that the rate of synthesis of two kinds of hemoglobin molecules is partially or wholly dependent on the

Table 1

In Vitro Biosynthesis of Chick Hemoglobins 1 and 2

Tracer	Composition of incubating media: Synthetic media	Plasma	Rate of incorporation: Hemoglobin 1	Hemoglobin 2	Ratio: Hemoglobin 1 / Hemoglobin 2
Leucine-U-C^{14}	HAH	-	292	203	1.43
	CH 1	-	250	162	1.53
	CH 2	-	209	172	1.21
Leucine-U-C^{14}	HAH	+	1973	1726	1.14
	CH 1	+	2554	1420	1.79
	CH 2	+	1930	1772	1.08
Alanine-1-C^{14}	HAH	-	89	84	1.03
	CH 1	-	110	71	1.54
	CH 2	-	69	56	1.22
Alanine-1-C^{14}	HAH	+	354	310	1.14
	CH 1	+	648	405	1.60
	CH 2	+	549	437	1.25
Glycine-2-C^{14}	CH 1	-	296	218	1.35
	CH 2	-	121	182	0.66

HAH, CH 1 and CH 2 represent the synthetic media prepared on the basis of the amino acid composition of human adult hemoglobin (Borsook, 1958) and chick hemoglobins 1 and 2 (Helm and Huisman, 1958). Plasma, whenever included in the incubating system, is in the ratio of 1:1 with the synthetic amino acid media. The rate of incorporation represents counts per minute per mg hemoglobin corrected to one micromole of the particular amino acid present in each component. The amino acid contents in μg/ml of incubating media HAH, CH 1, and CH 2 respectively are as follows: l-leucine - 131, 132, and 150; l-alanine - 45, 89.5, and 75.5; glycine - 100, 30.5, and 29.

composition of the physiological milieu, although it may also be influenced by other factors; viz. redox potential, oxygen tension, etc. It is quite possible that the environmental condition in the in vivo systems, e.g. bone marrow cells, etc., perhaps represses the incorporation rate of radioactive amino acids in chick hemoglobin 1 or increases that in hemoglobin 2. This idea seems to corroborate the earlier observation that with the embryonic development hemoglobin 2 increases while the other decreases (Saha, 1956).

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