On the mechanism of hemoglobin formation: Iron-inserting enzyme in duck erythrocytes

The authors have reported in the preceding paper on the extraction of the active principle which accelerates the incorporation of radioactive iron into protoporphyrin from duck-erythrocyte stroma with sodium cholate[1]. Two possibilities were discussed there that the active principle is either globin itself or a new enzyme. To solve this problem some purification of the principle was carried out by chromatography on Amberlite IRC 50. The estimation of the activity was made by incubating the preparation with radioactive iron, protoporphyrin and cysteine at 37°. The measurement of radioactive heme was done by essentially the same procedure as described in the preceding paper[2]. The reaction product obtained by the incubation was also analyzed by chromatography on Amberlite IRC 50 to know whether the radioactive heme-proteins synthesized were hemoglobin or not.

Thoroughly washed duck-erythrocyte stroma was extracted with 0.5 % sodium cholate in 0.05 *M* phosphate, pH 7.0. The extract showed two main peaks on chromatography on Amberlite IRC 50 equilibrated with 0.05 *M* phosphate, pH 6.0. Elutions were made by 0.1 *M* phosphate, pH 7.4. One was a fast-running component which appeared almost at the top of the effluent with almost colorless to straw yellow appearance and contained no hemoglobin (non-hemoglobin fraction). The other was a hemoglobin fraction. The ability to incorporate radioactive iron into protoporphyrin was found exclusively in the non-hemoglobin fraction (Table I). Thus, the active principle is not globin which behaves like hemoglobin on the column, as revealed by a preliminary experiment using native globin prepared according to JOPE[3].

TABLE I

DISTRIBUTION OF ACTIVITY

	*Activity** *counts/min*	E_{280}	E_{540}
Original cholate extract	5,300	29.4	7.5
Hemoglobin fraction**	60	8.4	2.4
Non-hemoglobin fraction**	2,300	1.7	negligible

* 1 ml of each sample was incubated for 3 h at 37° in air with [^{59}Fe]ferric ammonium citrate (1 μg Fe, $2.25 \cdot 10^6$ counts/min), protoporphyrin (0.04 μmole) and cysteine (0.2 μmole) in final vol. of 2 ml. The radioactive heme was extracted with HCl–acetone and transferred to a chloroform layer. Its radioactivity was measured by a well-type scintillation counter.

** Separated chromatographically on Amberlite IRC 50.

The radioactive heme formed in the cholate extract behaved identically with hemoglobin both by ion-exchange chromatography (Fig. 1) and by zone electrophoresis. However, a part of the radioactive heme was not bound to globin as was shown by the finding that the specific activity decreased after the chromatographical purification of the reaction product: for example, the specific activity of the reaction product, 7650 counts/min/mg hemin, became 5830 counts/min/mg hemin after chromatography. The radioactive heme of high specific activity other than hemoglobin was adsorbed firmly on the resin. On the other hand, the column chromatography of the reaction product obtained by the incubation of the non-hemoglobin fraction (prepared as in

Table I) showed the presence of only a small amount of radioactive heme in the effluent. Most of the radioactive heme was found at the upper portion of the column, just like the non-hemoglobin heme of high specific activity stated above. The nature of this heme fraction is not clear at present, though it may be somewhat like the firmly bound microsomal iron in reticulocytes reported by RABINOVITZ AND OLSON[4]. Free heme, which is essentially insoluble in neutral pH, behaved similarly on the column.

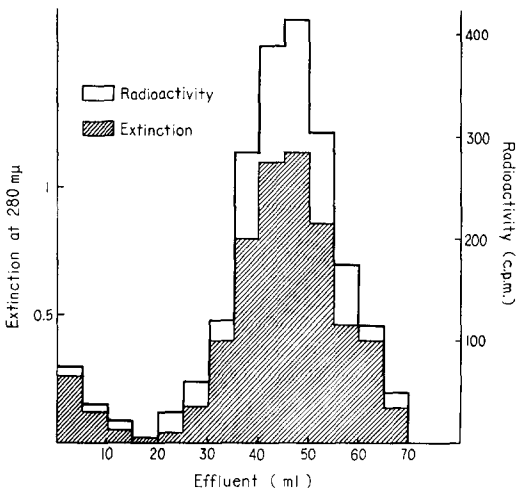

Fig. 1. Chromatographical analysis of radioactive heme synthesized by the cholate extract. The measurement of the radioactivity was as in Table I. Column (1.4 × 5 cm) of Amberlite IRC 50 CG 2, equilibrated with 0.05 *M* phosphate, pH 6.0. Elution was made by 0.1 *M* phosphate, pH 7.4.

The findings that the active principle free from globin can insert radioactive iron into protoporphyrin to form heme not bound to globin, whereas the hemolysate or the cholate extract forms hemoglobin, suggests the following mechanism of hemoglobin synthesis: the heme which was formed by the active principle may be transferred to pre-formed free globin. This is not in accordance with the suggestion of ERIKSEN[5] that iron is incorporated into a protoporphyrin-globin complex to form hemoglobin. The existence of free globin in duck erythrocytes was also suggested by the fact that the ratio of the specific activities of [2-^{14}C]glycine in heme and globin was always more than unity and decreased as the incubation proceeded[6].

Recently, several investigators have claimed that the process of heme synthesis from protoporphyrin and iron is an enzymic reaction[7].

However, KRUEGER, MELNICK AND KLEIN[8], who first prepared the active extract, hesitated to call it an enzyme, because the amounts of heme formed were not sufficiently large compared to the amount of the protein. According to the hypothesis of Eriksen, globin is able to accelerate the incorporation of iron into protoporphyrin, and HEIKEL, LOCKWOOD AND RIMINGTON[9] observed this accelerating effect of globin. If the active principle were globin itself, it could not be called an enzyme, but if the active protein itself is not the apo-protein of the heme-protein to be formed, it may be necessary at least to call the reaction catalytic. The active principle in rat-liver mitochondria which was extracted by cholate and purified by $(NH_4)_2SO_4$ fractionation, was proposed by the authors to be called an enzyme, as the active protein was

separated from the heme-proteins to be formed[2]. Similarly, the active fraction in Table I may have a right to be called an enzyme, although the naming may be postponed until the exact mechanism of the reaction is elucidated.

SHIGEKI MINAKAMI
YOSHIKO KAGAWA
YOSHIKI SUGITA
YOSHIMASA YONEYAMA
HARUHISA YOSHIKAWA

Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo (Japan)

1 Y. KAGAWA, S. MINAKAMI AND Y. YONEYAMA, *J. Biochem.*, 46 (1959) 771.
2 S. MINAKAMI, *J. Biochem.*, 45 (1958) 833.
3 E. M. JOPE, *Nature*, 164 (1949) 622.
4 M. RABINOVITZ AND M. OLSON, *Nature*, 181 (1958) 1665.
5 L. ERIKSEN, *Ciba Symposium on Porphyrin Biosynthesis and Metabolism*, London, 1955, p. 185.
6 Y. SUGITA, *J. Jap. Biochem. Soc.*, 31 (1959) 318.
7 R. F. LABBE, *Biochim. Biophys. Acta*, 31 (1959) 589.
8 R. C. KRUEGER, J. MELNICK AND J. R. KLEIN, *Arch. Biochem. Biophys.*, 64 (1956) 302.
9 T. HEIKEL, W. H. LOCKWOOD AND C. RIMINGTON, *Nature*, 182 (1958) 1119.

Received June 16th, 1959

Lactic oxidase *Tetrahymena pyriformis*: The over-all reaction mechanism

In a previous communication, the authors described an unusual lactic oxidase in the protozoan *Tetrahymena pyriformis*[1]. It was reported that pyruvate was the end-product of L-lactate oxidation, and that in crude preparations the enzyme did not (a) exhibit a requirement for DPN with O_2 as electron acceptor, (b) reduce DPN or oxidize DPNH in the presence of lactate or pyruvate, or (c) suffer appreciable inhibition by cyanide, amytal, 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone, lecithinase A (crotoxin), or an inhibitor obtained from the protozoan[2]. Since the latter 5 agents markedly inhibit the DPN-linked β-hydroxybutyric and glutamic oxidases—and except for amytal in relatively low concentration, succinic oxidase as well—it was apparent that, unlike the conventional lactic dehydrogenases, the *Tetrahymena* enzyme is not joined to the terminal electron-transport chain(s) involved in the oxidation of DPNH and succinate. This note outlines evidence for the mechanism of the over-all lactic oxidase reaction and presents some additional properties of the crude enzyme. Methods for the growth and collection of the protozoa and the preparation of cell-free homogenates have been described[3,4]. Lactic oxidase activity was measured by following O_2 consumption at 30° as indicated previously[1].

KEILIN AND HARTREE[5] have demonstrated that the addition of ethanol to such primary oxidizing systems as xanthine oxidase, uricase, glucose oxidase, and D-amino acid oxidase, already containing catalase, doubled the total O_2 uptake by these systems, since H_2O_2 formed in the primary reactions was utilized for the secondary or coupled oxidation of ethanol to acetaldehyde instead of being decomposed catalatically, liberating O_2. Similar experiments have been carried out with the lactic

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide.