

tions from normal serum was enhanced by prior treatment of serum donor with pituitary growth hormone; adrenocorticotrophic hormone or thyrotrophic hormone had no such effect. 2. Inhibitory fractions had gross properties of  $\beta$ -lipoproteins and could be prepared free of lipides having electrophoretic mobility of albumin or  $\alpha$ -globulins. Average ratio of total lipide to protein was .25 for fractions from diabetic rat serum and .11 for fractions from normal rat serum; phosphatide/total lipide ratio was lower in such fractions than in parent sera. Relation of inhibitory activity to lipide content requires further clarification, as inhibitory activity may be lost by delay during preparation of fractions without significant change of lipide content or electrophoretic behavior of fractions. 3. Inhibitory fractions interfered with stimulatory action of insulin on diaphragm muscle. The fraction from 1 ml of diabetic or normal rat serum nullified the stimulatory effect of at least .0004-.001 unit of insulin.

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Received December 15, 1958. P.S.E.B.M., 1959, v101.

## Net Synthesis of Heme from Protoporphyrin and Iron by Extracts of Duck Erythrocytes.\* (24814)

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Hemin obtained from incubated mixtures containing labeled ionic iron, protoporphyrin, and preparations of avian erythrocytes was found to contain appreciable labeled iron; when the porphyrin or cell preparation was omitted from mixtures, or mixtures were not incubated, little labeled iron was recovered as hemin(1-4). From these findings and others, in particular the heat-lability of the cell preparations, it was inferred that in avian erythrocytes protoporphyrin and ionic iron are the

immediate precursors of protoheme and that some component(s) of the erythrocytes, participates in heme formation. The inferences are reasonable; however, the evidence, at least for formation of heme from the presumed precursors, would be supplemented by establishing net synthesis of heme from protoporphyrin and iron in the presence of red-cell preparations. In particular, net synthesis would rule out the possibility that the recovery of labeled iron as hemin indicated above arose from artifact. The present report provides evidence

\* Supported by the U. S. Atomic Energy Comm.

for net synthesis of heme from iron and protoporphyrin in the presence of soluble material from duck erythrocytes.

**Methods.** Extracts of red cells were made as follows(2): at 1-4° cells were mixed with 2 volumes of water and let stand 30 minutes. One part of 1.72 *M* potassium chloride was added per 100 parts of added water. The mixture was centrifuged at about  $3 \times 10^4 \times g$  for 30 minutes. The sediment was washed twice in the centrifuge with 0.156 *M* potassium chloride and suspended in an equal volume of the dilute chloride. Tween 20, 0.4 ml/100 ml of suspension, was added. The mixture was let stand overnight and then centrifuged at  $3 \times 10^4 \times g$ . The supernate was used in the tests for heme synthesis. Test for synthesis was carried out as follows: In the cold sufficient solid potassium bicarbonate was added to the extract to make concentration 0.1 *M*. The mixture was gassed with 5% CO<sub>2</sub> in nitrogen for 5 minutes. In each of a number of 25 ml beakers was placed 28 micromoles of cysteine hydrochloride plus equivalent bicarbonate, 1 ml of solution containing about 250 millimicromoles of protoporphyrin(5), and 1 ml of solution containing a roughly equivalent amount of iron. Both solutions were prepared just before use. The iron, as ferrous ammonium sulfate alone or, when radioactive iron (Fe<sup>59</sup>) was used, as a mixture of the sulfate and ferric chloride, was dissolved in 10<sup>-4</sup> *M* hydrochloric acid plus 0.13 *M* potassium chloride. The porphyrin was dissolved in 0.015 *M* ammonium hydroxide plus 0.1 *M* bicarbonate. Shortly after addition of the other materials, a 12 ml portion of the gassed extract was added to each beaker. The test mixtures were then gassed with the carbon dioxide in a Dubnoff bath with shaking at 5° or less for 10 minutes. One half of each mixture was removed and treated for a given assay. The remainder under the gas was incubated at 37° for 30 minutes, then treated for the assay. The pH of the test mixture at room temperature initially and at the end of incubation was about 8.0, the optimum for heme formation(2). Inorganic iron in the samples of mixture was estimated by treating protein-free filtrates(6) with reductant and

o-phenanthroline(7). Porphyrin and heme were extracted from other samples with acid ethyl acetate(8). The porphyrin was removed from the acetate with 3 *N* hydrochloric acid and assayed in an Evelyn colorimeter(9) standardized spectrophotometrically(10). After porphyrin was removed, the acetate, which contained the heme, was treated in one of the following ways: (a) It was extracted with 10 ml portions of 1 *N* ammonium hydroxide until heme appeared in the alkali. The portion of alkali containing heme and 2 further portions were combined. (b) It was mixed with a little water, heated to remove the ethyl acetate, and neutralized with alkali. The heme in the solutions obtained by (a) and (b) was converted to reduced cyanide hemochromogen and assayed in an Evelyn colorimeter standardized spectrophotometrically(11). (c) The solution of heme in the acetate was digested with sulfuric acid and peroxide and assayed for iron(7). (d) When radioactive iron was used, radioactivity of the ethyl acetate mixture or of the ammoniacal extract (*cf.* (a) above) was determined. Heme formation was estimated from this activity and initial levels of inorganic and active iron. Heme formation was also estimated from these levels and the activity recovered as hemin(2).

Representative *results* are given in Table I. As indicated by Exp. 1-9, the decrease in inorganic iron and in porphyrin during incubation of complete test mixtures were in fair agreement with each other and the increase in heme. In the absence of added porphyrin (*cf.* Experiment 7*b*), there was essentially no change in level of iron or heme during incubation or indication of porphyrin formation. Without added iron a little porphyrin disappeared (*cf.* Experiment 7*c*). This may represent heme formation since the test mixtures contained a little inorganic iron initially, some of which disappeared during incubation. As indicated earlier by use of labeled iron(1-4), no appreciable heme formation occurred in the cold, (*cf.* Experiments 4 and 5), with preparations heated to 100° (*cf.* Experiment 8), or in absence of the cell preparation (*cf.* Experiment 10).

The relatively direct estimates of heme

TABLE I. Synthesis of Heme from Protoporphyrin and Inorganic Iron in Presence of Extracts of Duck Erythrocytes. Experimental details given in text. In each experiment a different sample of blood was used for preparation of the extract. In Exp. 1, 5 and 8 the iron used contained  $\text{Fe}^{59}$  giving about  $2.5 \times 10^4$  counts/min. An increase in amount of material designated +, a decrease -.

| Exp.     | Component of test mixture assayed | Change in amt of component/7 ml of test mixture during 30-min. incubation |                   | Estimates of heme used; exp. conditions other than indicated above                                                                                                                                                 |
|----------|-----------------------------------|---------------------------------------------------------------------------|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|          |                                   | Amt of component present in 7 ml test mixture initially                   | $\mu\text{moles}$ |                                                                                                                                                                                                                    |
| 1        | Porphyrin                         | 235                                                                       | - 80              | Heme estimated from $\text{Fe}^{59}$ in ammoniacal extr. and in hemin.                                                                                                                                             |
|          | Iron                              | 250                                                                       | - 64              |                                                                                                                                                                                                                    |
|          | Heme                              |                                                                           | +79               |                                                                                                                                                                                                                    |
|          | "                                 |                                                                           | +78               |                                                                                                                                                                                                                    |
| 2        | Porphyrin                         | 248                                                                       | - 86              | Heme from ethyl acetate estimated as hemochromogen.                                                                                                                                                                |
|          | Iron                              | 265                                                                       | - 88              |                                                                                                                                                                                                                    |
|          | Heme                              | 1800                                                                      | +120              |                                                                                                                                                                                                                    |
| 3        | Porphyrin                         | 214                                                                       | - 66              | Heme from ammoniacal extr. estimated as hemochromogen.                                                                                                                                                             |
|          | Iron                              | 260                                                                       | - 71              |                                                                                                                                                                                                                    |
|          | Heme                              | 1440                                                                      | +40               |                                                                                                                                                                                                                    |
| 4        | Porphyrin                         | 240                                                                       | - 70 (+ 1)        | Heme estimated as iron in ethyl acetate. Results in parentheses obtained with test mixtures kept at $2^\circ$ for 30 min.                                                                                          |
|          | Iron                              | 262                                                                       | - 67 (+ 2)        |                                                                                                                                                                                                                    |
|          | Heme                              | 1280                                                                      | +80 (- 1)         |                                                                                                                                                                                                                    |
| 5        | Porphyrin                         | 241                                                                       | - 65 (- 2)        | Heme estimated from $\text{Fe}^{59}$ in ethyl acetate. Results in parentheses obtained with test mixtures kept at $2^\circ$ for 30 min.                                                                            |
|          | Iron                              | 259                                                                       | - 59 (+ 3)        |                                                                                                                                                                                                                    |
|          | Heme                              |                                                                           | +72 (+ 4)         |                                                                                                                                                                                                                    |
| 6        | Porphyrin                         | 242 ( 246)                                                                | - 49 (- 51)       | Heme estimated from iron in ethyl acetate. Results in parentheses obtained with test mixtures that included preparation of globin(15).                                                                             |
|          | Iron                              | 248 ( 252)                                                                | - 45 (- 46)       |                                                                                                                                                                                                                    |
|          | Heme                              | 1350 (1370)                                                               | +50 (+50)         |                                                                                                                                                                                                                    |
| 7a       | Porphyrin                         | 221                                                                       | - 81              | Heme estimated from iron in ethyl acetate. In <i>b</i> porphyrin omitted. In <i>c</i> iron omitted.                                                                                                                |
|          | Iron                              | 245                                                                       | - 72              |                                                                                                                                                                                                                    |
|          | Heme                              | 1770                                                                      | +70               |                                                                                                                                                                                                                    |
| <i>b</i> | Porphyrin                         | 1                                                                         | - 1               |                                                                                                                                                                                                                    |
|          | Iron                              | 245                                                                       | + 3               |                                                                                                                                                                                                                    |
|          | Heme                              | 1780                                                                      | - 5               |                                                                                                                                                                                                                    |
| <i>c</i> | Porphyrin                         | 224                                                                       | - 5               |                                                                                                                                                                                                                    |
|          | Iron                              | 10                                                                        | - 4               |                                                                                                                                                                                                                    |
|          | Heme                              | 1770                                                                      | 0                 |                                                                                                                                                                                                                    |
| 8        | Porphyrin                         | 242                                                                       | - 89 (- 2)        | Heme estimated from $\text{Fe}^{59}$ in hemin. Results in parentheses obtained with extr. heated to $100^\circ$ for 7 min.                                                                                         |
|          | Iron                              | 258                                                                       | - 80 (+ 3)        |                                                                                                                                                                                                                    |
|          | Heme                              |                                                                           | +92 (+ 4)         |                                                                                                                                                                                                                    |
| 9        | Porphyrin                         | 239                                                                       | - 66              | Heme estimated as iron in ethyl acetate. Cell preparation prepared by use of ammonium sulfate.                                                                                                                     |
|          | Iron                              | 250                                                                       | - 68              |                                                                                                                                                                                                                    |
|          | Heme                              | 102                                                                       | +71               |                                                                                                                                                                                                                    |
| 10       | Porphyrin                         | 235 (237)                                                                 | - 3 (- 2)         | Heme estimated as iron in ethyl acetate. Erythrocyte preparation omitted from test mixtures; comparable salt mixture + detergent was substituted. Results in parentheses obtained with mixtures containing globin. |
|          | Iron                              | 250 (255)                                                                 | + 2 (+ 4)         |                                                                                                                                                                                                                    |
|          | Heme                              | 0                                                                         | + 1 (- 2)         |                                                                                                                                                                                                                    |

formation used in Experiments 2-4, 6, and 7 were of limited accuracy since the heme formed in these and similar tests did not exceed 8% of that present initially. Increase in the ratio of heme formed to initial heme was obtained with cellular material prepared as follows: In the cold, extract obtained as de-

scribed above was mixed with 0.1 volume of 0.5 *M* potassium phosphate and 0.5 volume of saturated ammonium sulfate, each at pH 7.2. The resulting precipitate was collected by centrifugation, dissolved in a volume of .1 *M* potassium bicarbonate plus .156 *M* chloride equal to one-fourth that of the extract, and

tested as described above. The final solutions prepared by the sulfate treatment, were usually about as active as the extracts from which they were prepared; thus, about one-fourth of the activity was recovered. As indicated by Exp. 9, the ratio of heme formed to initial heme was increased by the sulfate treatment. Similar results were obtained using other estimates of heme. Although the concentration of hemoglobin, indicated by the heme content, in the solutions obtained by the sulfate treatment was less than in the extracts, the activity per unit of protein, as indicated earlier for the simpler cell preparation(2), was not sufficient to allow unequivocal assignment of the activity to a catalyst.

**Discussion.** The present results establish net synthesis of heme from protoporphyrin and ionic iron in the presence of material from duck erythrocytes and indicate, as reported earlier(1-4), that the material from the erythrocytes is necessary for the synthesis. However, it appears that under certain conditions(12-14), notably in the presence of a detergent and globin(14), heme is formed at neutral pH and moderate temperature without addition of cellular material. Under present experimental conditions globin had no effect on heme synthesis in the presence of red-cell extracts (*cf.* Experiment 6) and alone (*cf.* Experiment 10) did not promote synthesis. Nevertheless, it is possible that the material from the avian erythrocytes in some relatively

non-specific way simply creates a suitable environment for heme synthesis.

**Summary.** Soluble preparations of duck erythrocytes were found to effect net synthesis of heme from protoporphyrin and iron.

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Received February 18, 1959. P.S.E.B.M., 1959, v101.

## Repopulation of Hematopoietic Tissues of X-Irradiated Mice by Cells From Leukemoid Blood. (24815)

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Congdon *et al.*(1) reported that injection of blood showing extreme granulocytosis induced by a tumor prolonged survival of mice that had been given a lethal dose of total-body x-irradiation. Congdon *et al.* suggested that the leukemoid blood might protect against early death by some mechanism other than repopulation. The leukemoid blood might pro-

vide a humoral factor which would stimulate recovery of irradiated hematopoietic tissues or large number of leukocytes might help combat infection. Later Smith and Congdon(2) found evidence indicating that injected blood cells probably repopulated at least some hematopoietic tissues. More conclusive evidence that repopulation takes place is reported here.