Role of Mitochondria in the in vitro Formation of Protoporphyrin and Haem

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Although the broad outline of the pathway of haem biosynthesis is now firmly established (1), certain points of detail remain to be elucidated. In particular, the transformation of coproporphyrinogen to protoporphyrin, involving oxidative decarboxylation of two carboxyethyl side chains (positions 2 and 4) to yield vinyl groups and the mechanism of insertion of iron into the porphyrin ring to form haem are still poorly understood. Both steps appear to be enzymically controlled but the number of enzymes involved and whether or not cofactors are necessary are unknown.

That mammalian reticulocytes are capable of synthesizing haem in vitro was first demonstrated by Reimann (2), in 1942 and has since been amply confirmed (3, 4). These cells contain mitochondria (5-8) as do also the synthetically-active basophilic stippled cells of lead poisoned animals (9). Sano (10) has shown that mitochondria participate at three stages in the biosynthesis of haem, namely (1) in the formation of δ-aminolaevulic acid (ALA) from glycine and active succinate (11-14), (2) in some step or steps in the synthesis of protoporphyrin from ALA and (3) in the incorporation of iron into the porphyrin ring. He found that in stage (2) an extract prepared from an acetone powder was as effective as intact mitochondria from rabbit liver, chicken erythrocytes, bone marrow or mesenteric lymph glands. Mitochondria from rat kidney, heart, muscle and intestinal mucosa were inactive.

Formation of Protoporphyrin

Falk, Dresel and Rimington (15) showed that haemolysates of chicken red cells produced large quantities of protoporphyrin and smaller amounts of coproporphyrin and uroporphyrin from added porphobilinogen (PBG). The supernatant fraction derived from centrifugation of such haemolysates (20,000 × g for 20 minutes) formed only uro- and coproporphyrins but protoporphyrin production was restored by adding a rat liver mitochondrial preparation. Mitochondria alone produced no porphyrin (16). Haemolysates of human red cells were found by Rimington and Booij (17) to form only traces of protoporphyrin from PBG but, here again, addition of liver mitochondria raised the yield fifteen or twenty fold. A system or fraction present in mitochondria appears to be necessary to complete the protoporphyrin-forming system of red cell haemolysates. Falk, Dresel and Rimington (15) found that oxygen was necessary for protoporphyrin production by the whole haemolysate of chicken red cells from PBG. Replacement of air by nitrogen markedly lowered the yield of this porphyrin suggesting that oxygen was involved as a hydrogen acceptor in the overall oxidative decarboxylation reaction.

The experiments which we now report have extended over the last two years and have been directed towards a more detailed elucidation of the role of mitochondria in systems derived from human and chicken red blood cells or from Rhodopseudomonas spheroides.

METHODS

Chicken Red Cell Haemolysate (CH)—This was prepared according to Dresel and Falk (18).

Chicken Red Cell Erythro (CRZ)—Heparinized
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chicken blood (10 ml.) was centrifuged at approximately 2000 x g for 10 minutes at 0°C, plasma removed and the cells suspended in 0.9% NaCl. After centrifuging as before, the saline and white cell layer was removed and the cells washed once more by 0.9% NaCl. Benzene (1 ml.) was added to the red cell mass which was shaken until haemolysis was complete (1 minute). An equal volume of glass-distilled water was then added and after thorough mixing by shaking for 1 minute, the enzyme-containing supernatant was separated by centrifuging for 10 minutes at room temperature and removing the benzene layer. It was filtered through paper and stored at 5°C. This clear preparation yielded no deposit when it was centrifuged for 15 minutes at 11,750 x g.

Preparation of Mitochondria—The weighed liver of a freshly killed rat was homogenised in 0.25 molar sucrose (10% w/v). After centrifuging at 2000 x g for 10 minutes at 5°C, the supernate was removed and centrifuged at 11,750 x g for 15 minutes at 0°C. The residue was suspended in 0.25 molar sucrose and again centrifuged under the same conditions. The final residue was suspended in a volume of glass-distilled water equal to half the weight of liver used and stored at -10°C.

Mitochondrial Supernatant—The suspension of mitochondria was frozen and thawed three times then centrifuged at 11,750 x g for 15 minutes at 0°C and the supernatant collected.

Acetone Powder of Mitochondria—A suspension of mitochondria in glass-distilled water (2 ml.) was shaken with ice-cold acetone (40 ml.) and left to stand at -10°C for 30 minutes. The mixture was filtered in the cold on a Buchner funnel and the residue washed with 20 ml. of ice-cold acetone, then dried in a vacuum desiccator. To prepare an extract, the powder was suspended in glass-distilled water (2 ml.), ground up thoroughly and the supernatant recovered after centrifuging at 11,750 x g for 15 minutes.

Coproporphyrinogen—A strong solution (2 ml.) of coproporphyrin III in 0.2 N NaOH was shaken in a glass-stoppered flask wrapped in black cloth with 4 g. freshly ground sodium amalgam for 5 minutes. The colourless solution was then filtered rapidly with the aid of suction through four layers of Whatman No. 42 filter paper on a sintered glass funnel into a small test tube. The pH was cautiously adjusted to approximately 7.5.

Porphobilinogen (PBG)—This was dissolved in phosphate buffer pH 7.4 (1 ml. of 0.1 molar KH₂PO₄ + 4 ml. of 0.1 molar Na₂HPO₄).

Incubation—Small test-tubes were immersed in a water-bath at 37°C for the times and under the conditions stated. For anaerobic experiments, evacuated Thunberg tubes were used containing alkaline pyrogallol in the side arm. Experiments were conducted in subdued light or darkness.

Analytical Methods—The contents of the tubes were mixed with ethyl acetate: acetic acid (3:1) and centrifuged for 5 minutes at 2000 x g. After removal of the supernatant into a small separatory funnel, the residue was washed repeatedly on the centrifuge with the acid mixture until the supernatant was no longer fluorescent. The bulked supernatants were shaken twice with saturated sodium acetate and these washings re-extracted by ethyl acetate until the latter was no longer fluorescent. It was then added to the main extract which was shaken once with 3% sodium acetate. Total porphyrin was then extracted by shaking with 15% HCl leaving haem in the organic phase. In experiments using Fe⁹², this was made up to 25 ml. and the radioactivity counted in a well-type scintillation counter.

The 15% HCl solution containing the porphyrins was neutralized to congo red with saturated sodium acetate and the porphyrins extracted by repeated shaking with ether. After one wash by 3% sodium acetate followed by one with water, the ether was shaken with small quantities of 0.36% HCl until all coproporphyrin was removed and then with 5% HCl to remove protoporphyrin. Optical density of each extract was then determined at 380 mµ, 430 mµ and at the Soret maximum (Dmax) using 1 cm. cells and a Unicam photoelectric spectrophotometer. Porphyrin content was calculated using the correction formula of Rimington and Sveinsson (19), viz:

$$\text{μg. Copro.} = (2D_{\text{max}} - D_{990} - D_{430}) \times \frac{1.5}{1.833} \times V$$

$$\text{μg. Proto.} = (2D_{\text{max}} - D_{990} - D_{430}) \times \frac{2.04}{1.67} \times V$$

where V is the volume of the extract in each case.

Control experiments showed that any porphyrinogen originally present was oxidized to porphyrin during the manipulations and included in the final extract.

Fe⁹²—This was obtained from the Radiochemical Centre, Amersham, and used as FeCl₃ diluted with sterile 0.9% NaCl.

All reagents were of analytical grade and only glass-distilled water was used.

RESULTS

Analytical Recovery experiments—Some absorption with consequent loss of porphyrin upon precipitated protein occurred whatever...
precipitant was used. Acetone was perhaps somewhat superior to the ethyl acetate: acetic acid mixture but was less convenient in fur-

**Table I**

Recovery of Coproporphyrin Added to Enzyme Preparation CBZ

<table>
<thead>
<tr>
<th>Porphyrin added (µg.)</th>
<th>Recovery (%)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.60</td>
<td>94.9</td>
<td>No enzyme added.</td>
</tr>
<tr>
<td>13.60</td>
<td>94.2</td>
<td>No enzyme added.</td>
</tr>
<tr>
<td>5.44</td>
<td>88.3</td>
<td>No enzyme added.</td>
</tr>
<tr>
<td>5.44</td>
<td>86.4</td>
<td>No enzyme added.</td>
</tr>
<tr>
<td>5.44</td>
<td>81.5</td>
<td>Precipitated immediately.</td>
</tr>
<tr>
<td>5.44</td>
<td>76.8</td>
<td>Precipitated immediately.</td>
</tr>
<tr>
<td>5.44</td>
<td>71.0</td>
<td>Precipitated immediately and left overnight at room temperature.</td>
</tr>
<tr>
<td>3.28</td>
<td>66.0</td>
<td>Precipitated immediately.</td>
</tr>
<tr>
<td>3.28</td>
<td>72.2</td>
<td>Precipitated immediately.</td>
</tr>
<tr>
<td>3.28</td>
<td>66.7</td>
<td>Acetone used as precipitant.</td>
</tr>
<tr>
<td>3.28</td>
<td>74.6</td>
<td>Acetone used as precipitant.</td>
</tr>
<tr>
<td>4.00</td>
<td>71.8</td>
<td>Acetone: HCl used as precipitant.</td>
</tr>
</tbody>
</table>

Some typical results are included in Table I.

**Porphyrins Formed from Porphobilinogen (PBG)**

by Different Enzyme Preparations—Chicken red-cell haemolysate contains all the enzymes necessary for the synthesis of haem from glycine. When δ-aminolaevulic acid or porphobilinogen is used as substrate, in addition to haem much protoporphyrin is produced together with lesser quantities of coproporphyrin. A similarly prepared haemolysate from human erythrocytes and PBG forms very little protoporphyrin unless mitochondria are also added. The preparation made with benzene from chicken red-cells, CBZ, also retains the enzymes necessary for the utilization of PBG but porphyrin production stops at the coproporphyrin stage unless mitochondria are added. Mitochondria alone produced

**Table II**

Properties of Different Enzyme Preparations Utilizing PBG as Substrate

Incubation for 15 hours at 37°C except when stated.

<table>
<thead>
<tr>
<th>ENZYME PREPARATION</th>
<th>Mitochondria</th>
<th>PBG (µg.)</th>
<th>Copro. (µg.)</th>
<th>Proto. (µg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken red-cell haemolysate, CH, 4 hrs.</td>
<td>100</td>
<td>1.44</td>
<td>8.32</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>15</td>
<td>0.16</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>+</td>
<td>0.06</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.11</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.02</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Chick red-cell, benzene method, CBZ</td>
<td>30</td>
<td>5.09</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.20</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+(heated)</td>
<td>0.05</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.11</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Human red-cell, benzene method</td>
<td>0.15</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.76</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.04</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+(heated)</td>
<td>0.15</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>
no porphyrin from PBG and heating destroyed their ability to complete the protoporphyrin-forming system. Some properties of the different enzyme preparations are recorded in Table II.

Properties of the Chick Red-Cell Preparation CBZ—The soluble enzyme system prepared from chick red cells by the benzene method was selected for further study. The optimum pH was found to be \( \sim 7.4 \). The time course of coproporphyrin production from PBG (60 µg.) is illustrated in Fig. 1, whilst Fig. 2 shows the increase of coproporphyrin and protoporphyrin with time when mitochondria are added to the preparation.

The preparation is moderately stable, 80% activity being retained during 15 days at 5°C.

When the enzyme preparation was ultrafiltered and the activities of resuspended residue and of ultrafiltrate, respectively, were separately measured, all the activity was found to reside in the residue with no indication of the requirement of any filterable cofactor (Table III).

### Table III

<table>
<thead>
<tr>
<th>Formation of Porphyrins from PBG (30 µg.) by Non-filterable Residue and Ultrafiltrate of Enzyme Preparation CBZ</th>
<th>Copro. (µg.)</th>
<th>Proto. (µg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole enzyme preparation</td>
<td>2.98</td>
<td>0.22</td>
</tr>
<tr>
<td>Non-filterable residue</td>
<td>3.32</td>
<td>0.54</td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Recombined fractions</td>
<td>2.57</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Role of Mitochondria in Protoporphyrin forma-
tion—The chick red-cell preparation CBZ made with the use of benzene carries porphyrin formation no further than to coproporphyrin. If rat liver mitochondria are added, the oxidative decarboxylation to protoporphyrin proceeds and it was thought that a closer study of this phenomenon might throw light upon the mechanism of the reaction.

Not all mitochondria were effective, those prepared from rat heart or kidney being inactive and rat spleen mitochondria only feebly active.

Liver mitochondria retain their activity during storage at -5°C for 8 weeks but are inactivated by 2 minutes heating at 100°C. Much of the activity is retained by an acetone powder preparation. Disruption by repeated freezing and thawing caused no loss of activity and after centrifuging at 11,750×g for 15 minutes, activity was recovered in the supernatant. Ultrafiltration of the latter showed that activity resided in the non-filterable residue; it was destroyed by heating to 100°C. These properties, which are illustrated in Table IV, indicate that the factor supplied by liver mitochondria is enzymic in nature and is not a thermostable co-factor.

It is now well established that the porphyrinogens rather than the porphyrins are the true intermediates in protoporphyrin and haem synthesis. Experiments were therefore carried out using coproporphyrinogen III as substrate with and without the addition of mitochondria or the supernate from frozen and thawed mitochondria.

From Table V it will be seen that the supernate was again as active as intact mitochondria in completing the protoporphyrin forming system.

In many enzymic systems, ferricyanide is able to replace oxygen as the final hydrogen acceptor; preliminary experiments were there-

<table>
<thead>
<tr>
<th>Preparation of fraction added</th>
<th>Copro. (µg.)</th>
<th>Proto. (µg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.03</td>
<td>0.21</td>
</tr>
<tr>
<td>Whole mitochondria (rat liver)</td>
<td>6.74</td>
<td>2.69</td>
</tr>
<tr>
<td>Whole mitochondria (rat liver)</td>
<td>3.03</td>
<td>0.98</td>
</tr>
<tr>
<td>Acetone powder of above</td>
<td>3.63</td>
<td>0.76</td>
</tr>
<tr>
<td>Supernatant after freezing and thawing, 0.1 ml.</td>
<td>1.28</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>0.2 ml.</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>0.3 ml.</td>
<td>0.43</td>
</tr>
<tr>
<td>None</td>
<td>6.05</td>
<td>0.16</td>
</tr>
<tr>
<td>Supernatant of frozen and thawed mitochondria (rat liver)</td>
<td>2.49</td>
<td>1.65</td>
</tr>
<tr>
<td>Non-ultrafilterable residue of above</td>
<td>1.88</td>
<td>2.39</td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>5.87</td>
<td>0.10</td>
</tr>
<tr>
<td>None</td>
<td>9.95</td>
<td>0.36</td>
</tr>
<tr>
<td>None</td>
<td>10.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Whole mitochondria (rat heart)</td>
<td>10.00</td>
<td>0.29</td>
</tr>
<tr>
<td>Whole mitochondria (rat spleen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.52</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>2.85</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>1.53</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>1.81</td>
<td>0.15</td>
</tr>
</tbody>
</table>
TABLE V
Formation of Protoporphyrin from Coproporphyrinogen III (C\textsuperscript{3}GN) in Presence of CBZ and Rat Liver Mitochondria or a Supernate Prepared from Them by Freezing and Thawing

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>Substrate</th>
<th>Copro. (\textmu g.)</th>
<th>Proto. (\textmu g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBZ + rat liver mitochondria</td>
<td>PBG (30 \textmu g.)</td>
<td>5.48</td>
<td>2.74</td>
</tr>
<tr>
<td></td>
<td>C\textsuperscript{3}GN</td>
<td>5.74</td>
<td>1.01</td>
</tr>
<tr>
<td>Mitochondria only</td>
<td></td>
<td>7.06</td>
<td>0.71</td>
</tr>
<tr>
<td>None</td>
<td>C\textsuperscript{3}GN</td>
<td>3.46</td>
<td>0.04</td>
</tr>
<tr>
<td>CBZ + rat liver mitochondria</td>
<td></td>
<td>2.42</td>
<td>1.16</td>
</tr>
<tr>
<td>Mitochondria only</td>
<td></td>
<td>3.14</td>
<td>0.09</td>
</tr>
<tr>
<td>CBZ + mitochondrial supernate</td>
<td>C\textsuperscript{3}GN</td>
<td>25.20</td>
<td>4.26</td>
</tr>
<tr>
<td>Supernate only</td>
<td></td>
<td>26.00</td>
<td>0.55</td>
</tr>
</tbody>
</table>

TABLE VI
Effect of Some Inhibitors on Conversion of PBG (30 \textmu g.) to Porphyrins by Preparation CBZ

<table>
<thead>
<tr>
<th>Addition</th>
<th>Copro. (\textmu g.)</th>
<th>Proto. (\textmu g.)</th>
<th>Total (\textmu g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.33</td>
<td>0.43</td>
<td>3.76</td>
</tr>
<tr>
<td>K ferricyanide (10\textsuperscript{-3}M)</td>
<td>0.19</td>
<td>0.09</td>
<td>0.28</td>
</tr>
<tr>
<td>Liver mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>//</td>
<td>4.3</td>
<td>5.9</td>
<td>10.2</td>
</tr>
<tr>
<td>//</td>
<td>4.2</td>
<td>4.6</td>
<td>8.8</td>
</tr>
<tr>
<td>// + NaF (10\textsuperscript{-2}M)</td>
<td>4.3</td>
<td>6.2</td>
<td>10.5</td>
</tr>
<tr>
<td>//</td>
<td>4.2</td>
<td>6.2</td>
<td>10.4</td>
</tr>
<tr>
<td>// + (5 \times 10\textsuperscript{-2}M)</td>
<td>3.3</td>
<td>6.5</td>
<td>9.8</td>
</tr>
<tr>
<td>//</td>
<td>3.4</td>
<td>6.7</td>
<td>10.1</td>
</tr>
<tr>
<td>None</td>
<td>2.44</td>
<td>0.18</td>
<td>2.62</td>
</tr>
<tr>
<td>Liver mitochondria</td>
<td>0.59</td>
<td>0.75</td>
<td>1.34</td>
</tr>
<tr>
<td>//</td>
<td>0.16</td>
<td>0.92</td>
<td>1.08</td>
</tr>
<tr>
<td>// + iodoacetate (10\textsuperscript{-3}M)</td>
<td>0.19</td>
<td>1.10</td>
<td>1.29</td>
</tr>
<tr>
<td>//</td>
<td>0.23</td>
<td>1.21</td>
<td>1.44</td>
</tr>
<tr>
<td>None</td>
<td>2.91</td>
<td>0.41</td>
<td>3.32</td>
</tr>
<tr>
<td>Liver mitochondria</td>
<td>0.53</td>
<td>1.30</td>
<td>1.83</td>
</tr>
<tr>
<td>//</td>
<td>0.39</td>
<td>2.39</td>
<td>2.78</td>
</tr>
<tr>
<td>// + o-phenanthroline (5 \times 10\textsuperscript{-3}M)</td>
<td>0.55</td>
<td>1.70</td>
<td>2.25</td>
</tr>
<tr>
<td>//</td>
<td>0.65</td>
<td>0.88</td>
<td>1.53</td>
</tr>
<tr>
<td>None</td>
<td>4.58</td>
<td>1.74\textsuperscript{1)}</td>
<td>6.32</td>
</tr>
<tr>
<td>KCN (5 \times 10\textsuperscript{-2}M)</td>
<td>2.91</td>
<td>2.79\textsuperscript{1)}</td>
<td>5.70</td>
</tr>
<tr>
<td>None</td>
<td>5.80</td>
<td>2.12\textsuperscript{1)}</td>
<td>7.92</td>
</tr>
<tr>
<td>KCN (5 \times 10\textsuperscript{-2}M)</td>
<td>4.68</td>
<td>3.08\textsuperscript{1)}</td>
<td>7.76</td>
</tr>
</tbody>
</table>

1) Uroporphyrin (\textmu g.)
fore carried out in which this substance was added to the CBZ system. Potassium ferriycyanide at $10^{-3}M$ concentration had a generally inhibitory effect, probably due to oxidation by it of coproporphyrinogen to coproporphyrin as the latter was formed in the system from the substrate (PBG). Ferricyanide could not, therefore, replace rat liver mitochondria.

The action of some other substances is also recorded in Table VI. Sodium fluoride was without significant effect up to a concentration of $10^{-2}M$. Since the CBZ preparation is capable of forming haem from protoporphyrin in the presence of mitochondria, one observes a decreased yield of total porphyrin when comparing experiments with and without addition of mitochondria although in the former case protoporphyrin production is considerably raised at the expense of coproporphyrin. Iodoacetate between $10^{-3}M$ and $10^{-2}M$ definitely enhanced protoporphyrin production.

The effect of cyanide was examined, aerobically and anaerobically on the CBZ preparation acting on PBG without added mitochondria and in these experiments yields of uroporphyrin and coproporphyrin only were determined, because Lockwood and Benson (20) have shown that in the presence of KCN this system forms coproporphyrinogen I; no further conversion to protoporphyrin would be expected since the enzymes responsible for this step act only on the III series isomer.

It will be seen from Table VI that KCN in a concentration of $5\times10^{-2}M$ had little effect upon the quantity of porphyrin produced from PBG except that the uro-copro conversion was slightly retarded both in aerobic and anaerobic conditions.

The most interesting effect upon the CBZ system was obtained with o-phenanthroline. Two actions appear to be discernable, an increase in the proportion of protoporphyrin to coproporphyrin formed but also a generally inhibitory effect, the total porphyrin yield decreasing progressively as the concentration of o-phenanthroline is raised.

**Formation of Haem**

The utilization of iron by red-cell haemolyzates to produce haem appears to be an enzymically catalysed reaction (21-24). Glutathione, ascorbic acid or cysteine act as co-factors (21, 23, 25) and it is probable that protoporphyrin rather than protoporphyrinogen is the immediate precursor (24).

The utilization of iron by intact avian erythrocytes and mammalian reticulocytes appears to follow a similar course (4) as does also haem synthesis by liver cell preparations (26-28).

There is, in addition, evidence suggesting that iron taken up by the reticulocyte is first bound to a protein constituent of the cell membrane from which it is released for haem formation (4, 29); binding to the microsomes may also be involved (30). Protoporphyrin may also react in a protein-bound form in haem synthesis (26, 28, 31).

A suitable system for investigation of the enzymic union of iron and protoporphyrin is provided by the chicken red-cell haemolyzate preparation CBZ, already described. Protoporphyrin was added in phosphate buffer, pH 7.4 (1 ml. of $0.1M$ KH$_2$PO$_4$ and 4 ml. of $0.1M$ Na$_2$HPO$_4$) and Fe$^{3+}$ as the citrate. After incubation at 37°C under the conditions specified in each experiment, haem was extracted completely by successive treatments with ethylacetate-acetic acid mixture (3:1). The combined extracts were treated as described under Methods and activity measured after dilution to 25 ml; the necessary background and blank reading were recorded.

**RESULTS**

The chicken red-cell preparation CBZ had relatively feeble activity, but this was slightly increased by the addition of ascorbic acid ($10^{-3}M$) and markedly augmented by the addition of a suspension of liver mitochondria (Table VII).

As in the experiments upon protoporphyrin formation, the activity for haem formation was next tested of a supernate prepared by freezing and thawing rat liver...
TABLE VII

Synthesis of Haem from Added Protoporphyrin and Fe59 by Chicken Red-Cell Enzyme Preparation CBZ alone and in Presence of Ascorbic Acid or Rat Liver Mitochondria

<table>
<thead>
<tr>
<th>CBZ</th>
<th>Mitochondria</th>
<th>Ascorbic acid ((10^{-3} M))</th>
<th>Protoporphyrin</th>
<th>Fe59 added ((\text{c.p.m.}))</th>
<th>Haem formed ((\text{c.p.m.}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>7832</td>
<td>544</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>124</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>37</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>115</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td>5.7 µg.</td>
<td>685</td>
<td>4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>4</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>78</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>92</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0</td>
<td>&quot;</td>
<td>&quot;</td>
<td>84</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>123</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>+</td>
<td>13.8 µg.</td>
<td>252</td>
<td>8</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>85</td>
</tr>
</tbody>
</table>

TABLE VIII

Comparison of the Activity of Rat Liver Whole Mitochondria with That of the Supernate Prepared from Them by Freezing and Thawing on Haem Synthesis in Presence of CBZ

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Mito. supernant</th>
<th>Substrate</th>
<th>Fe59 added ((\text{c.p.m.}))</th>
<th>Haem formed ((\text{c.p.m.}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0</td>
<td>Protop.</td>
<td>7832</td>
<td>544</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>32</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>PBG (30 µg.)</td>
<td>&quot;</td>
<td>528</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>106</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>&quot;</td>
<td>&quot;</td>
<td>935</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>113</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>&quot;</td>
<td>&quot;</td>
<td>938</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>&quot;</td>
<td>&quot;</td>
<td>130</td>
</tr>
</tbody>
</table>

TABLE IX

Haem-forming Activity of Rat Liver Mitochondria Preparations; Effect of Anaerobic Conditions and of Heating

<table>
<thead>
<tr>
<th>System</th>
<th>Gas phase</th>
<th>Protop. ((\text{pg.}))</th>
<th>Fe59 added ((\text{c.p.m.}))</th>
<th>Haem formed ((\text{c.p.m.}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver mito.</td>
<td>Air</td>
<td>40</td>
<td>478</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(heated)</td>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>Rat liver mito. (whole)</td>
<td>Air</td>
<td>&quot;</td>
<td>7832</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(supernate)</td>
<td>&quot;</td>
<td>4</td>
</tr>
</tbody>
</table>
mitochondria three times and then centrifuging at 11,750×g for 15 minutes at 0°C. This was added to the CBZ preparation using either protoporphyrin or porphobilinogen as substrate. Compared with whole mitochondria, the supernate was virtually devoid of activity (Table VIII).

All these experiments suggested that rat liver whole mitochondria alone were capable of catalysing efficient synthesis of haem from iron and protoporphyrin. A further series of experiments confirmed this; their activity was destroyed by heating and was favoured by anaerobic conditions (Table IX).

The optimum pH for haem synthesis, with protoporphyrin as substrate, was found to be 7.1 (Fig. 3). The time course of the reaction is illustrated in Fig. 4 which shows a linear increase of haem during the first 6 hours. The effects of increasing iron and protoporphyrin respectively are shown in Figs. 5, 6 and 7.

Sodium fluoride was investigated for inhibitory activity but found to be without action at concentrations up to 10⁻¹ M with either protoporphyrin or coproporphyrinogen as substrate (Table X).
The findings of Bénard, Gajdos and Gajdos-Tárók (32) with this substance can not, therefore, be attributed to an effect either upon the incorporation of iron into protoporphyrin or upon the oxidative decarboxylation of coproporphyrinogen to form protoporphyrin.

**FIG. 5.** Relation between haem formed and Fe³⁹ added in the system: Rat liver mitochondria + protoporphyrin (36.8 μg.) + ascorbic acid (10⁻³ M).

**FIG. 6.** Relation between haem formed and protoporphyrin added in the system: Rat liver mitochondria + Fe³⁹ (768 c.p.m.) + ascorbic acid (10⁻³ M) + phosphate buffer pH 7.4 + protoporphyrin (up to saturation level at 67.5 μg.).

**FIG. 7.** Relation between haem formed and protoporphyrin added in the system: Rat liver mitochondria + Fe³⁹ (768 c.p.m.) + ascorbic acid (10⁻³ M) + phosphate buffer pH 7.4 + protoporphyrin (up to saturation level at 67.5 μg.).

**DISCUSSION**

Chicken red cells are capable of synthesizing protoporphyrin and also haem from suitable precursors. The preparation (CBZ) made from them by haemolyzing with benzene and discarding the insoluble material by centrifugation can not carry the synthesis beyond the stage of coproporphyrinogen. This system is completed, however, by the addition of rat liver mitochondria.

The chemical mechanism by which coproporphyrinogen is transformed into protoporphyrinogen or protoporphyrin is not yet known. The step involves the oxidative decarboxylation of two propionic acid side chains (β positions 2 and 4) and could be envisaged as proceeding first by dehydrogenation to form 2,4-diacrylyl deuteroporphyrin and then by decarboxylation of the latter. Alternatively, β-oxidation of the two propionic acid residues could lead to a porphyrin structure with two β-ketonic-acid side chains.
The latter, being inherently unstable, might then lose carbon dioxide. The porphyrin resulting would be 2,4-diacetyl deuteroporphyrin but this does not appear to be an intermediate in protoporphyrin biosynthesis.

It was hoped that a study of the part played by mitochondria in the coproporphyrinogen→protoporphyrin (ogen) transformation might throw light upon the chemical mechanism involved. The finding that a supernatant prepared from frozen and thawed mitochondria is as effective as the intact particles in completing the system, renders it unlikely that a fully functional electron transport chain is necessary. Perhaps the mitochondrial fraction supplies a particularly suited hydrogen acceptor; no artificial hydrogen acceptor has yet been found capable of completing the system. Since certain mitochondria, such as those of rat heart are ineffective, enzymological comparison of these with mitochondria from rat liver might shed further light upon this problem.

Iron incorporation into protoporphyrin also requires a system present in rat liver mitochondria and in this case a supernate prepared from them is inactive. From the work of others, it seems possible that the iron and possibly the protoporphyrin also, may be bound to protein before enzymic combination is effected.

**SUMMARY**

1. The preparation is described of an enzymic system (CBZ) from chick red cells which forms coproporphyrin but very little protoporphyrin from porphobilinogen. Activity resides entirely in the non-ultrafilterable fraction. Some properties of the system have been investigated.

2. Addition to this system of rat liver mitochondria increases protoporphyrin formation at the expense of coproporphyrin. The supernatant prepared by freezing and thawing rat liver mitochondria and centrifuging exhibits this activity as does also the extract from acetone-dried rat liver mitochondria. Activity is destroyed by heating.

3. Mitochondria from rat heart and kidney do not increase protoporphyrin formation when added to CBZ and porphobilinogen. Mitochondria from rat spleen are only very feebly active in this respect.

4. Ferricyanide strongly inhibits porphyrin formation from porphobilinogen by CBZ and rat liver mitochondria; sodium fluoride is without effect but addition of iodoacetate increases protoporphyrin production.

α-phenanthroline appears to favour protoporphyrin formation but to have also a generally inhibitory effect upon the system. Cyanide tested aerobically and anaerobically had no significant effect upon uroporphyrin and coproporphyrin production by CBZ acting alone upon porphobilinogen.

5. Haem formation by the CBZ system has been investigated using either protoporphyrin as substrate or coproporphyrinogen plus rat liver mitochondria or porphobilinogen plus rat liver mitochondria. CBZ alone has only slight and somewhat variable activity in iron incorporation. This is increased by addition of ascorbic acid. Rat liver mitochondria have quite considerable activity which is also increased by addition of ascorbic acid but the most efficient system was constituted by CBZ plus rat liver mitochondria plus ascorbic acid.

6. The supernate from frozen and thawed and centrifuged rat liver mitochondria is almost entirely inactive as regards iron incorporation.

7. Anaerobic conditions favour haem formation from protoporphyrin and rat liver mitochondria, heating destroys the activity. Sodium fluoride is without effect.

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