Effects of Ehrlich Ascites Tumor on Iron Incorporation into Heme*

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SUMMARY

The Ehrlich ascites tumor takes up intravenous Fe\textsuperscript{59} and utilizes much of this for cellular heme formation. \textit{In vivo} experiments showed that the amount of iron utilized by liver and marrow for heme synthesis was directly related to the amount of iron taken up by these tissues. \textit{In vitro} experiments revealed no difference in iron-protoporphyrin chelation by liver mitochondria from control and tumor-bearing mice. There was likewise no evidence of insufficient iron for heme synthesis in these mitochondria. Large injections of iron had no effect on liver catalase activity in control or tumor-bearing mice. It was concluded that any decrease in liver heme synthesis due to a tumor could possibly be related to a decreased \textit{δ}-aminolevulinic acid dehydrase activity but not to any effect at the site of iron-protoporphyrin chelation.

One of the most frequently observed biochemical effects which a cancer produces on the host is a decrease in liver catalase activity. This effect has been attributed to the liberation of a toxic substance by the tumor (3, 8, 10). Nakahara and Fukuoka first isolated a tumor tissue fraction which depressed liver catalase activity \textit{in vivo}, and the concept of a cancer toxin, "toxohormone," was proposed (9). Reviews of the extensive investigations on toxohormone which followed have been published (8, 10). Evidence obtained in intact animals has indicated that this decrease in liver catalase activity may be due to an impaired biosynthesis of the enzyme and, more specifically, to its heme prosthetic group (10). It has been concluded from various studies that the site of inhibition is the last reaction in heme biosynthesis—that is, the incorporation of iron into protoporphyrin (2, 12).

Many observations have been made of alterations in iron metabolism in the tumor-bearing host (4, 5, 15). Changes in porphyrin metabolism have also been reported in tumor-bearing and toxohormone-treated animals. These include increased liver, blood, and urinary porphyrins (12, 16) and decreased \textit{δ}-aminolevulinic acid dehydrase activity in liver (17).

Because of these observed tumor effects on iron and porphyrin metabolism and on heme-containing catalase, further investigation of heme biosynthesis, especially at the sub-cellular level, was indicated. Most previous studies concerned with cancer and heme biosynthesis have been confined to \textit{in vitro} experiments. The present report describes some investigations with mice bearing the Ehrlich ascites tumor. Iron utilization specifically for heme biosynthesis not only \textit{in vitro} but also in cell-free preparations was observed.

MATERIALS AND METHODS

Swiss mice of either sex weighing 20–22 gm. were used. Two-tenths ml. injections of Ehrlich ascites tumor (strain ELD) were given intraperitoneally. Transplants were made, and experimental procedures were performed 7–10 days after tumor implant. All animals were maintained on a stock diet. For the \textit{in vitro} experiments each animal received intravenously 0.5 \textmu c. Fe\textsuperscript{59}Cl\textsubscript{2} (about 0.3 \textmu g. Fe) prior to sacrifice. Mice were sacrificed by decapitation. The livers were immediately removed and made into a 10 per cent homogenate in 0.25 M sucrose. Mitochondria were separated by centrifugation, washed 3 times with water, and suspended in four volumes of 0.1 M Tris(hydroxy-methyl)aminomethane buffer, pH 8.2 (6).
Homogenates were incubated at 38° C. in air with either δ-aminolevulinic acid or protoporphyrin as substrate and Fe59Cl3 as a tracer. Periodically aliquots were withdrawn. Hemin was isolated by the method of Labbe and Nishida (7) with washed red blood cells to supply carrier heme. Radioactivity measurements were made with a well-type scintillation detector. Aliquots of liver homogenates were counted to determine total radioactivity. Whole femurs were removed, autoclaved, freed of soft tissue, and counted intact. The bones were cut into small pieces, and hemin was isolated with the aid of carrier as for liver homogenate.

Iron-protoporphyrin chelating enzyme activity was measured either by Fe59 incorporation or by protoporphyrin disappearance (6). Catalase activity was determined by the method of Dille and Watkins (1) with incubation at 0° C. for periods of less than 2 minutes. Iron was determined quantitatively as described in Sandell (13). Liver protoporphyrin was isolated and determined essentially by the method of Schwartz and Watson (14). Tumor cells and fluid were separated by centrifugation. The cells were washed 3 times with isotonic saline, which was sufficient to give constant radioactivity.

**RESULTS**

The total iron content of whole tumor was 4.8 μg/gm of tumor, and 83 per cent of this was in the cells (Table 1). A 4- to 5-gm. tumor thus contained half as much iron as the whole liver. Turnover of iron in the tumor fluid and cells is illustrated in Chart 1. Data for the fluid are qualitatively similar to those for serum. Within 2 hr. 12 per cent of injected Fe59 was recovered in the tumor. After 5 hr. the whole tumor contained a constant 1.4 per cent of the injected dose per gram of tumor, irrespective of its age. Although the cell:fluid ratio in the tumor varied, it averaged near unity. Fe59 incorporated into tumor cellular heme paralleled the iron uptake curve for cells, but the hemin radioactivity per gram of cells was only 10 per cent of the total radioactivity in the cells.

The tumor cells were assayed for iron-protoporphyrin chelating enzyme activity and compared with normal mouse liver on a basis of protein concentration. As seen in Chart 2, tumor cells were 63 per cent as active as normal liver tissue. However, since a tumor may be several times the size of the liver, it is possible that the whole tumor may actually synthesize somewhat more heme per unit of time than does the whole liver. These enzymatic activities in liver and tumor cell homogenates were additive on mixing, and the tumor cells did not affect the liver under the conditions of the assay (Table 2).

Liver catalase activity was decreased to 42 per cent of controls in mice bearing Ehrlich ascites tumor (Table 1), a finding in agreement with many previous reports. Because of the relatively large

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control mice</th>
<th>Tumor-bearing mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (k0/gm liver)</td>
<td>1.21 (13)†</td>
<td>0.51 (13)†</td>
</tr>
<tr>
<td>Protoporphyrin (μg/gm liver):</td>
<td>0.73 (8)</td>
<td>1.01 (9)</td>
</tr>
<tr>
<td>Range</td>
<td>0.39–1.44</td>
<td>0.24–1.90</td>
</tr>
<tr>
<td>Total iron:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (μg/gm liver)</td>
<td>71 (8)</td>
<td>40 (8)</td>
</tr>
<tr>
<td>Liver mitochondria (μg/μg protein)</td>
<td>0.39 (4)</td>
<td>0.23 (4)</td>
</tr>
<tr>
<td>Whole tumor (μg/gm tumor)</td>
<td>4.8 (4)</td>
<td></td>
</tr>
<tr>
<td>Tumor cells (μg/gm cells)</td>
<td>9.0 (4)</td>
<td></td>
</tr>
<tr>
<td>Tumor fluid (μg/gm fluid)</td>
<td>1.8 (4)</td>
<td></td>
</tr>
</tbody>
</table>

*8- to 11-day tumors.
†Number of samples averaged or included in a pool.

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1δ-Aminolevulinic acid was supplied through the courtesy of Dr. John R. Dice of Parke, Davis and Company, Detroit, Mich.
iron utilization by the tumor, the possibility that an iron deficiency in the liver may be responsible for the decreased liver catalase activity was investigated. To observe the effect of iron on liver catalase large doses of an iron-dextran preparation (18 mg Fe/mouse) were administered to control and tumor-bearing mice at the time of tumor implantation. In neither group did the iron injections significantly alter the liver catalase activity.

With α-aminolevulinic acid added as the heme precursor liver homogenate from tumor-bearing mice incorporated Fe⁵⁹ into heme at a rate 30 per cent below control values (Chart 3). This may represent nothing more than the lower α-aminolevulinic acid dehydrase activity in livers of tumor-bearing animals. Also shown are comparative data with added protoporphyrin as substrate. The initial rate of heme biosynthesis from protoporphyrin was apparently decreased in liver homogenate from tumor-bearing mice to about the same extent as with α-aminolevulinic acid as substrate.

### Table 2

<table>
<thead>
<tr>
<th>Mg. Protein Added</th>
<th>M₄ Moles Protoporphyrin Utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse liver homogenate</td>
<td>Tumor cell homogenate</td>
</tr>
<tr>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>6.8</td>
<td>1.8</td>
</tr>
<tr>
<td>6.8</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Conditions: 30 μmoles protoporphyrin, 24 μmoles FeSO₄, 150 μmoles Tris buffer (pH 8.2), 40 μmoles ascorbic acid, tissue preparation, H₂O to 1.0 ml.; incubated in air at 38°C for 60 min.

### Chart 2

- Relative activities of normal liver and tumor cell homogenates for catalyzing iron-protoporphyrin chelation.
  - Conditions: ~5 mmoles protoporphyrin, ~4 mgmole FeSO₄, 140 mmoles Tris buffer, 40 μmoles ascorbic acid, homogenate, and H₂O to 1.0 ml.; incubated under nitrogen at 38°C for 60 min.

### Chart 3

- Incorporation of iron-⁵⁹ into heme by liver homogenates from control and tumor-bearing mice.
  - Conditions: 65 ml. 10 per cent liver homogenate in phosphate buffer, pH 7.4, 2.6 μc. Fe⁵⁹, 155 μmole δ-aminolevulinic acid or 1.3 μmole protoporphyrin; incubated in air at 38°C, 10-ml. aliquot withdrawn periodically for heme isolation.

However, the character of these curves changed after a period of time. According to experience in other assays it may be expected that most of the substrate (protoporphyrin) would have been consumed by the time that the incorporation of Fe⁵⁹ had reached a maximum. By contrast α-aminolevulinic acid was added in such excess that α-aminolevulinic acid dehydrase, and possibly other enzymes active in heme biosynthesis should have been saturated throughout the incubation period.

Since iron incorporation has been suggested as the site at which the tumor affects heme formation
in the liver, the enzymatic chelation of iron by protoporphyrin was singled out for additional in vitro studies. Because the chelating enzyme is localized in liver cell mitochondria (11), this fraction was isolated from the livers of control and tumor-bearing mice to facilitate study of the enzyme. As seen in Chart 4 mitochondria from control and tumor-bearing mouse livers utilized protoporphyrin at the same rates. In this assay the optimal concentrations of iron were added, and the possibility existed that the enzyme system was normally functional but must operate in vivo in the tumor-bearing mice with insufficient iron substrate. Therefore mitochondria were assayed in the presence and absence of exogenous iron to determine whether a deficiency of available iron existed in those derived from the tumor-bearing mice (Table 1), a value in agreement with a previous report (16). However, significance of the results may be questioned in this case due to the extreme overlapping of data. Similar porphyrin assays on thoroughly perfused livers showed no such change in protoporphyrin concentrations between control and tumor-bearing mouse tissues.

![Chart 4](chart_4.png)

**Chart 4.**—Rate of iron-protoporphyrin chelation in liver mitochondria prepared from control and from tumor-bearing mice. Conditions: 20 μmoles protoporphyrin, 24 μmoles FeSO₄, 130 μmoles Tris buffer, 20 μmoles ascorbic acid, 0.2 ml. mitochondria, and H₂O to 0.5 ml.; incubated under nitrogen at 38°C.

 These data are plotted in Chart 5. It is seen that the amount of protoporphyrin utilized per given amount of enzyme (mitochondria) was the same whether the mitochondria were derived from control or from tumor-bearing animals. This relationship held true in both the presence and absence of added iron. It is noteworthy that both reaction rates were increased to the same extent in the presence of added iron, suggesting a comparable endogenous iron supply available for heme formation.

To examine further the possibility of inhibited iron-protoporphyrin chelation in the livers of tumor-bearing mice, liver protoporphyrin concentrations were determined. In those animals bled only by decapitation prior to removal of the liver there was an average 40 per cent increase in the free protoporphyrin in the livers of tumor-bearing mice (Table 1), a value in agreement with a previous report (16). However, significance of the results may be questioned in this case due to the extreme overlapping of data. Similar porphyrin assays on thoroughly perfused livers showed no such change in protoporphyrin concentrations between control and tumor-bearing mouse tissues.

![Chart 5](chart_5.png)

**Chart 5.**—Effect of mitochondria concentration on iron-protoporphyrin chelation in the presence and absence of exogenous iron. Conditions: 40 μmoles protoporphyrin, 24 μmoles FeSO₄, 250 μmoles Tris buffer, 40 μmoles ascorbic acid, mitochondria, and H₂O to 1.0 ml.; incubated under nitrogen at 38°C for 15 min. (Mitochondria protein concentration: Control, 10.7 mg/ml; tumor-bearing, 11.6 mg/ml.)

**Table 3.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Per cent of injected dose in tissue</th>
<th>Counts per min.† in hemin</th>
<th>Tumor-bearing Control ×100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.5</td>
<td>1880</td>
<td>70</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>8.0</td>
<td>1350</td>
<td>74</td>
</tr>
<tr>
<td>Marrow (femurs):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.70</td>
<td>4650</td>
<td>38</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>0.65</td>
<td>2350</td>
<td>41</td>
</tr>
</tbody>
</table>

* Measurements were made 12 hours after Fe⁵⁹ injection.
† Counts/min in hemin represents the radioactivity in 8 mg. of crystallized hemin. About 40 mg. of carrier was used for the isolation. The hemin was isolated from 5 ml. of 10 per cent liver homogenate or from eight femurs.

Evidence obtained in vivo also indicates that the process of iron incorporation into heme is not impaired in tumor-bearing mouse livers. The utilization of injected Fe⁵⁹ for heme synthesis is directly related to the amount of Fe⁵⁹ taken up by the tissues (Table 3). The tumor-bearing mouse
livers took up 70 per cent as much of an injected dose of Fe$^{59}$ as controls, and the Fe$^{59}$ activity in recovered hemin was 74 per cent of controls in these same livers. Similar measurements on marrow showed that femurs of tumor-bearing mice took up 38 per cent as much of the injected Fe$^{59}$ as did controls, while the hemin activity in these same femurs was 41 per cent of that in controls.

**DISCUSSION**

An impaired utilization of iron for heme biosynthesis was originally suggested by Fukuoka and Nakahara (2) as an explanation of decreased liver catalase activity in tumor-bearing animals. Three different types of evidence seem to argue against this explanation. First, large injections of iron did not alter catalase activity in either control or tumor-bearing mice, although there have been differing results concerning the effect of iron administration on catalase activity. These conflicting results have been discussed by Nakahara and Fukuoka (10), who have suggested varying amounts of toxohormone as a possible cause for the different effects of administered iron on liver catalase activity. Second, the rate of iron-protoporphyrin chelation was identical in both types of liver mitochondria with or without added iron. This was in spite of the fact that total iron content of those prepared from tumor-bearing mouse livers was less. Therefore, in isolated mitochondria there appears to be no deficiency in available iron substrate. Third, erythrocyte catalase is not lowered in tumor-bearing animals, even though in these same animals the marrow iron uptake was decreased appreciably more than the liver uptake. With subcellular fractions it was clearly shown that the iron-protoporphyrin chelating enzyme activities were similar in liver mitochondria from both control and tumor-bearing mice. Nearly the same results were obtained in comparing the catalysis of iron-protoporphyrin chelation by liver homogenates. The slight difference in the curve obtained from tumor-bearing mouse liver homogenate may not be significant. On the other hand, the possibility of a readily soluble inhibitor's being washed out of the mitochondria or diluted below a highly effective concentration in the homogenate was not ruled out in these particular experiments. Indeed, there is evidence of an iron-protoporphyrin chelation inhibitor in the ascitic tumor fluid (unpublished observation). However, further characterization must be done before any significance can be attached to this finding.

The *in vitro* incorporation of Fe$^{59}$ into heme was directly related to the amount of radio-iron in the tissue in both liver and marrow. This finding is not indicative of inhibited heme synthesis at the stage of iron incorporation in either of these tissues. Any impairment in porphyrin or heme synthesis in the livers of tumor-bearing animals could be explained on a basis of the decreased $\delta$-aminolevulinic acid dehydrase activity as first reported by Tschudy and Collins (17) and confirmed in these studies. The decreased incorporation of Fe$^{59}$ into heme with $\delta$-aminolevulinic acid as substrate is of the same order of magnitude as the decrease in $\delta$-aminolevulinic acid dehydrase activity in the livers of tumor-bearing mice.

There are conflicting results concerning the increased free protoporphyrin concentrations in the livers of tumor-bearing animals (12). With unperfused livers, the present work showed some increase in protoporphyrin in many instances. However, with well perfused livers no change in protoporphyrin content of liver tissue was detectable. Although porphyrin determinations were not carried out on mouse blood, it has been reported that the erythrocyte protoporphyrin concentration in tumor-bearing rats is about doubled (16). This fact may help to account for the increased liver protoporphyrin described earlier (12), since there was no mention of these livers having been perfused to remove the blood. Other experimental differences which could lead to conflicting results include the type of tumor and the animal being used.

A combination of results from *in vivo* and *in vitro* experiments indicate that there is probably no impairment in iron incorporation into heme in livers of tumor-bearing mice. Therefore, the decreased liver catalase activity could not be explained on a basis of inhibited heme synthesis at the site of iron incorporation. Although the possibility of a soluble inhibitor of heme biosynthesis was not completely ruled out, it seems that the decreased liver catalase in tumor-bearing animals is more probably related to fundamental changes induced in liver cell metabolism by the tumor than to the inhibition of a specific reaction in heme biosynthesis.

**REFERENCES**

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