PHENOBARBITAL-INDUCED SYNTHESIS OF
THE MICROSOMAL DRUG-METABOLIZING ENZYME
SYSTEM AND ITS RELATIONSHIP TO THE
PROLIFERATION OF ENDOPLASMIC MEMBRANES

A Morphological and Biochemical Study

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ABSTRACT
Liver microsomes, isolated from rats which had been treated with phenobarbital in vivo,
were found to exhibit increased activities of oxidative demethylation and TPNH-cytochrome
c reductase and an increased amount of CO-binding pigment. Simultaneous administration
of actinomycin D or puromycin abolished the phenobarbital-induced enzyme synthesis.
Increased rate of P32 incorporation into microsomal phospholipid was the first sign of
phenobarbital stimulation and appeared 3 hours after a single injection of this drug. Micro-
somes were divided into smooth-surfaced and rough-surfaced vesicle fractions. The frac-
tion consisting of smooth-surfaced vesicles exhibited the greatest increase in protein content
and oxidative demethylation activity after phenobarbital administration in vivo. Ultra-
structural studies revealed that drug treatment also gave rise to proliferation of the endo-
plasmic reticulum in the hepatic parenchymal cells, first noticed after two phenobarbital
injections. The phenobarbital-induced synthesis of the metabolizing enzymes is discussed
with special reference to the relationship to the stimulated synthesis of the endoplasmic
membranes.

INTRODUCTION

The liver microsomal fraction, isolated after
homogenization and differential centrifugation,
contains free RNP particles and two kinds of
vesicles: rough, with RNP particles attached to

1Abbreviations used are: RNP, ribonucleoprotein;
DPNH and TPNH, di- and triphosphopyridine
nucleotide, reduced forms; i.p., intraperitoneally;
RNA, ribonucleic acid; DNA, deoxyribonucleic acid;
ER, endoplasmic reticulum; DOC, deoxycholate,
IDP, inosine-5′-diphosphate; ATP, adenosine-5′-
the outer surface, and smooth, which lack these
particles (1). Liver microsomes are known to
catalyze a number of TPNH-dependent hydroxyla-
tion reactions, among them the oxidative demethy-
lation of various drugs (cf. review, 2). TPNH-
cytochrome c reductase (3–5) and the CO-binding
pigment (6, 7) are probably involved in oxidative
demethylation (8–10). Pretreatment of rats in
triphosphate; G-6-P, glucose-6-phosphate; FA, form-
aidehyde.
rize with certain drugs, e.g. phenobarbital, induces an increased rate of drug hydroxylation (11-13) and also leads to increased numbers of endoplasmic membranes (14). It is the purpose of this paper to present data which indicate that phenobarbital acts by the specific induction of the synthesis of TPNH-cytochrome c reductase and the CO-binding pigment, and that this substrate induction is sensitive to actinomycin D and puromycin. The phenobarbital-induced enzyme synthesis will be discussed with special reference to the production of the enzyme-bearing endoplasmic membranes, as studied by chemical methods and in the electron microscope.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200 to 300 gm) were used. All animals were starved overnight and killed by decapitation. The livers were excised and placed at once in cold 0.25 M sucrose. They were weighed, cut into small pieces, and washed clean of blood by rinsing with cold sucrose.

Microsomes were prepared according to the method described by Ernster et al. (15).

The experimental animals were given one daily injection of 100 mg of phenobarbital per kg body-weight intraperitoneally (i.p.). The controls received the same amount of 0.9 per cent NaCl. Actinomycin D was also administered i.p. in an amount of 8 ng per 100 gm of body-weight.

For the electron microscopic investigations, small pieces of the liver from experimental animals and controls were fixed for 1.5 to 2 hours at 0-4°C in 2 per cent osmium tetroxide, buffered with purified s-collidine (16) or a phosphate buffer (pH 7.4) (17). After dehydration in a graded series of ethanol solutions, starting with 70 per cent, the tissues were immersed in two changes of propylene oxide and were then immersed in Epon 812 (18). Thin sections were stained with lead hydroxide (19) and examined in a Siemens Elmiskop I electron microscope.

Protein was measured by the method of Lowry et al. (20). RNA was determined according to Ceriotti (21). Lipids were extracted (22) and the phosphorus content estimated (23). The value obtained was multiplied by 25 to obtain the total amount of phospholipid (15).

Oxidative demethylation activity was assayed with aminopyrine as substrate. The incubation system contained microsomes, 5 mM aminopyrine, 0.05 M tris buffer (pH 7.5), 50 mM nicotinamide, 5 mM MgCl₂, 0.5 mM TPN, and a TPNH-generating system consisting of 5 mM dl-isocitrate, 0.01 mM MnCl₂, and enough isocitric dehydrogenase to reduce 0.32 μmoles TPN per minute, in a final volume of 2 ml. The time of incubation was 20 minutes and the temperature 37°C. The amount of formaldehyde formed was measured by the Nash reaction (24).

Oxygen consumption was measured with a Clark oxygen electrode. Measurements were started with microsomes and 0.3 mM TPNH, in a final volume of 3 ml. After recording the TPNH oxidase activity, the drug was added to a final concentration of 5 μM. The temperature was 30°C.

All other enzyme assays were performed according to previously described methods (15, 25).

Abbreviations

BC, bile capillary.
C, cytosome.
CM, cell membrane.
CS, cytosegresome.
erg, rough-surfaced ER.
erg, smooth-surfaced ER.
Gl, glycogen.
M, mitochondrion.
Mβ, microbody.
N, nucleus.
S, sinusoid.

The electron micrographs in plates 1 to 6 are from thin sections of Epon-embedded tissues; the sections were stained with lead hydroxide prior to examination in the microscope and show the appearance of hepatic parenchymal cells.

FIGURE 1 Control (intraperitoneal injections of physiologic saline for 5 days). Long, slender profiles of rough-surfaced ER (erg) tend to be arranged in parallel arrays. Smooth-surfaced ER appears as short tubular or vesicular images. Transitions between rough- and smooth-surfaced ER (unattached arrow) are relatively frequent. × 15,000.

FIGURE 2 Intraperitoneal injections of phenobarbital for 3 days. As compared with the control, considerably increased numbers of tubular and vesicular images, believed to represent proliferated smooth-surfaced ER (erg), are present in the cytoplasm. × 28,000.
The thyroidectomized rats were a generous gift from Dr. J. R. Tata, Mill Hill, London. Actinomycin D was made available to us by Merck, Sharp & Dohme, International. All chemicals employed were standard commercial products.

A part of this work has already been reported briefly (26).

RESULTS

A. Electron Microscopy

The cytoplasm of hepatic parenchymal cells from control animals given physiologic saline i.p. (Fig. 1) showed an appearance similar to that in normal rat and mouse. In experimental animals given phenobarbital alone, alterations of the endoplasmic reticulum (ER) were noted in some of the animals sacrificed 2 days after the beginning of the injections, and in all of those sacrificed on the 3rd day and later. The earliest changes (observed in animals sacrificed on the 2nd and 3rd day) consisted in proliferation of the ER (Fig. 2). Smooth-surfaced tubular and vesicular profiles of ER were more abundant than in control animals, but otherwise showed the same appearance as in the latter. Unequivocal alterations in the structure or disposition of the rough-surfaced ER were not observed. Moderate numbers of "free" RNP particles were present in the cytoplasmic ground substance. On the 4th and 5th days (Figs. 3 to 5), the cytoplasmic ground substance was filled with tightly packed smooth- and rough-surfaced profiles of the ER. Rough-surfaced ER appeared to be more abundant than in control animals, and large areas of cytoplasm occupied by conglomerations of rough-surfaced tubular profiles arranged in parallel arrays were often encountered (Fig. 4). Smooth-surfaced ER usually appeared as circular or oval vesicles. Both smooth- and rough-surfaced ER often showed dilatation to a variable degree. Significant alterations in cytoplasmic organelles other than those of the ER were not observed.

A moderate proliferation of the ER occurred in hepatic parenchymal cells of experimental animals which had received both phenobarbital and actinomycin D (Fig. 6). This alteration was first observed on the 3rd day. Dilatation and extreme proliferation of the ER, as noted in animals given phenobarbital alone, was not present. Administration of actinomycin D alone did not cause any appreciable alterations in liver cell structure.

B. Biochemistry

Phenobarbital treatment resulted in increased amounts of microsomal protein, RNA, and phospholipid (Fig. 7). The phospholipid content was already significantly higher after one phenobarbital injection and exhibited a greater increase than RNA and protein.

Fig. 8 shows the response of several microsomal enzymes to phenobarbital treatment. After five injections, there was a 4- to 5-fold increase over controls in the aminopyrine-demethylating and TPNH-cytochrome c reductase activities and in the amount of CO-binding pigment, all as calculated on the protein basis. The specific IDPase activity, measured in the presence of 0.1 per cent DOC, and the content of cytochrome b₅, as calculated per mg protein, were about the same as in the controls, while the specific activities of glucose-

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Figure 3 Four intraperitoneal injections of phenobarbital. The cytoplasmic ground substance contains numerous vesicles, most of which are smooth-surfaced. The rough-surfaced ER (erg) shows dilatation. × 15,000.

Figure 4 Four intraperitoneal injections of phenobarbital. Area of cytoplasm containing numerous cisternae of rough-surfaced ER (erg) arranged in a parallel fashion. Most of the cisternae are expanded at their ends where some become smooth-surfaced (unattached arrow). The cytoplasm surrounding the stacked cisternae is mainly composed of smooth-surfaced vesicular profiles. In the right upper corner is an area containing ER which seems to have been segregated from the remainder of the cytoplasm by a system of roughly parallel membranes. Such images have been termed "cytosegresomes" and probably contain acid phosphatase (46). × 30,000.
6-phosphatase, ATPase, and DPNH-cytochrome c reductase were lower in the treated animals than in the controls.

Twelve hours after one single dose of phenobarbital, there was a significant increase in the rate of oxidative demethylation and the concentration of the participating enzymes (Fig. 9). The rate of ³²P incorporation into microsomal phospholipid, however, exhibited more than a 2-fold increase already 3 hours after the administration of phenobarbital, and reached its maximum 12 hours after the injection.

In untreated rats, as well as in mice and guinea pigs, the TPNH-cytochrome c reductase and oxidative demethylation activities and the amount of CO-binding pigment have been found to be equally distributed within microsomal subfractions; i.e. rough- and smooth-surfaced vesicles (27). Fig. 10 shows that following stimulation with phenobarbital there was a greater increase in the amount of protein and oxidative demethylation activity in the fraction containing smooth vesicles as compared to the one predominantly composed of rough vesicles; the latter, however, also exhibited a highly significant response to phenobarbital stimulation which is in agreement with previous findings (28).

Fig. 11 shows the effect of actinomycin D on drug-induced enzyme synthesis. This antibiotic exerted a strong inhibitory effect at the beginning of the treatment; however, following repeated injections of phenobarbital, it was no longer possible to prevent an increase in enzymatic activities with simultaneous administration of actinomycin D. Puromycin completely abolished the phenobarbital-stimulated increase in the concentration of CO-binding pigment and oxidative demethylation activity (Table I).

There was a 3- to 4-fold increase in the rate of ³²P incorporation into microsomal phospholipid 12 hours after a single phenobarbital injection (Fig. 12). No significant increase over the controls was found in this incorporation rate when actinomycin D was given alone. Simultaneous administration of phenobarbital and this antibiotic caused an increased rate of ³²P incorporation into microsomal lipid phosphorus; although this stimulation of the incorporation rate was found to be significantly lower than the stimulation caused by giving phenobarbital alone. Treatment with phenobarbital also increased the rate of ³²P incorporation into mitochondrial phospholipid, to a much lesser extent, however. It cannot be excluded that the stimulation, at least in part, depended on contamination of the mitochondrial fraction with microsomes.

Table II shows the stimulation of the TPNH-dependent oxygen uptake caused by addition of various drugs to microsomes isolated from phenobarbital-treated and control animals. For all the drugs added, the increase in oxygen consumption was found to be stimulated several times by repeated phenobarbital administration in vivo. It may be noted that the microsomal TPNH-oxidizing activity, measured in the absence of substrate, was not significantly increased by phenobarbital treatment. The molar amount of formaldehyde formed from the oxidatively demethylated drugs was the same as the amount of oxygen consumed.

There are many similarities between the response of rat liver to phenobarbital stimulation and the effects of administration of thyroid hormone to a thyroidectomized rat (cf. review, 29); e.g., increased liver weight, increased protein synthesis, increased TPNH-cytochrome c reductase activity, sensitivity to actinomycin D, and the relatively long time lag before measurable changes occur. In order to study the possible role of the thyroid hormone in drug-induced enzyme synthesis, thyroidectomized rats that had received
FiguRe 7  Effect of phenobarbital treatment on microsomal protein, RNA, and phospholipid. Mean values (in mg per gm liver) of six phenobarbital-treated rats are plotted, expressed as percentage of the mean values obtained from a control group of six rats. Phenobarbital injections are marked with arrows. The averages ± standard deviations for the phenobarbital-treated groups (Exp.) and the control groups (Con.) were the following:

<table>
<thead>
<tr>
<th>No. of phenobarbital treatments</th>
<th>Protein (mg/gm liver)</th>
<th>RNA (mg/gm liver)</th>
<th>Phospholipid (mg/gm liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.1 ± 1.46 ± 0.38</td>
<td>4.82 ± 0.38</td>
<td>6.75 ± 1.27</td>
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<tr>
<td></td>
<td>23.9 ± 1.54 ± 0.38</td>
<td>4.62 ± 0.32</td>
<td>10.00 ± 1.27</td>
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<tr>
<td>2</td>
<td>23.1 ± 1.34 ± 0.34</td>
<td>4.50 ± 0.34</td>
<td>6.40 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>27.7 ± 1.28 ± 0.41</td>
<td>5.31 ± 0.41</td>
<td>10.77 ± 2.00</td>
</tr>
<tr>
<td>3</td>
<td>24.1 ± 1.30 ± 0.29</td>
<td>4.28 ± 0.29</td>
<td>6.22 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>31.4 ± 1.32 ± 0.56</td>
<td>5.56 ± 0.40</td>
<td>11.70 ± 2.10</td>
</tr>
<tr>
<td>4</td>
<td>22.8 ± 1.38 ± 0.37</td>
<td>4.30 ± 0.37</td>
<td>6.05 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>33.1 ± 2.22 ± 0.71</td>
<td>6.94 ± 0.71</td>
<td>12.70 ± 2.72</td>
</tr>
<tr>
<td>5</td>
<td>22.0 ± 1.50 ± 0.46</td>
<td>4.40 ± 0.40</td>
<td>6.30 ± 1.40</td>
</tr>
<tr>
<td></td>
<td>35.0 ± 3.00 ± 0.90</td>
<td>7.08 ± 0.90</td>
<td>15.10 ± 3.20</td>
</tr>
</tbody>
</table>

It is well documented that treatment of rats with certain drugs induces an increased tolerance to the action not only of the specific drug used for pretreatment but also of a whole group of compounds which share the property of being hydroxylated by liver microsomal enzymes in the presence of TPNH and oxygen (11–13, 30). It has been postulated that the inducing agents act by causing an increased synthesis of the microsomal drug-metabolizing enzymes (13, 28, 31). Administration of ethionine was shown to inhibit the drug-induced increase in the activities of the metabolizing enzymes (13, 31), and Gelboin and Sokoloff reported that treatment with phenobarbital, which is one of the most potent inducing agents, stimulated the incorporation of amino acids into microsomal proteins of cell-free liver preparations (32). It was recently shown that phenobarbital stimulation of the activities of several drug-metabolizing enzymes is associated with increased numbers of endoplasmic membranes (14, 28).

Earlier results suggested that TPNH-cytochrome c reductase and the CO-binding pigment are involved in liver-microsomal drug-hydroxylating reactions (8–10). Strong evidence for the involvement of these two enzymes in oxidative demethylation was obtained in the present study. Repeated administration of phenobarbital to rats caused a ca. 1.5-fold increase in the amount of microsomal membrane constituents—protein, RNA, and phospholipid—while the activities of aminopyrine demethylation and TPNH-cytochrome c reductase and the amount of CO-binding pigment, all as calculated on the protein basis, exhibited a 4- to 5-fold increase over the controls. The amount of cytochrome b₅, calculated per mg protein, did not change, while DPNH-cytochrome c reductase showed a lower specific activity in the drug-treated animals; this was also true for ATPase and glucose-6-phosphatase. The fact that treatment with phenobarbital causes a several-fold increase in the concentration of only one of the microsomal hemoproteins, the CO-binding pigment, and in the activity of only one of the microsomal flavoproteins TPNH-cytochrome c reductase, indicates that the induction is a specific substrate-caused de novo synthesis of the metabolizing enzymes.

However, stimulation with phenobarbital also leads to proliferation of the ER. It would thus appear that the newly synthesized enzymes are...
dependent upon the production of new membranes in order to function. This view is further supported by the increased rate of $\Delta P_i$ incorporation into microsomal phospholipids, which was the first sign of phenobarbital stimulation.

The fastest and greatest increase in the substrate-induced enzyme levels was found in the fraction containing smooth-surfaced vesicles. This is in agreement with the results of Remmer and Merker (28). An increased amount of endoplasmic membranes was found by electron microscopy in hepatic parenchymal cells after two to three injections of phenobarbital. The earliest alteration was the appearance of small smooth-surfaced vesicular and tubular profiles in the cytoplasm. Following further injections, an increase in the amount of rough-surfaced membranes was also observed. It is possible that drug treatment first gives rise to a synthesis of smooth membranes; some of these may later become coated with RNP particles.

Microsomes catalyze a TPNH-dependent $O_2$
FIGURE 9  Increased rate of Pi incorporation into microsomal phospholipid, and increase in oxidative demethylation and TPNH-cytochrome c reductase activities and in the amount of CO-binding pigment after one phenobarbital injection. Pi incorporation is plotted as cpm per mg lipid phosphorus of the treated rats as percentage of controls. 1 mc Pi was given i.p. to each rat 1 hour before it was killed. ---C, Pi incorporation. ---X, Demethylation. ---A, CO-binding pigment. ---O, TPNH-cytochrome c reductase.

uptake, which is known as the TPNH oxidase (33). This O2 uptake is not inhibited by CO, as is drug hydroxylation (34). Addition of a hydroxyl acceptor increases the microsomal TPNH-dependent O2 uptake, and this increase is CO sensitive. The increase in oxygen consumption, caused by the addition of various drugs, was found to be stimulated several-fold by repeated phenobarbital administration in vivo.

Actinomycin D, which is known to block the DNA-dependent synthesis of messenger-RNA (35-37), initially abolished the phenobarbital-induced enzyme synthesis. This result is in agreement with the findings of Gelboin and Blackburn for the stimulatory effect of 3-methylcholanthrene on microsomal amino acid incorporation and benzpyrene hydroxylase activity (38) and those of Salas et al. for glucose-induced synthesis of liver glucokinase (39). The effects of many doses of phenobarbital could not be totally abolished by simultaneous administration of actinomycin D, which may suggest that the ability of the liver to detoxicate this antibiotic is increased by repeated administration. Puromycin inhibition occurs at the stage of assembly of new protein at the ribosome (40). In the dose given, puromycin totally abolished the drug-induced enzyme synthesis, which is in agreement with the results of Conney and Gilman (41) and a further evidence for an actual synthesis of new enzyme protein.
The phenobarbital effect on the phospholipid synthesis, measured as increased rate of $\text{P}_{i}$ incorporation into microsomal phospholipids, was initially and partly inhibited by simultaneous injection of actinomycin D. In this case, however, the inhibitory action of the antibiotic has been too weak to allow any final conclusions to be drawn. In this connection, it should be mentioned that Mueller has reported that actinomycin D inhibits the estrogen-stimulated phospholipid synthesis in perfused rat uterus (42).

A large number of inducible enzyme systems has been discovered and studied in bacteria. Enzymes that attack exogenous substrates have generally been found inducible in these organisms. Very often the presence of a substrate induces the synthesis not of a single, but of several enzymes sequentially involved in its metabolism (43). At present, it is difficult to perform a direct comparison of the findings reported above with the results of Monod and co-workers from the study of the "lactose" system of *E. coli* (cf. review, 44). There are, however, important similarities between these two systems. The substrates induce a de novo synthesis of enzyme protein. Actinomycin D inhibits this synthesis. Removal of the inducer results in cessation of enzyme synthesis. However, further studies of the kinetics of phenobarbital induction have to be performed before any conclusions concerning a possible analogy with bacterial systems can be drawn.

This work has been supported by grants from the Swedish Cancer Society and the "Stiftelsen Therese och Johan Anderssons Minne, Stockholm."

The authors wish to thank Miss Hjördis Berg, Miss Margareta Sparthan, and Miss Gesa Thies for their skilful technical assistance.

Received for publication, August 3, 1964.

### Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxidative demethylation activity</th>
<th>CO-binding pigment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mg/gm liver mg/mg liver protein</td>
<td>FA/min/mg protein</td>
</tr>
<tr>
<td>None</td>
<td>26.4</td>
<td>2.62</td>
</tr>
<tr>
<td>Phenobarbital*</td>
<td>27.8</td>
<td>10.70</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>30.0</td>
<td>2.76</td>
</tr>
<tr>
<td>+Puromycin†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Three injections of phenobarbital were given, once every 24 hours.
† Simultaneously with each one of the three phenobarbital injections, 12.5 mg of puromycin per 100 gm of body-weight were injected i.p.

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**Figure 12** Effect of phenobarbital and actinomycin D on the rate of $\text{P}_{i}$ incorporation into phospholipids of microsomes and mitochondria. C, control; Ph, phenobarbital; A, actinomycin D. Phenobarbital and actinomycin D were administered 12 hours before decapitation. Controls were injected simultaneously with the same volume of 0.9 per cent NaCl. All rats received 1 mc of $\text{P}_{i}$ 1 hour before they were killed. Mitochondria were isolated according to Ernster and Löw (45).
TABLE II
Effect of Phenobarbital Treatment on the Microsomal TPNH-Dependent Oxygen Uptake, as Stimulated by Various Drugs

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Additions</th>
<th>Oxygen consumption (μmoles O₂ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>TPNH</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>TPNH + aminopyrine</td>
<td>5.35</td>
</tr>
<tr>
<td>2</td>
<td>TPNH</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>TPNH + dimethylnitrosamine</td>
<td>6.15</td>
</tr>
<tr>
<td>3</td>
<td>TPNH</td>
<td>3.90</td>
</tr>
<tr>
<td></td>
<td>TPNH + codeine</td>
<td>5.84</td>
</tr>
<tr>
<td>4</td>
<td>TPNH</td>
<td>3.72</td>
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<tr>
<td></td>
<td>TPNH + phenobarbital</td>
<td>5.00</td>
</tr>
</tbody>
</table>

TABLE III
Effect of Phenobarbital Treatment on the Oxidative Demethylation System of Thyroidectomized Rats and Thyroidectomized + Triiodothyronine (T₃)-Treated Rats

<table>
<thead>
<tr>
<th>No. of phenobarb. treatm.</th>
<th>Protein activity</th>
<th>TPNH cytochrome c oxidase activity</th>
<th>CO-binding pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/gm liver</td>
<td>μmoles FA/min./mg protein</td>
<td>μmoles TPNH oxi.d./min./mg protein</td>
</tr>
<tr>
<td>Non-thyroidectomized</td>
<td>25.2</td>
<td>2.95</td>
<td>0.029</td>
</tr>
<tr>
<td>Thyroidectomized</td>
<td>16.6</td>
<td>3.30</td>
<td>0.018</td>
</tr>
<tr>
<td>Thyroidectomized + 1 T₃ inj.*</td>
<td>19.0</td>
<td>3.45</td>
<td>0.036</td>
</tr>
<tr>
<td>Thyroidectomized + 3 T₃ inj.*</td>
<td>20.2</td>
<td>4.70</td>
<td>0.036</td>
</tr>
</tbody>
</table>

* 45 μg of triiodothyronine was injected subcutaneously on the 3rd day before beginning of phenobarbital stimulation.
‡ 45 μg of triiodothyronine was injected subcutaneously on the 3rd, 6th, and 8th day before beginning of the phenobarbital stimulation.

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