Hormonal Effects Upon in vitro Cholesterol Synthesis

By Nancy L. Noble, Ph.D. and Robert J. Boucek, M.D.

This report concerns a comparison of the rates at which labeled acetate is converted into digitonin-precipitable substance by sponge connective tissue and the liver. The effects of the gonads and the thyroid gland on the in vitro incorporation of C\(^14\)-labeled acetate as cholesterol were also explored.

Connective tissue isolated from the albino rat by the sponge implantation technic is rich in lipids and has a high concentration of cholesterol. It has been demonstrated that aortic tissue of the rabbit and chicken can synthesize cholesterol in vitro from C\(^14\)-labeled acetate. Connective tissue-sponge biopsies from the albino rat synthesize cholesterol in vitro at a rate which is comparable to that reported for aortic tissue.

Considerable interest in the mechanism of cholesterol accumulation in the arterial wall has resulted from the observations that a distorted serum lipid partitioning occurs in human atherosclerosis and that serum lipids may be altered by the administration of certain hormones. The earliest biochemical changes in the intima as atherosclerosis develops in the human are increases in the amount of collagen and in the binding of hexosamine by the scleroprotein of intimal connective tissue. There is a sex difference in the lipid partitioning in the human atheroma. Furthermore, atherosclerotic changes in arteries occur more commonly in the human male than in the female. Thus, the relationship of the effects of sex hormones upon cholesterol synthesis in connective tissue to the problem of atherosclerosis is fundamental.

The purpose of this investigation was to study (a) the incorporation of C\(^14\)-labeled acetate into cholesterol and other digitonin-precipitable substances in slices of connective tissue and liver, and (b) the effects of certain glandular ablations and the administration of hormones upon this in vitro incorporation.

Methods

Connective tissue was obtained from male and female rats of approximately 9 months of age by the sponge-biopsy technic previously described. In all the experimental studies, the connective tissue biopsy and liver were removed from the pretreated or control rat and immediately placed into cold oxygenated Krebs-Ringer bicarbonate solution of pH 7.4. The synthesis of cholesterol from acetate was studied by a modification of the technic described by Siperstein et al.

Tissue slices weighing 500 to 750 mg. were placed in the incubation flask containing 5 ml. of Krebs-Ringer bicarbonate solution at pH 7.4, and 0.5 ml. of C\(^14\)-carboxyl-labeled sodium acetate in physiologic saline (0.82 mg. sodium acetate or 2.22 \(\times\) 10\(^7\) c.p.m.* was added. The flask was gassed with a mixture of 95 per cent O\(_2\) and 5 per cent CO\(_2\) and incubated in this atmosphere for 11 hours at 37.5 C. with continuous agitation. Liver slices were run simultaneously and in the same manner. Following alcoholic saponification of the contents of the incubation flask, cholesterol was extracted with petroleum ether, carrier cholesterol was added and the digitonide was precipitated. The suspension was filtered on Whatman filter paper no. 42, and the filter paper and precipitate were mounted on a planchet and counted. The digitonides isolated from the sponge-connective tissue and liver were counted at reliable per cent error levels of 2.6 and 0.52 per cent respectively. The radioactivity was corrected for self-absorption.

Because in some of the experimental conditions there were changes in the water content of the biopsy-connective tissue, the radioactivity of the digitonide isolated from the connective tissue was calculated on a content basis of per gram of dry sponge implant and is reported as c.p.m. per gram sponge implant. The radioactivity of the liver digitonide is reported as c.p.m. per gram tissue weight.

Hormones were injected intramuscularly daily for 21 days into groups of intact male and female rats with sponge implants of 90 days of age. A sponge-biopsy was removed before treatment was

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* c.p.m., counts per minute.
### Table 1.—Labeled Digitonin-Precipitable Substance

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Relaxin</th>
<th>Estrogen</th>
<th>Testosterone</th>
<th>Progesterone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Connective tissue,</strong> e.p.m. per Gm. sponge implant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>7,019 ± 3,785</td>
<td>4,751* ± 1,887</td>
<td>11,073* ± 2,823</td>
<td>8,038 ± 3,949</td>
<td>9,425 ± 2,851</td>
</tr>
<tr>
<td>female</td>
<td>4,658 ± 3,228</td>
<td>2,491* ± 1,402</td>
<td>2,412 ± 946</td>
<td>2,958 ± 929</td>
<td>2,417 ± 941</td>
</tr>
<tr>
<td><strong>Liver,</strong> e.p.m. per Gm. tissue weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>107,346 ± 45,442</td>
<td>133,021 ± 55,746</td>
<td>178,166* ± 21,013</td>
<td>150,488 ± 12,058</td>
<td>118,054 ± 21,583</td>
</tr>
<tr>
<td>female</td>
<td>142,364 ± 53,258</td>
<td>131,421 ± 25,301</td>
<td>135,992 ± 19,549</td>
<td>115,364 ± 32,651</td>
<td>119,816 ± 24,198</td>
</tr>
</tbody>
</table>

*Supplied by Warner-Chilecott Laboratories, Morris Plains, N. J.

### Results

A significant difference was observed between the rates of in vitro cholesterol synthesis in the connective tissue biopsies from the male and female rats (table 1). The value of 7019 ± 3785 e.p.m./Gm. of sponge implant for the tissue of the male was significantly greater than the value of 4638 ± 3228 for the tissue of the female rat. No significant difference was found in the cholesterol synthesis in the liver slices obtained from the two sexes.

The effects of the hormones, relaxin, estrogen, testosterone, and progesterone, upon cholesterol synthesis of connective tissue and liver are recorded in table 1. Pretreatment of the rat with testosterone or progesterone caused no significant alteration of cholesterol synthesis in the sponge-connective tissue. Estrogen injections significantly increased the synthesis in the connective tissue of the male rat but caused no significant change in the female tissue. The only hormone which significantly affected synthesis in connective tissue obtained from both sexes was relaxin. This hormone, which is obtained from the ovaries of pregnant sows, caused a decrease in the incorporation of C14-acetate into the digitonin-precipitable substance.

Estrogen pretreatment increased the incorporation of the labeled acetate into liver cholesterol in the male rat but had no effect on this tissue of the female. The other hormones, relaxin, testosterone, and progesterone, did not alter the cholesterol synthesis in the liver slices of the pretreated rats.
The effects of the thyroid and compounds related to thyroid activity upon cholesterol synthesis in connective tissue and liver slices are shown in table 2. The incorporation of labeled acetate into the digitonin-precipitable substance of sponge-biopsy connective tissue was not altered in either sex by thyroid ablation, by the feeding of desiccated thyroid to the hypothyroid rat or to the intact rat, or by the administration of tetraiodothyroacetic acid. Thyroid-stimulating hormone administered parenterally to intact rats significantly increased cholesterol synthesis in the connective tissue of the female rat but did not affect the synthesis in tissue from the male.

The radioactivity of the digitonide isolated from the livers of the hypothyroid female rats was significantly reduced when compared with that of the intact animals. Tetraiodothyroacetic acid administration significantly increased cholesterol synthesis in the liver slices of the male rat.

Castration and oophorectomy effected significant reductions in the cholesterol synthesis of connective tissue (table 3) when compared with the values for the intact rats. Castration reduced cholesterol synthesis from 7019 ± 3785 c.p.m./Gm. sponge implant to 3697 ± 2358 while removal of the ovaries caused a decrease from 4638 ± 3228 c.p.m./Gm. sponge implant to a value of 2596 ± 1051. Administration of estrogen to the gonadectomized animals caused increases in cholesterol synthesis. The sponge-connective tissues which were removed from the two gonadectomized groups after the 20-day period of estrogen and relaxin administration had reduced rates of cholesterol synthesis, which were the same as the pretreatment values.

DISCUSSION

Tissues in general appear to be capable of incorporating acetate into cholesterol at different rates.8-13 The rate of incorporation of C14-acetate into the digitonin-precipitable substance was less in the connective tissue slices of the rat than in liver slices. When the rates in the two tissues were compared on the basis of the concentration of noncollagenous protein, the conversion of acetate to cholesterol in connective tissue was about 1.6 per cent that of the liver.4 The capacity of arterial tissue to incorporate labeled acetate into cholesterol

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**Table 2.—Labeled Digitonin-Precipitable Substance**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Thyroid ablation</th>
<th>Thyroid ablation + desiccated thyroid</th>
<th>Desiccated thyroid</th>
<th>Tetraiodothyroacetic acid</th>
<th>Thyroid stimulating hormone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective tissue, c.p.m. per Gm. sponge implant male</td>
<td>7,019 ± 3785 (30)</td>
<td>4,354 ± 1,364 (6)</td>
<td>4,750 ± 3,546 (5)</td>
<td>8,824 ± 3,567 (6)</td>
<td>8,724 ± 3,583 (6)</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>4,638 ± 2,274 (55)</td>
<td>4,794 ± 3,359 (6)</td>
<td>5,866 ± 3,539 (6)</td>
<td>4,028 ± 2,100 (6)</td>
<td>7,573* ± 2,049 (6)</td>
<td></td>
</tr>
<tr>
<td>Liver, c.p.m. per Gm. tissue weight male</td>
<td>107,346 ± 48,442 (13)</td>
<td>110,252 ± 7,988 (5)</td>
<td>112,159 ± 6,141 (5)</td>
<td>208,743* ± 38,183 (3)</td>
<td>121,525 ± 37,367 (4)</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>142,364 ± 53,258 (15)</td>
<td>112,159 ± 52,370 (6)</td>
<td>110,252 ± 50,244 (6)</td>
<td>117,280 ± 52,370 (3)</td>
<td>112,159 ± 39,658 (4)</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference.

**Table 3.—Labeled Digitonin-Precipitable Substance**

<table>
<thead>
<tr>
<th>Biopsy-Connective Tissue</th>
<th>C.P.M. per Gm. sponge implant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Castrated...</td>
<td>3697 ± 2381</td>
</tr>
<tr>
<td>Spayed...</td>
<td>2596 ± 1051</td>
</tr>
</tbody>
</table>

*Significant difference.
varied but was generally 0.5 to 3 per cent that observed in liver slices.\(^3\)

The effects of hormonal pretreatment on the rates of cholesterol synthesis in the slices of connective tissue and liver were not generally the same. Synthesis in the liver was significantly altered by the administration of tetraiodothyroacetic acid and by thyroid ablation, but synthesis in connective tissue slices was unaffected. Pretreatment of the male rat with estrogen resulted in an increase in cholesterol synthesis in both the liver and connective tissue. The hormones, relaxin and thyroid stimulating hormone, which affected synthesis in connective tissue slices, did not alter liver synthesis. Thus, the mechanism by which cholesterol synthesis in connective tissue is regulated appears to differ from that for the liver.

The concentration of cholesterol was determined in the connective tissue biopsy and liver of each experimental animal, and it was found that none of the experimental conditions significantly altered the concentration of this lipid in either tissue. It would thus seem that cholesterol synthesis in connective tissue and liver in these experimental animals was not directly related to the cholesterol concentration in the two tissues.

The rate at which C\textsuperscript{14}-acetate was incorporated into the digitonin-precipitable substance of the connective tissue biopsy of the untreated male rat was greater than that in the female tissue. The fact that there was no significant difference between the cholesterol concentrations of the connective tissue biopsies from the male and female rats of this study would again suggest a lack of relationship between cholesterol concentration and cholesterol synthesis in connective tissue.

The studies in which the rats were pretreated with estrogen or testosterone indicate that the sex difference in cholesterol synthesis in connective tissue can not be explained on the basis of the effect of a single hormone. Testosterone did not alter the incorporation of labeled acetate in either sex while estrogen significantly increased the rate in the tissue of the male but did not affect the rate in the female tissue. The removal of the major source of testosterone or estrogen by castration or oophorectomy respectively caused a significant reduction in cholesterol synthesis in the connective tissue, and the subsequent administration of estrogen caused a return in both sexes toward the synthesis rates of the intact rats. The augmentation effect by estrogen pretreatment in the intact male and in the gonadectomized animal is similar to that reported on the increase in acetate incorporation into cholesterol in the perfused calf aorta after the addition of estrone to the perfusate.\(^4\)

The lack of an effect of estrogen pretreatment upon cholesterol synthesis in the biopsy-connective tissue of the intact female rat may be related to the level of endogenous estrogen and to its effect upon the fibroblasts of connective tissue. Only in the oophorectomized rat or in the male rat did exogenous estrogen have an effect upon cholesterol synthesis.

Relaxin was the only hormone studied which caused a reduction in the rate of cholesterol synthesis in the connective tissue of both sexes. A combination of relaxin and estrogen was administered to groups of intact male and female rats, and the augmentation of in vitro cholesterol synthesis in connective tissue which had been observed in the male rat after estrogen pretreatment did not occur. In fact, cholesterol synthesis in connective tissue slices from these relaxin- and estrogen-treated male and female rats was significantly depressed. Thus, the effect of relaxin upon the in vitro cholesterol synthesis in connective tissue would seem to dominate that of estrogen.

The mechanism by which relaxin effects this reduction in cholesterol synthesis in connective tissue is not apparent. Relaxin pretreatment did not alter the concentration of cholesterol in connective tissue.\(^5\) Following relaxin treatment, the mast cells of sponge-connective tissue were increased in number, and the metachromatic granules of these cells were reduced in number and appeared to be clumped tightly about the nucleus. Heparin administration was the only other treatment studied in this laboratory which similarly altered the morphology of mast cells. However, heparin did not effect an increase in mast cell number in connective tissue. Heparin is thought to be a product of the mast cell\(^1\) and has been reported
to enhance the oxidation of fats.\textsuperscript{17} Perhaps the effect of relaxin upon the incorporation of acetate into cholesterol in connective tissue is mediated through its effect upon mast cells.

Hypothyroidism in rats, as reported by others,\textsuperscript{18, 19} caused a significant reduction in hepatic synthesis of cholesterol in the female rat, and after the feeding of desiccated thyroid to the hypothyroid female rats, the incorporation of acetate into cholesterol of the liver was increased toward the value of the intact animal. The failure of the administration of desiccated thyroid or thyroid stimulating hormone to alter cholesterol synthesis in liver slices from an intact animal is not explicable since the rate of cholesterol synthesis in the liver varies directly with thyroid activity.\textsuperscript{15} Tetraiodothyroacetic acid reportedly lowers plasma cholesterol concentration without producing a detectable increase in metabolic rate.\textsuperscript{19} However, liver slices from male rats pretreated with tetraiodothyroacetic acid had a greater rate of cholesterol synthesis than slices from the untreated rats. Thyroid stimulating hormone was the only one of the thyroid group substances which altered cholesterol synthesis in connective tissue, and it caused a significant increase in the tissue of the female.

\textbf{SUMMARY}

Connective tissue obtained by sponge biopsy from the albino rat incorporated C\textsuperscript{14}-sodium acetate into digitonin-precipitable substance in vitro at a rate which was 1.6 per cent of that of the liver.

The radioactivity of the digitonin-precipitable substance of connective tissue of the male rat was greater than that of the female. Gonadectomy reduced cholesterol synthesis in connective tissue of both sexes. Pretreatment of the intact male rat and the gonadectomized animal with estrogen caused an increase in cholesterol synthesis.

The administration of relaxin effected a decrease in the radioactivity of the digitonide of connective tissue of the intact male and female rats and of the estrogen-treated castrated and spayed rats.

Cholesterol synthesis in connective tissue slices was not altered by thyroid ablation, by the feeding of desiccated thyroid to the hypothyroid rat or to the intact rat, or by the administration of tetraiodothyroacetic acid, progesterone, or testosterone to intact animals. Thyroid stimulating hormone pretreatment caused an increase in cholesterol synthesis in connective tissue of the female rat but did not affect synthesis in the male.

Hormonal effects upon in vitro cholesterol synthesis in the connective tissue and liver were not generally similar. Synthesis in the liver was altered by thyroid ablation and by the administration of estrogen and tetraiodothyroacetic acid.

\textbf{SUMMARY IN INTERLINGUA}

Tessuto conjunctive, obtenite ab rattos albin per biopsia a spongia, incorporava acetato de natrium a C\textsuperscript{14} in vitro a in un substantia precipitabile per digitonina con un intensitate amontante a 1,6 pro cento del activitate corrispondente del hepatе.

Le radioactivitate del substantia precipitate per digitonina ab tessuto conjunctive de rattos esseva plus grande in le caso de animales mascule que in le caso de animales feminin. Go- nadectomia reduceva le synthese de cholesterol in le tessuto conjunctive de ambe sexos. Pretramiento con estrogeno in intacte rat- tos mascule e in rattos e rattas gonadectomisate causava un augmento del synthese de cholesterol.

Le administration de relaxina effectuava un reduction del radioactivitate del digitonide de tessuto conjunctive in le caso de intacte rattos e rattas e de gonadectomisate rattos e rattas post pretramiento con estrogeno.

Le synthese de cholesterol in sectiones de tessuto conjunctive non esseva alterate per thyroidectomia, per le administration de desic- cate extracto thyroide a rattos hypothyroide o intacte, o per le administration de acido tetra- iodothyroacetic, de progesterona, o de testosterona a animales intacte. Pretramiento con hormones a stimulation thyroide causava un augmento del synthese de cholesterol in le tessuto conjunctive de rattos feminin sed non afficeva ille synthese in rattos mascule.

Le effectos hormonal super le synthese in vitro de cholesterol in tessuto conjunctive e in
tessuto hepatic non eseva generalmente identic. In le caso de tessuto hepatic, le synthese esseva alterate per thyroidectomia e per le administration de estrogeno e de acido tetraiodothyroacetic.

REFERENCES

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NANCY L. NOBLE and ROBERT J. BOUCEK

Circ Res. 1957;5:573-578
doi: 10.1161/01.RES.5.5.573
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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