Squalene Feeding in Experimental Atherosclerosis

By David Kitchevsky, Ph.D., Arden W. Moyer, Ph.D., Walter C. Tesar, B.S., John B. Logan, B.S., Raymond A. Brown, Ph.D., and Gilbert Richmond, B.S.

The hydrocarbon squalene, which has been suggested as a precursor in the biosynthesis of cholesterol, was fed to rabbits to see whether cholesterol thus synthesized would be efficacious in producing atheromatous lesions. Rabbits on a 3 per cent squalene diet for seven weeks showed an increase in liver weight and liver nonsaponifiable material but no more atheroma than did controls. Animals on the same diet for 14 weeks showed an increase in the cholesterol-rich lipoproteins as determined in the ultracentrifuge. Addition of squalene in no way altered the atherogenic propensities of the cholesterol diet.

In 1926 Channon observed that the livers of rats maintained on a diet containing the unsaturated C30H50 hydrocarbon squalene showed a threefold increase in nonsaponifiable material and a concomitant 100 per cent increase in cholesterol content. The same year Heilbron, Kamm and Owens suggested that this compound might be an intermediate in the biologic synthesis of cholesterol. In 1934, Robinson suggested a scheme for the direct conversion of squalene to cholesterol.

Quite recently, considerable interest has been revived in the role played by squalene in the biogenesis of cholesterol. With the aid of radioisotopes, Langdon and Bloch have shown that squalene is, indeed, a normal constituent of rat liver, and its function as a precursor in cholesterol biosynthesis has been established. Another route of cyclization to cholesterol, different from that suggested by Robinson, has been proposed. Degradation studies tend to favor this latter scheme.

The findings that the arterial tissue can synthesize cholesterol in vitro and that liver slices taken from cholesterol-fed rats show a great reduction in their power to convert acetate to cholesterol have prompted the suggestions that exogenous cholesterol may not play an important part in the development of atherosclerosis. In contrast to these findings, it has been demonstrated that, in the intact rabbit, cholesterol synthesis proceeds in the face of massive cholesterol feeding. The importance of exogenous cholesterol as a factor in the development of atherosclerosis has been emphasized by Gofman and has been discussed in a recent review by Moses. Squalene represents what is presumably the most efficient precursor of cholesterol. Since any cholesterol synthesized from squalene may be regarded as endogenous cholesterol, a means exists for testing the effect of endogenous cholesterol in the development of atherosclerosis.

METHODS

Five groups of male rabbits weighing between 1.5 and 2 Kg. were used. Group I received a normal diet. In the other four groups the normal diet was augmented as follows: groups II and III, with 3 per cent squalene in corn oil, group IV, with 3 per cent cholesterol in corn oil, and group V, with 3 per cent squalene plus 3 per cent cholesterol in corn oil. In a preliminary report corn oil was shown to produce no atherosclerosis when added to the normal diet. Channon found that the amount of cholesterol necessary to give results similar to those observed after squalene feeding is the molar equivalent of the squalene fed; inasmuch as the molecular weights of squalene and cholesterol (410 and 386, respectively) are fairly close together, these compounds were fed in approximately equimolar quantities. The rabbits were maintained on these diets for seven

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Table 1.—Degree of Atheroma in Groups of Rabbits Fed Various Diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet*</th>
<th>0</th>
<th>±</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>+++++</th>
<th>Average Atheroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (N)</td>
<td>9/10</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0.05</td>
</tr>
<tr>
<td>II</td>
<td>X + Squalene—7 Weeks</td>
<td>5/8</td>
<td>2/8</td>
<td>1/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0.25</td>
</tr>
<tr>
<td>III</td>
<td>X + Squalene—14 Weeks</td>
<td>6/8</td>
<td>2/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0.13</td>
</tr>
<tr>
<td>IV</td>
<td>X + Cholesterol</td>
<td>0/8</td>
<td>0/8</td>
<td>2/8</td>
<td>2/8</td>
<td>2/8</td>
<td>2/8</td>
<td>2.50</td>
</tr>
<tr>
<td>V</td>
<td>X + Cholesterol and Squalene</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
<td>5/10</td>
<td>2/10</td>
<td>2.70</td>
</tr>
</tbody>
</table>

* All supplements represent 3 per cent of the total diet.

weeks, with the exception of group III which was maintained on a squalene diet for 14 weeks, then sacrificed. The livers were weighed, dissolved in concentrated alcoholic potassium hydroxide and assayed for total nonsaponifiable material, cholesterol14 and, in the case of group II, for squalene15. The aortas were examined visually for atherosclerotic lesions and these were graded on a 0–4 plus scale, in the order of increasing severity. Ultracentrifugal examination of the serum for β-lipoproteins was carried out according to the method described by Gofman.16

Squalene was purchased from Distillation Products, Inc., control #Q693, and distilled before use. The boiling point of the material (225 degrees C. at 2 mm.) and its index of refraction (nD15 1.4953) correspond with those determined by Heilbron.17 The hexahydrochloride melted at 103–109 degrees, corresponding with the 103–110 degrees which has been reported.19 Paper chromatography of the redistilled squalene on "Quilon" impregnated paper20 gave a single spot with an Rf value of 0.73 (the value 0.71 had previously been reported19). Only redistilled squalene was used in these feedings.

RESULTS AND DISCUSSION

That squalene does not cause atheromatous lesions when fed in corn oil, and, moreover, that fed together with cholesterol it does not inhibit cholesterol-induced atherosclerosis is shown by the data presented in table 1.

Atheroma observed in the two groups receiving squalene correspond to those seen in rabbits maintained on normal chow plus corn oil.14 The slight increase over the normal values may reflect the effect of added dietary fat. In another group of five rabbits maintained on 3 per cent squalene for seven weeks, the average atheroma was 0.10. No importance can be attached to the slight difference in average atheroma between the groups on cholesterol and cholesterol plus squalene, since

Table 2.—Averages of Nonsaponifiable Material in Livers of Groups of Rabbits Fed Various Diets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Diet*</th>
<th>Average liver weight (gms)</th>
<th>Total nonsaponifiable material (per cent)</th>
<th>Cholesterol (per cent)</th>
<th>Squalene (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>68 (48–96)‡</td>
<td>0.2 (0.1–0.3)</td>
<td>0.2 (0.1–0.3)</td>
<td>0.1 (0.01–0.2)</td>
</tr>
<tr>
<td>II</td>
<td>X + Squalene—7 weeks</td>
<td>91 (62–131)</td>
<td>0.6 (0.2–1.3)</td>
<td>0.2 (0.1–0.3)</td>
<td>--</td>
</tr>
<tr>
<td>III</td>
<td>X + Squalene—14 weeks</td>
<td>67 (48–75)</td>
<td>3.1 (1.1–7.7)</td>
<td>1.7 (0.8–3.9)</td>
<td>--</td>
</tr>
<tr>
<td>IV</td>
<td>X + Cholesterol</td>
<td>90 (69–103)</td>
<td>10.5 (8.7–13.4)</td>
<td>8.2 (4.2–16.9)</td>
<td>--</td>
</tr>
<tr>
<td>V</td>
<td>X + Cholesterol and Squalene</td>
<td>99 (73–116)</td>
<td>15.2 (7.7–30.8)</td>
<td>8.4 (4.2–16.9)</td>
<td>--</td>
</tr>
</tbody>
</table>

* All supplements represent 3 per cent of the total diet. † Percentages are based on wet liver weight. ‡ Range of determinations.
SQUALENE FEEDING IN ATHEROSCLEROSIS

Table 3.—Average S/ Values (mg. Per Cent) for Groups of Rabbits on Various Diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet*</th>
<th>S/ Class of Lipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–11 12–20 21–35 35–100 100–400</td>
</tr>
<tr>
<td>I</td>
<td>Normal (X)</td>
<td>80 18 30 45 50</td>
</tr>
<tr>
<td>II</td>
<td>N + Squalene—7 Weeks</td>
<td>71 30 22 20</td>
</tr>
<tr>
<td>III</td>
<td>N + Squalene—14 Weeks</td>
<td>156 152 95 50 34</td>
</tr>
<tr>
<td>IV</td>
<td>N + Cholesterol</td>
<td>370 925 1,240 910 545</td>
</tr>
<tr>
<td>V</td>
<td>N + Cholesterol and Squalene</td>
<td>190 1,030 1,065 1,075 615</td>
</tr>
</tbody>
</table>

* All supplements represent 3 per cent of the total diet.

similar differences have been observed in groups of rabbits on a high cholesterol diet. The data obtained in working up the liver nonsaponifiable fractions are given in table 2. All values are based upon wet weight of liver. There was considerable variation from rabbit to rabbit in the percentages of nonsaponifiable liver materials. In table 3 the extremes for each group are listed. In general, however, cholesterol feeding, or prolonged squalene feeding, caused a marked increase in liver nonsaponifiable material. In the case of the animals receiving squalene for seven weeks, the three-fold increase was similar to that observed in rats by Channon. The differences in the nonsaponifiable content of the livers of the rabbits on squalene and cholesterol were very marked, even though the liver weights were approximately the same. The large increase in the weights of the livers of cholesterol-fed animals would seem to be almost entirely ascribable to dietary cholesterol.

The apparent S/ values of the serum lipoproteins are presented in table 3. At the start of the experiment, the average S/ lipoprotein levels for all animals were as follows: S/ 0–11, 54 mg. per 100 cc.; S/ 12–20, 23 mg. per 100 cc.; S/ 21–35, 25 mg. per 100 cc.; S/ 35–100, 26 mg. per 100 cc.; and S/ 100–400, 30 mg. per 100 cc.

It has been shown that the cholesterol content of lipoprotein molecules is inversely proportional to their S/ rate, falling from 30 per cent at S/ 0–8 to 5 per cent at S/ 40,000. A more recent report asserts that cholesterol accounts for 45 per cent of the S/ 6 fraction. The animals on the seven-week squalene diet showed no increase in their serum lipoproteins.

As squalene feeding continued, there was an increase in the serum levels of the S/ 0–11, 12–20 and 21–35 lipoproteins. Perhaps upon greatly prolonged squalene feeding the other groups might also have shown an increase. In the group on the 14-week squalene diet, it is assumed that cholesterol synthesis went on for the entire period, yet the levels of serum lipoproteins and the severity of atheroma did not remotely approach those observed in the cholesterol-fed animals. Thus, while a diet designed to give the most efficient cholesterol synthesis gave, after a comparatively long time, a rise in the levels of those lipoproteins which contain more cholesterol, it never resulted in as high levels as those obtained upon direct cholesterol feeding. Furthermore, this diet gave no evidence of causing the atheroma observed in cholesterol-fed animals.

Inasmuch as the squalene diet may be regarded as a source of endogenous cholesterol, it would appear that in our experiments exogenous cholesterol played a more important role in the development of atherosclerosis than did endogenous cholesterol.

SUMMARY

1. Rabbits maintained on a diet containing squalene showed an increase in liver weight and in liver nonsaponifiable material, but no more atheroma than did controls.

2. Addition of squalene to a cholesterol diet did not lessen the severity of the cholesterol-induced atherosclerosis.

3. Endogenous cholesterol, as represented here by that synthesized from squalene, did not cause atherosclerosis; exogenous cholesterol did.

REFERENCES

DAVID KRITCHEVSKY, ET AL.


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