Attempts to Induce Stomach Tumors

III. The Effects of (a) A Residue of Cholesterol Heated to 300° C., and (b) Δ3,5-Cholestadiene*

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In recent years Roffo has described (8-11) the production of gastropapillomatosis of the forestomach, adenocarcinoma of the glandular stomach, and mesenteric and hepatic sarcoma in rats by feeding various fats or cholesterol heated to 350° C. for half an hour. Results obtained in this laboratory by feeding heated fats (1, 7) and heated cholesterol (5) to Wistar rats have already been published. No adenocarcinomas have occurred in our experiments; the feeding of heated fats led to an induced avitaminosis A associated with papillomatosis of the forestomach, but no hyperplastic lesions were seen in either part of the stomach after ingestion for 2 years of cholesterol heated to 300° C. for half an hour. The noncarcinogenicity of cholesterol heated to 200° C. or to 300° C. has been demonstrated also by Steiner, Steele, and Koch (13) who found no tumors up to 18 months when the heat products were injected subcutaneously into mice.

(a) Experiments with the Residue

The possibility arose of increasing any carcinogenic activity in heated cholesterol by the elimination of some noncarcinogenic products of heating. It was observed in early preparations of heated cholesterol that the molten mass, after having been heated for half an hour to 300° C., was no longer entirely soluble in ethanol. If the suspension was filtered hot, a white substance could be separated that had a melting point of about 192° C. and gave a carmine-red color with antimony trichloride in chloroform solution only on long standing. It was at first thought that this substance would be the β-cholesterol that Diels and Linn (2) isolated from cholesterol heated in the same way but poured into acetone instead of ethanol. This compound, however, was supposed to melt at 160° C. It was subsequently noted that Mauthner and Suida (6) had obtained an ethanol-insoluble derivative from cholesterol by heating with anhydrous copper sulphate to 200° C. This compound melted at 188° to 192° C. and was dicholesterol ether, formed by elimination of 1 molecule of water between 2 molecules of cholesterol. Since, moreover, dicholesterol ether was shown by Wokes (17) to give a red color with antimony trichloride in chloroform solution only on long standing, it was concluded that the compound obtained by pyrolysis of cholesterol at 300° C. was dicholesterol ether. Thus one substance of known constitution could easily be removed from the mixture of heat products.

Diels and Linn (2) asserted that 50 per cent of the cholesterol that they heated to 310° C. for half an hour was converted to cholestenone. Heilbron and Sexton (4) also obtained this ketone by distillation of cholesterol. This compound was therefore reasonably certain to be present in the mixture of heat products. Diels and Linn used cold methanol to extract the cholestenone and this procedure was adopted here.

A quantitative experiment showed that, on average, 84 per cent of the cholesterol was recoverable unchanged at any one heating. According to Diels and Linn (3), at least 75 per cent was converted to cholestenone and “β-cholesterol,” while Roffo described total destruction of cholesterol heated to 350° C. for half an hour. Actually, in our quantitative experiment, about 8 per cent of the cholesterol used was isolated as dicholesterol ether; nothing separated from the ethanol solution on cooling, i.e., no “β-cholesterol” was obtained. The weight of A4-cholestenone obtained corresponded to 32 per cent of the original cholesterol.

Feeding of residue.—The methanol-insoluble residue was a dark red translucent gum. It was dissolved in chloroform to make a 20 per cent solution, and this solution was dropped on to rat-cake (15), 0.1 ml. on each piece, as described in Part I (5). One piece of this rat-cake, i.e., 20 mgm. of residue, was fed daily to each of a group of 24 Wistar rats. These rats also

* Because of the difficulties of international communication the author has not read proof of this article.

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received an adequate supply of untreated rat-cake and water *ad libitum*, while milk and green-stuff were fed once weekly.

**Results with residue.**—Four of the 24 rats died before 200 days. Of the rest, the first died at 405 days; 7 others before 500 days; 10 between 500 and 600 days, and the remainder at 653 and 687 days respectively. Of these 20 rats, one died from pneumonia; the remainder all had more or less severe lung abscesses. The hemorrhagic erosions reported in Part I were also frequently seen here and presumably are not related to the feeding of the residue. In the 20 rats surviving 400 days, all except 3 had normal forestomachs. The rat dying of pneumonia at 547 days showed a slight, unevenly distributed hyperkeratosis; one dying at 548 days showed a generalized hyperkeratosis with a slight hyperplasia tending to form minute papillae; that dying at 653 days showed an area of localized hyperkeratosis and hyperplasia, but the last rat to die, at 687 days, showed no abnormality of the forestomach. A condition of generalized slight hyperkeratosis and hyperplasia was seen in one rat fed whole heated cholesterol (5); this minor degree of change was seen in 3 of 12 controls and cannot be ascribed to the heat products of cholesterol.

(b) **EXPERIMENTS WITH Δ3,5-CHOLESTADIENE**

Beside the intermolecular dehydration of cholesterol to form dicholesteryl ether, intramolecular dehydration might occur to yield one or more cholestadienes. According to Staveley and Bergmann (12), a cholestadiene, m.p., 79°C, arises on heating cholesterol with kieselguhr or on heating cholesterilene, which was then dehydrogenated to pseudocholestanene; this substance they demonstrated to be Δ3,5-cholestadiene. Moreover, Heilbron and Sexton (4) regarded the formation of pseudocholestenene on distilling cholesterol of normal atmospheric pressure as due to dehydration of the cholesterol to cholesterilene, which was then dehydrogenated to pseudocholestenene. Δ3,5-Cholestadiene gives an immediate carmine-red color with antimony trichloride in chloroform and the products of heating cholesterol to 300°C. were tested in this way for the diene. Several fractions, especially first filtrates from chromatograms, gave an immediate color, but it was not found possible to isolate any cholestadiene.

The absence of any considerable quantity of Δ3,5-cholestadiene in the products of heating cholesterol to 300°C. was rather against the theory advanced by Veldstra (16) that the effective agent in heated fats or heated cholesterol was this particular diene. It was observed in this Department by Beck (1) that lard heated to 330°C. for half an hour gave a brownish red color with antimony trichloride in chloroform. It seemed possible that this color was due to a diene but masked by the colored material in heated fat. A quantity of heated lard was, therefore, saponified and the nonsaponifiable fraction tested for dienes. However, only a brownish red color could be obtained, even with the early chromatogram filtrates, which would have contained any diene present in the fraction. It seems unlikely, therefore, that any diene is formed in lard by heating to 330°C.

Strong's work (14) would seem to indicate that neither Δ3,5- nor Δ2,4-cholestadiene is a powerful carcinogen, since no tumors were obtained by painting or by injecting either of these hydrocarbons. Hence the presence of traces of either diene could hardly account for the lesions that Roffo described. However, Veldstra (16) states that Waterman obtained 4 papillomas of the stomach in an unspecified number of mice by feeding Δ3,5-cholestadiene for one year, and it seemed desirable to test this substance by mouth in rats.

**Feeding Δ3,5-cholestadiene.**—Δ3,5-Cholestadiene was prepared by the method of Eck and Hollingsworth (3) from cholesterol, which was either nonfluorescent (in chloroform solution) or had been rendered nonfluorescent by purification *via* the acetate. A product melting at 79°C to 80°C, and having $\left[\alpha\right]_D^{11} - 110°$ or greater is readily prepared by refluxing 50 gm. cholesterol in 200 ml. xylene with 50 gm. anhydrous copper sulphate for 4 hours, filtering, and transferring the product to petroleum ether (b.p., 60°C to 80°C.) solution, passing this solution through a large column of activated alumina, to remove other substances, and recrystallizing the diene from ethanol.

The diene was dissolved in chloroform to give a 25 per cent solution and was dropped on to rat-cake, as previously described for the heated cholesterol solutions. One piece of impregnated rat-cake, *i.e.*, 25 mgm. of diene, was fed daily to each of 24 Wistar rats maintained on the same basal diet as was used in previous experiments. In case this diene was the substance responsible for the induced avitaminosis A seen in rats fed fats heated to 330°C. (1), half this group of rats received a supplement of carrot, about 2 gm. per rat, 3 times a week.

**Results with Δ3,5-cholestadiene.**—No tumors were seen in any rats. Apart from the usual hemorrhagic erosions of the glandular zone, the only abnormality seen in the stomach was some generalized hyperkeratosis in rat 70, dying at 416 days, and in rat 72, dying at 449 days, both of which had received the supplements of carrot. No tendency to papillomatosis was seen in the group receiving no carrot, although 4 of these died between 400 and 500 days, and 2 between 500 and 600 days, while 6 survived more than 600 days, the last being sacrificed on account of poor...
condition at 689 days. In the carrot-fed group, 7 rats outlived those showing hyperkeratosis, the last dying at 691 days. Rat 72 was the first rat seen in this Department to have a stone in the bladder; this will be described below.

There is thus no evidence that Δ3,5-cholestadiene has any injurious effect on the stomachs of rats when fed at the relatively high level of 25 mgm. daily for nearly 2 years. Moreover, there was no evidence of consis-

(2) Quantitative: ¹

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of weight on ignition</td>
<td>54.56 per cent</td>
</tr>
<tr>
<td>Proportion of calcium as CaO</td>
<td>1.69 &quot; &quot;</td>
</tr>
<tr>
<td>Magnesium as MgO</td>
<td>15.04 &quot; &quot;</td>
</tr>
<tr>
<td>Phosphorus as P₂O₅</td>
<td>29.90 &quot; &quot;</td>
</tr>
<tr>
<td></td>
<td>101.19 &quot; &quot;</td>
</tr>
<tr>
<td>Nitrogen as NH₃ (microkjeldahl)</td>
<td>5.49 per cent</td>
</tr>
</tbody>
</table>

There was also a trace of iron.

Rat 72.—This rat died at approximately 18 months of age, after 440 days' feeding with Δ3,5-cholestadiene. All organs appeared normal to the naked eye except the kidneys. The bladder contained a large, smooth stone measuring 17 mm. along its greatest axis, and 13 mm. and 9 mm. respectively in the other two planes; the weight was 1.83 gm.

Analyses.—(1) Qualitative: The stone proved to be entirely inorganic; no urates, carbonates, oxalates, cystine, or cholesterol were present. The analysis corresponds very closely to that for Mg NH₃PO₄·6H₂O in which, however, part of the magnesium has been replaced by calcium.

The bladder itself showed loss of mucosa, presumably due to irritation by the stone. Submucous fibrosis was present and numerous papillae (not neoplastic) projected into the lumen. Part of a section is shown in the accompanying photomicrograph.

SUMMARY

1. Rats on an adequate basal diet fed the residue left from cholesterol heated to 300° C. after the removal of dicholesteryl ether and Δ4-cholestenone, at

¹ The author is greatly indebted to Dr. John A. Mair, of the Chemistry Department, Glasgow University, for the quantitative analyses.
a level of 20 mgm. daily for 2 years, showed no tumor of the forestomach nor of the glandular zone.

2. Other rats fed Δ3,5-cholestadiene at a level of 25 mgm. daily for 2 years, plus an adequate basal diet, showed no tumor in either part of the stomach. It seems unlikely that this diene is concerned in the avitaminosis A induced by feeding heated fats to rats.

3. A large, inorganic bladder stone is reported in 1 rat of this series.

REFERENCES


Attempts to Induce Stomach Tumors. III. The Effects of (a) A Residue of Cholesterol Heated to 300° C., and (b) \[\Delta\]
3,5-Cholestadiene

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