Effects of Dietary Proteins, Methionine and Vitamins on Plasma Lipids and Atherogenesis in Cholesterol-Fed Cockerels

By JEREMIAH STAMLER, M.D., RUTH PICK, M.D. AND LOUIS N. KATZ, M.D.

With the assistance of Mrs. D. Century and Mr. P. Johnson

Methionine deficiency in the presence of a high-fat, high-cholesterol diet aggravates atherogenesis in the cockerel. High-protein, high-vitamin supplementation suppresses the atherogenic effect of a cholesterol-fat-containing diet. High-protein supplementation alone had a suppressive effect; high-vitamin supplementation had no effect. However, the combined protein and vitamin supplement was the most effective.

RECENT work in this department led to the tentative conclusion that patterns of cholesterolemia and atherogenesis in man and experimental animals may be influenced by the total dietary pattern. An hypothesis was put forward, suggesting that dietary imbalance—excess of some nutrients (e.g., total calories, total fats, saturated fats, refined carbohydrates) and inadequacy (absolute or relative) of others (e.g., proteins, vitamins, minerals, essential fatty acids)—may be particularly pernicious in inducing hypercholesterolemia and atherosclerosis.

The present experiments were undertaken to test this hypothesis in chicks, particularly with respect to inter-related effects of lipid, cholesterol, amino acids, proteins and vitamins.

METHODS

Four series of experiments were accomplished, utilizing a total of 180 cockerels (10 birds per group) (table 1). The department's established technics for studies on experimental atherosclerosis were used throughout. One-day-old Hy-line chicks were obtained from a commercial hatchery and reared in a battery brooder. The experimental periods were ages 16 to 21, 14 to 21, 8 to 15, and 13 to 23 weeks in series 39, 41, 46, and 47 respectively.

In all series chicks were fed a diet supplemented with 5 per cent cottonseed oil and cholesterol (1, 2, 2, and 1 to 2 per cent in series 39, 41, 46, and 47 respectively). Over-all, two types of diets were utilized, one with commercial chick starter mash as its base, the other with purified ingredients (table 1). In the latter, two types of purified basal proteins were used, casein-gelatin (series 39, 41, and 46) or soy protein (series 46). In the control groups on purified ration, the protein level was 35 per cent (groups 2 and 2D, table 1); in the high-protein groups on purified ration, it was increased to 63 per cent (groups 3 and 3D); in the reduced-protein group, it was decreased to 20 per cent (group 4, series 39). The sulfhydryl amino acid d,l-methionine was incorporated in the purified rations of all groups, except the low-methionine groups (table 1). In the control groups, it was given at the level of 0.50 to 0.75 per cent of the ration; in the high-protein groups, 1.50 to 2.50 per cent. Choline and inositol (0.2 to 0.3 and 0.1 to 0.2 per cent, respectively, for the control groups on purified ration) were increased to 1.0 to 1.5 per cent and 0.5 to 1.0 per cent, respectively, in the high-protein groups. In the high-vitamin groups, premixes of multiple fat-soluble and water-soluble vitamins were incorporated in the ration at levels five times as great as for the control groups on purified ration. Complete details concerning the vitamin and salt mixtures utilized, as well as other aspects of the diets, are available from the authors upon request.

In series 39, the effects of high cholesterol-high
DIETARY PROTEINS, METHIONINE AND VITAMINS

TABLE 1.—General Experimental Design

<table>
<thead>
<tr>
<th>Series no. and cholesterol oil supplement*</th>
<th>Group no.</th>
<th>Over-all dietary characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>1</td>
<td>Control—Mash</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Control—Purified</td>
</tr>
<tr>
<td>-1 C-O</td>
<td>3</td>
<td>High protein—High vitamin—Purified</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Reduced protein—Purified</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Low methionine—Purified</td>
</tr>
<tr>
<td>41</td>
<td>1</td>
<td>Control—Mash</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Control—Purified</td>
</tr>
<tr>
<td>-2 C-O</td>
<td>3</td>
<td>High protein—High vitamin—Purified</td>
</tr>
<tr>
<td>46</td>
<td>1</td>
<td>Control—Mash</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Control—Purified—Casein and gelatin</td>
</tr>
<tr>
<td>-1-2 C-O</td>
<td>3</td>
<td>High protein—High vitamin—Purified</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Borderline—Low methionine—Purified (D)†</td>
</tr>
<tr>
<td>47†</td>
<td>1</td>
<td>Control—Mash</td>
</tr>
<tr>
<td>-0.5 C-O</td>
<td>3</td>
<td>High protein—High vitamin—Mash</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>High protein—Mash</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>High vitamin—Mash</td>
</tr>
</tbody>
</table>

- 0.5 C-O, 1 C-O and 2 C-O are mash or purified feed supplemented with 0.5, 1 or 2 per cent cholesterol plus 5 per cent cottonseed oil. In series 46, mash birds ate 1 C-O, purified diet chicks ate 2 C-O.
- In this series, each group was made up of 7 or 8 birds at the start of the experimental feeding regimen. Two birds from each group were sacrificed after 5 weeks.
- The purified ration of this group contained the usual soy protein (not α-protein) at the 35 per cent level, but methionine supplementation was omitted.

fat combined with low protein, low methionine or high protein-high vitamin diets were explored (table 1). Low methionine diet was accomplished by use of α-protein (25 per cent), a soy protein low in this amino acid, plus omission of methionine in the ration (series 39, group 5, table 1). High protein-high vitamin experiments were repeated in series 41 and 46.

In series 46, high cholesterol-high fat combined with high protein-high vitamin was also studied, using commercial mash as the ration base. Series 47 repeated this design, extending it to include analysis of the separate effects of high proteins and high vitamins individually (table 1). In these experiments with commercial mash, the protein supplement was casein, soy protein, defatted liver, defatted fish meal, and Brewer's yeast, 7 per cent of each; the vitamin supplement was identical with that used in the purified ration.

RESULTS

Effects of Reduced Protein or Low Methionine Content in a Purified Ration Containing Cholesterol Oil. These dietary combinations were studied in series 39 and 46, groups 4, 5 and 5D (tables 2 and 3). The reduced protein diet (series 39, group 4) was associated with a lower feed intake and weight gain. Plasma lipid levels were essentially similar in the reduced protein and control groups. A higher incidence of thoracic aorta lesions was observed in the reduced protein

TABLE 2.—Feed Intake, Body Weight and Plasma Lipids in Cockerels on Purified Diets

<table>
<thead>
<tr>
<th>Series no. and group no.</th>
<th>Type diet</th>
<th>Feed intake (Gm./chick/day)*</th>
<th>Terminal weight (Gm.)</th>
<th>Terminal total cholesterol (mg. %)</th>
<th>C/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>39-2</td>
<td>Control</td>
<td>70</td>
<td>1910 ± 75</td>
<td>201 ± 190</td>
<td>0.84</td>
</tr>
<tr>
<td>39-3</td>
<td>High protein—High vitamin</td>
<td>65</td>
<td>1731 ± 113</td>
<td>152 ± 9</td>
<td>0.93</td>
</tr>
<tr>
<td>39-4</td>
<td>Reduced protein</td>
<td>58</td>
<td>1279 ± 43</td>
<td>215 ± 65</td>
<td>0.91</td>
</tr>
<tr>
<td>39-5</td>
<td>Low methionine</td>
<td>74</td>
<td>1906 ± 68</td>
<td>381 ± 59</td>
<td>0.97</td>
</tr>
<tr>
<td>41-2</td>
<td>Control</td>
<td>78</td>
<td>1912 ± 65</td>
<td>228 ± 11</td>
<td>1.00</td>
</tr>
<tr>
<td>41-3</td>
<td>High protein—High vitamin</td>
<td>46</td>
<td>1557 ± 77</td>
<td>263 ± 26</td>
<td>1.25</td>
</tr>
<tr>
<td>46-2</td>
<td>Control</td>
<td>71</td>
<td>1296 ± 38</td>
<td>321 ± 20</td>
<td>1.16</td>
</tr>
<tr>
<td>46-2D</td>
<td>Control—Soy protein</td>
<td>78</td>
<td>1465 ± 68</td>
<td>195 ± 11</td>
<td>1.80</td>
</tr>
<tr>
<td>46-3D</td>
<td>High protein—High vitamin (Soy)</td>
<td>68</td>
<td>1270 ± 28</td>
<td>146 ± 5</td>
<td>1.00</td>
</tr>
<tr>
<td>40-5D</td>
<td>Borderline—Low methionine</td>
<td>81</td>
<td>1355 ± 42</td>
<td>258 ± 14</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Data collected on group as a whole.
† Phospholipids were determined on pooled specimens of plasma. The C/P ratio is the ratio of total cholesterol to phospholipids.
‡ Standard error of mean.
Effects of Soy Protein vs. Casein-Gelatin in a Purified Ration Containing Cholesterol Oil. This experiment was accomplished in series 39, 41, 46 (tables 2 and 3). Feed intake and weight gain tended to be less in high protein-high vitamin groups, compared with controls. In 2 of the 3 series of experiments, hypercholesterolemia was less marked in the high protein-high vitamin groups. Except for one bird in series 46 with a minimal lesion, thoracic aorta atherogenesis was absent in high protein-high vitamin chicks of all series. No coronary lesions were noted in these birds in series 39 and 46, in contrast to the controls. Coronary atherogenesis was also less marked in the experimental groups in series 41 (table 3).

Effects of High Protein-High Vitamin Supplementation of a Purified Ration Containing Cholesterol Oil. Feed intake tended to be slightly less in the high protein-high vitamin groups, as was expected in view of this ration's greater caloric content (table 4). Gain in weight was greater in

TABLE 3.—Atherogenesis in Cockerels on Purified Diets

<table>
<thead>
<tr>
<th>Series no. and group no.</th>
<th>Gross thoracic aorta atherogenesis</th>
<th>Microscopic coronary atherogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent with lesions</td>
<td>Per cent with lesions grade 1 or &gt;2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39-2</td>
<td>22.2</td>
<td>0</td>
</tr>
<tr>
<td>39-3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>39-4</td>
<td>77.8</td>
<td>0</td>
</tr>
<tr>
<td>39-5</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>41-2</td>
<td>20.0</td>
<td>0</td>
</tr>
<tr>
<td>41-3</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>46-2</td>
<td>33.3</td>
<td>11.1</td>
</tr>
<tr>
<td>46-2D</td>
<td>20.0</td>
<td>0</td>
</tr>
<tr>
<td>46-3D</td>
<td>10.0</td>
<td>0</td>
</tr>
<tr>
<td>46-5D</td>
<td>13.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Lesions in the thoracic aorta were graded on an arbitrary scale from 0-4.

This is the average of the grades for those birds with lesions; chicks graded 0 were excluded in calculating this mean.

Two standard Sudan-stained sections of the heart were examined microscopically, and a count was made of all arterioles and arteries visualized, and the percentage of those exhibiting atherosclerotic plaques. This is the mean per cent for the group, and is an index of severity of atherosclerotic involvement.

group; atherogenesis was otherwise essentially similar in the two groups.*

The low methionine group exhibited a level of feed intake and weight gain comparable to that of the control group. Cholesterolemia, cholesterol/phospholipid (C/P) ratio, incidence of thoracic and coronary lesions, and severity of coronary lesions were all increased in the low methionine group. The borderline-adequate methionine group (series 46, 5D) was not significantly different from its matched control.

Effects of High Protein-High Vitamin

*It should be noted that the control group on purified ration ingested protein at the 35 per cent level (25 per cent casein + 10 per cent gelatin). The reduced protein group consumed 20 per cent protein (10 per cent casein + 10 per cent gelatin). In later experiments, lowering of protein to 10 to 15 per cent—a frank low protein ration—resulted in marked increase in hypercholesterolemia and atherogenesis in cockerels on a high fat-high cholesterol ration.*
the experimental groups. Hypercholesterolemia, aorta and coronary ath- 
erogenesis were less marked in the high protein-high vitamin 
supplemented group.

Effects of Separate Supplementation ivith 
High Protein or High Vitamin in Commercial 
Mask Containing Cholesterol Oil. This single 
supplementation resulted in lower feed in-
takes and weight gains (series 47, groups 6 
and 7) (table 4). In contrast to the high 
protein-high vitamin group (3M), both the 
high protein-alone and the high vitamin-alone 
groups had levels of hypercholesterolemia as 
high as or higher than the controls. Corre-
spondingly, the high vitamin-alone group had 
coronary atherogenesis as marked as the 
control (table 5). In the group supplemented 
with high protein-alone, incidence of coronary 
atherogenesis was markedly lower than in 
the controls, and was similar to that of the 
high protein-high vitamin group.

Effects of Cholesterol OH Supplementation 
of Purified Ration vs. Commercial Chick 
Starter Mash. In all series, cholesterol sup-
plementation at the same (series 39 and 41) 
or twofold greater (series 46) levels in the 
purified, compared with the commercial mash, 
group yielded less marked hypercholesterol-
elmia and atherogenesis (group 1, series 46, 
tables 4 and 5 and group 2, series 46, tables 
2 and 3).

DISCUSSION
The findings of these experiments indicate 
that high protein-high vitamin supplementation 
suppressed hypercholesterolemia and 
atherogenesis in cholesterol-oil-fed cockerels. 
This was true with both purified and com-
mercial mash diets. Vitamin supplementation 
one alone had no such effect. Protein 
supplementation alone apparently suppressed 
coronary atherogenesis in a manner similar 
to high protein-high vitamin. However, 
protein alone apparently had less definitive 
and clear-cut effects than the combination. 
It would seem, therefore, that the high pro-
tein diet is the decisive factor responsible 
for these results, but that high vitamin intake 

has an adjuvant, synergistic influence. These 
results are consistent with the concurrent 
independent observations in chicks by other 
workers. Antiatherogenic effects of protein 
have apparently not been hitherto reported.

These experiments further demonstrate that 
insufficiency of methionine intake aggravated 
the hypercholesterolemic and atherogenic 
effects of cholesterol-oil ingestion. They also 
confirm in cockerels observations previously 
reported in monkeys and rats.

The present results further indicate that 
addition of cholesterol to the chick diet in-
duced less hypercholesterolemia and ath-
erogenesis in a purified than in a regular mash 
ration. The mechanism of this effect is not 
apparent from the data of this experiment. 
It may be a resultant of the greater protein 
intake with the purified ration (35 vs. ap-
proximately 20 per cent). However, the 
results with 20 per cent protein in a purified 
ration (series 39, group 4) do not support 
this suggestion, since enhancement of hyper-
cholesterolemia was not observed. These data 
are difficult to interpret, however, in view of 
the differences in feed intake and growth 
pattern. This problem was explored further.

Finally, the data of these experiments fur-
ther suggest that different results may be ob-
tained with qualitatively different proteins 
in a cholesterol-oil-supplemented purified 
ration. Thus, hypercholesterolemia and ath-
erogenesis were less marked with soy protein 
that with casein-gelatin (series 46, groups 
2 and 2D). Similar findings were obtained 
independently by other workers.

| Table 5.—Atherogenesis in Cockerels on Mash Diet |
|---|---|---|---|---|
| Series | Gross thoracic aorta | Microscopic coronary atherogenesis |
| no. | Per cent with lesions | Per cent with lesions | Per cent of vessels with lesions (all birds) |
| no. | Mean grade birds with lesions | Mean grade birds with lesions |
| 46-1 | 90 | 0 | 1.1±0.2 |
| 46-3M | 60 | 0 | 4±0.1 |
| 47-1 | 0 | 0 | 0.4±0.1 |
| 47-3M | 0 | 0 | 0 |
| 47-6 | 17 | 0 | 0.5±0 |
| 47-7 | 17 | 0 | 0.3±0 |
| 46-1 | 100 | 90.9 | 21.2±4.6 |
| 46-3M | 90.9 | 11.3±2.7 |

*Data on the other groups involved in this com-
parison are available from the authors upon request.
The relevance (if any) of these findings for the problem of possible interrelationships among diet, cholesterolemia and atherogenesis in man is dealt with elsewhere.5

SUMMARY
Inadequate methionine intake led to increased hypercholesterolemia, coronary and aorta atherosclerosis in chicks on a cholesterol-oil-supplemented purified ration.

High protein-high vitamin supplementation partially suppressed hypercholesterolemia and atherogenesis in birds on cholesterol oil rations, of both the purified and commercial mash varieties. High protein supplementation alone tended to produce some of these effects, whereas high vitamin supplementation alone did not.

Chicks fed a purified ration developed less marked hypercholesterolemia and atherogenesis than birds on a commercial feed, even when the former ingested greater quantities of cholesterol. In purified rations containing cholesterol oil, cholesterolemia and atherogenesis tended to be less marked with soy protein than with casein-gelatin (both at the 35 per cent level).

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We are deeply indebted to Dr. H. M. Scott, University of Illinois, Urbana, Illinois for advice concerning the composition of the purified diets. It is a pleasure to express appreciation to the members of the experimental atherosclerosis technical team of the Cardiovascular Department, Miss Mildred Michael, Mrs. Charlene Thompson, Mrs. Eva Miller, Mrs. Montez Vankinscott (research technicians, chemistry) and Messrs. David Bryant and Grady Crowley (laboratory assistants).

SUMMARIO IN INTERLINGUA
Inadequate nivello dietari de methionina resultava in augmentos de hypercholesterolemia e de atherosclerosis coronari e aortic in gallettos recipiente un dieta purificate con supplementos de cholesterol e oleo.

Alte supplementos de proteina e de vitamina resultava in un suppression partial del hypercholesterolemia e del atherogenesis in aves recipiente dietas a cholesterol e oleo, tanto del typo purificate como etiam de provenientia commercial. Alte supplementos de proteina sol (i.e. sin supplementation de vitaminas) tendeva a producer certes de iste effectos, durante que alte supplementos de vitamina (sin supplementation proteinic) non monstrava un tal tendentia.

Gallettos recipiente un dieta purificate disveloppava minus marcate grades de hypercholesterolemia e de atherogenesis que gallettos recipiente un dieta commercial, mesmo in casos in que le dieta purificate contineva plus alte quantitates de cholesterol. In dietas purificate e supplementate per cholesterol e oleo, le cholesterolemia e le atherogenesis tendeva a esser minus marcate con proteina de soja que con caseina e gelatinia (in ambe casos a nivello de 35 pro cento).

REFERENCES
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