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## Hypercholesteremia and Atherosclerosis Induced in Rabbits by Purified High Fat Rations Devoid of Cholesterol.* (23800)

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Investigations in humans(1-6) have recently established that a difference in cholesterol levels can be induced by dietary use of certain vegetable oils and some fish oils as compared to hard animal fats and hydrogenated fats. A large dietary intake of certain hard animal fats or hydrogenated fat is capable of elevating blood cholesterol levels. Conversely highly unsaturated vegetable oils either have no such effect or will actually lower plasma cholesterol levels. Speculation as to agents in fats or oils which affect cholesteremia has included: saturated or unsaturated fatty acids(1), essential or polyunsaturated fatty acids(7), beta-sitosterol(8) and short chain fatty acids(1). Following the classic studies of Anitschkow(9) numerous investigators have produced hypercholesteremia and aortic atheroma in rabbits by adding cholesterol to commercial rabbit feeds. Kritchevsky *et al.*(10) showed that type of fat was important in determining extent of this effect. They found that, in the presence of exogenous cholesterol, partially hydrogenated vegetable shortening produced higher blood cholesterol values and more severe aortic lesions than did corn oil. In the absence of dietary cholesterol, Kritchevsky found negligible atheroma production with either vegetable shortening (Primex) or corn oil when both were fed at 9% of the diet. This latter finding led Deuel and Rieser(11) to the conclusion that, "These data likewise refute the concept that fat *per se* has an atherogenic effect." Steiner and Dayton(12) more recently reported that blood cholesterol levels could be elevated 4-5 times in rabbits without dietary cholesterol by prolonged feeding of diets containing 50-75% ground peanuts. They also reported small areas of gross aortic atherosclerosis in 2 of 33 rabbits after 5 to 12 months, thus suggesting that atherosclerosis could be induced by diets low in cholesterol. Use of diets of known controlled chemical composition makes possible more accurate control of all dietary components than can be attained with the usual rabbit diet. In this paper we describe 2 rabbit experiments using the purified diet of Thacker(13).

*Methods.* Male New Zealand albino rabbits averaging 1.8 kg in weight were housed in individual cages. They were divided into groups of comparable weight. Diet consumption was estimated daily and body weight weekly. Rabbits were bled from the marginal ear vein before initial feeding and at suitable

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TABLE I. Composition of Purified Diets.

| Ingredient | % composition |
|---|---|
| Alphacel* | 10., 9.75, 9.25 or 8. |
| Casein† | 25. |
| Dextrin | 39.9 |
| Cholesterol‡ | .0, .25, .75 or 2. |
| Fat§ | 20. |
| Macro minerals | 5. |
| Minor " | .1 |
| | 100. |

* Nutritional Biochemicals Corp., Cleveland, O.

† Commercial grade: Borden Co., N. Y.

‡ Cholesterol levels varied at expense of Alphacel.

§ Hydrogenated shortening (Primex), hydrogenated coconut oil (Hydrol) or safflower oil.

*Macro minerals, g* — $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$ 308.2, $Ca(H_2PO_4)_2$ 104.7, $K_2HPO_4$ 218.7, KCL 124.7, NaCl 77.0, $CaCO_3$ 68.5, $3\,MgCO_3 \cdot Mg(OH)_2 : 3H_2O$ 35.1, $MgSO_4$ 38.3.

*Minor minerals, g*—$FeC_6H_5O_7 \cdot 5H_2O$ 453.85, $CuSO_4 \cdot 5H_2O$ 28.15, $MnSO_4 \cdot H_2O$ 16.5, KI 1.5.

Vitamins added as follows: mg or I.U./100 g diet—$B_1$ HCl .07, $B_2$ .6, dl calcium pantothenate 1.5, $B_6$ HCl .7, niacin 20., choline chloride 100, betaine chloride 100, inositol 10, para-aminobenzoic acid .2, folic acid .1, d biotin .05, vit. A acetate 665 I.U., $B_{12}$ .005, $D_2$ 850 I.U., E 7.5 I.U., menadione .075.

intervals thereafter. Heparin was used as an anticoagulant. Plasma cholesterol was estimated by a modified method using the Abell (14) procedure for saponification and extraction except that 3 Skellysolve B extractions were used and the entire supernatant was removed after each extraction. After evaporation of combined supernatants to dryness under nitrogen at 60°C, the residue was taken up in chloroform to standard volume, filtered, and cholesterol content determined on aliquots by the method of Kenney (15) at 420 *mu* and color development at 37°C for 30 min. Aortae were stained with Sudan IV and graded for gross atherosclerotic lesions by 2 independent observers using a scale of 0-4 (ranging from none to severe). For grading purposes aortae were divided into 2 areas *i.e.* above intercostals, and intercostals and below. Dietary fat (Table I) consisted of partially hydrogenated vegetable shortening (Primex†), hydrogenated coconut oil (Hydrol‡) or safflower oil§ (containing about 70% linoleic acid). Fat in these diets accounts for approximately 40% of caloric value. When cholesterol was present it was mixed with other dry ingredients instead of being dissolved in fat. It is necessary to train rabbits to eat this purified diet by mixing it with 3% of alfalfa meal for the first week. *I. Cholesterol-containing diets*: Seventy-two rabbits were divided into 6 groups of 12 each and fed *ad lib*. Three groups were fed 20% Primex diet with cholesterol levels of 0.25, 0.75 and 2.0% respectively. Three other groups were fed 20% safflower oil diet with the same 3 levels of cholesterol. *II. Cholesterol-free diets*: Twenty rabbits were divided into 2 groups of 10 each. One group was fed 20% Hydrol diet and the other 20% safflower oil diet. All rabbits were pair-fed, that is daily food ingestion was limited to an amount which all rabbits would eat.

*Results. I. Cholesterol-containing diets.* Body weight, food consumption, mortality, and aortae atheroma data are presented in Table II. Animals on the 2% cholesterol diets were sacrificed and autopsied after 67 days, the 0.75% cholesterol group after 75 days, and the 0.25% cholesterol group after 103 days. No differences in the degree of lesions between survivors on various diets could be detected, however, mortality was most severe for all groups on hydrogenated shortening. At all 3 levels of dietary cholesterol rabbits on safflower oil gained weight more rapidly and ate more food than did rabbits on shortening.

In addition to poor growth and food consumption, many animals on hydrogenated shortening diet showed loss of hair, suggestive of essential fatty acid deficiency. Some rabbits on each diet developed a jaundiced condition. The cause of jaundice under these conditions is not understood but is perhaps related to hypercholesteremia commonly occurring with biliary obstruction.

Changes in average blood cholesterol values for the groups are shown in Fig. 1. Even though rabbits on hydrogenated shortening diet ate less food than their counterparts receiving safflower oil and thus ingested less cholesterol, their average plasma cholesterol levels were higher than those of safflower oil

† Procter and Gamble, Cincinnati, O.

‡ Durkee Famous Foods, Chicago, Ill. Iodine value approximately 4.

§ Pacific Vegetable Oil Corp., San Francisco, Calif.

TABLE II. Aortae Grading, Body Weight, Food Consumption, and Mortality of Rabbits Fed Cholesterol-Supplemented Diets.

| Diet | | Avg terminal | Avg | Avg food | |
|---|---|---|---|---|---|
| Fat | Cholesterol (%) | aortae grading* | terminal wt (kg) | consumption (g/rabbit/day) | % mortality† |
| 20% hydrogenated shortening | 2. | .96‡ | 1.77 | 65 | 33 |
| | .75 | 1.05‡ | 1.96 | 57 | 67 |
| | .25 | 1.03‡ | 2.57 | 66 | 25 |
| 20% safflower oil | 2. | 1.19‡ | 2.65 | 84 | 8 |
| | .75 | 1.07‡ | 2.78 | 71 | 33 |
| | .25 | 1.19‡ | 3.22 | 87 | 0 |

* Mean of areas (above intercostals; intercostals and below).
† Initially each group contained 12 animals.
‡ Difference between hydrogenated shortening and safflower oil groups not statistically significant.

rabbits. This was true for all 3 dietary regimens.

Statistical analysis of differences between groups (by t test) was applicable only when plasma cholesterol determinations were done on individuals (after 45 days). Analysis of such data demonstrated that in most cases differences in plasma cholesterol levels between hydrogenated shortening and safflower oil groups were significant ($P<0.05$). One exception was found in groups on diets containing 0.25% cholesterol. At end of experimental period, plasma cholesterol levels were not significantly different between these 2 groups.

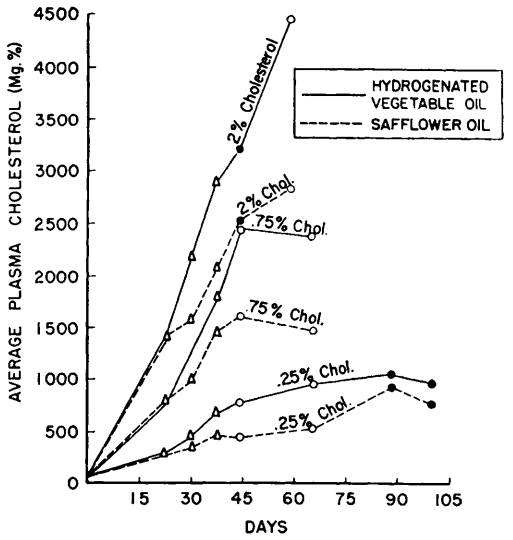


FIG. 1. Effect of exogenous cholesterol on plasma cholesterol levels (hydrogenated shortening *vs* safflower oil). △—Pooled plasma not subject to statistical analysis. ○—Statistically significant difference between groups at a given amount of exogenous cholesterol ($P < 0.05$). ●—Differences between groups not statistically significant.

*II. Cholesterol-free diets.* Because the above experiment did not distinguish between types of fat as to effects on production of atheroma, it was thought that perhaps all 3 levels of exogenous cholesterol so overwhelmed the animal that atheroma were produced in presence of either type of fat. Accordingly, an experiment was designed to compare hydrogenated coconut oil, an almost completely saturated fat, against safflower oil in the absence of dietary cholesterol. Plasma cholesterol values for this experiment are shown in Table III. After 100 days on experimental diets, plasma cholesterol level was elevated 12 times over normal (0 days) by 20% hydrogenated coconut oil diet and only 1.5 times by 20% safflower oil diet. All animals on hydrogenated coconut oil surviving to termination of experiment had clear-cut aortic atheromatous lesions with average degree of severity of 1.4, whereas only 4 of 10 rabbits on safflower oil showed any aortic involvement, as manifested by slight sudanophilia. Difference in appearance between stained aortae from the 2 dietary groups was visually dramatic.

For histologic studies‖ aortae were fixed in neutral 10% formalin, sectioned and stained with hematoxylin-eosin, Schiff's periodic acid, Mallory's aniline blue connective tissue stain, Wilder's silver reticulum stain and Unna's orcein stain for elastic fibers. To be able to examine as much as possible of lesions present in the aorta the whole length of the aorta

‖ We are indebted to Dr. R. J. Stein for histological examination of the aortae.

TABLE III. Individual Plasma Cholesterol Levels and Degree of Atheroma of Rabbits on Cholesterol-Free Diets.

| Diet | Plasma cholesterol (mg %) | | | Terminal aortae grading | |
|---|---|---|---|---|---|
| | 0 days | 52 days | 100 days | Above intercostals | Intercostals and below |
| 20% hydrogenated coconut oil | 43 | 476 | 567 | 1.3 | .6 |
| | 107 | 751 | 1062 | 1.9 | 1.7 |
| | 66 | 226 | 750 | 2.2 | .4 |
| | 51 | 412 | 719 | 1.9 | .9 |
| | 66 | 771 | 653 | 3.0 | 1.0 |
| | 68 | 459 | dead | | |
| | 36 | dead | " | | |
| | 59 | 555 | 729 | 1.7 | 1.0 |
| | 59 | 756 | dead | | |
| | 69 | 386 | 627 | .8 | .9 |
| Avg | 62 | 532 | 730 | 1.9 | .9 |
| 20% safflower oil | 88 | 143 | 136 | .0 | .0 |
| | 43 | 117 | 101 | .0 | .05 |
| | 116 | 118 | 113 | .0 | .0 |
| | 106 | 143 | 141 | .1 | .1 |
| | 39 | 109 | 62 | .0 | .0 |
| | lost | 116 | 139 | .0 | .0 |
| | 125 | 213 | 156 | .1 | .0 |
| | 45 | 79 | 75 | .05 | .0 |
| | 45 | 119 | 86 | .0 | .0 |
| | 74 | 149 | 135 | .0 | .0 |
| Avg | 76 | 131 | 114 | .03 | .02 |

(arch, thoracic and abdominal aorta) was rolled upon itself like a deflated fire-hose, tied around its circumference with black surgical silk and sections taken through the whole. In this manner one was able to examine the various regions of the aorta in one slide (Fig. 2-a). Microscopically, the lesions exhibited marked intimal changes and to a lesser degree changes within the media. As shown in Fig. 2 (b,c,d) the intima was markedly thickened and almost entirely composed of oval and round foam-like cells. These cells contained nuclei that were round and some of which were vesicular with a scanty amount of chromatin material and others definitely pyknotic. Cytoplasm stained very lightly with eosin and exhibited a fibrillar-like architecture. Lining this intimal thickening a few normal appearing endothelial cells could be seen. The histology of this intimal change was characteristic of a true experimental atherosclerosis. Changes in the media were characterized by thickening and splitting of the elastic fibers with appearance of more than usual cellularity of the collagen between these elastic membranes.

Of the original 10 animals per group, 3 on hydrogenated coconut oil failed to survive the experimental period while all survived on safflower oil. Rabbits on the hydrogenated coconut oil diet evidenced considerable loss of hair and a general "ruffled" appearance (Fig. 3). Those on safflower oil were robust in appearance and would have consumed much larger quantities of diet if their intake had not been limited. Two animals on hydrogenated coconut oil developed jaundice while no jaundice occurred in the animals on safflower oil. The over-all average feed efficiency (food eaten/wt. gained) for rabbits on safflower oil was 3.92 (2.64-5.22) as compared to 5.78 (3.42-13.32) for rabbits on hydrogenated coconut oil.

*Discussion.* This study indicates that relative ability of different dietary fats to induce atheromatous lesions can be more clearly differentiated by cholesterol-free dietary procedure than by traditional cholesterol-feeding. This experimental technic obviates feeding unphysiologically high levels of exogenous cholesterol and reflects the cholesterolgenic property of the diet itself.

The difference in these experiments between safflower oil and hydrogenated coconut oil

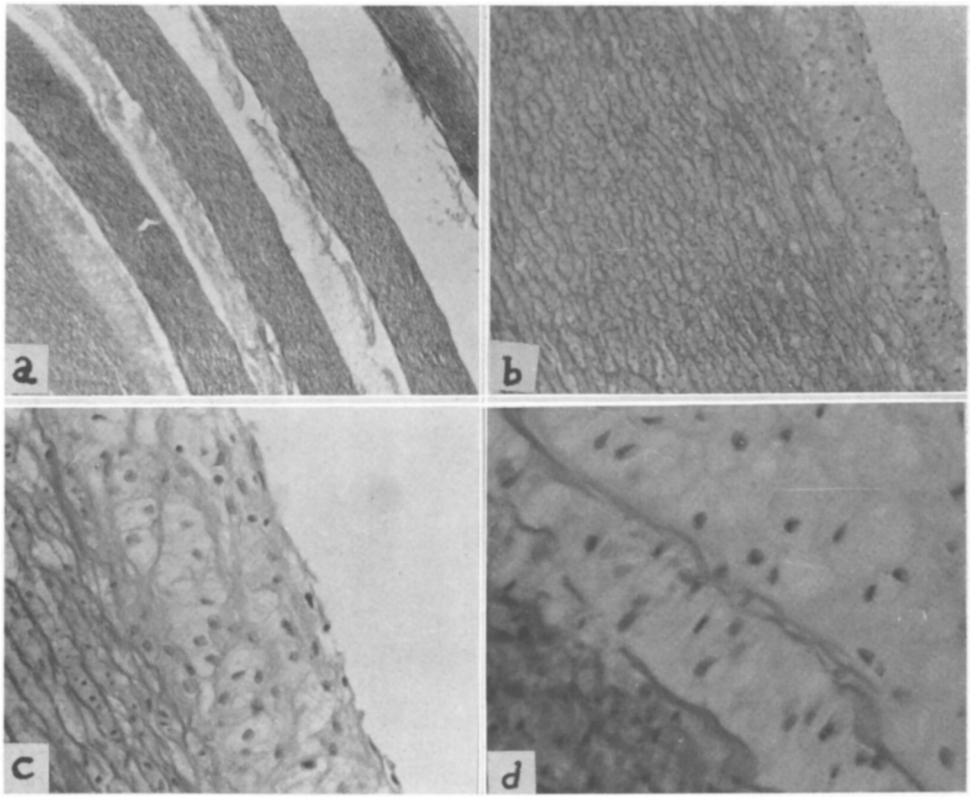


FIG. 2. Sections of aortae from rabbits fed cholesterol-free diets containing hydrogenated coconut oil.

might be due to (a) a hypercholesteremic effect of short chain saturated fatty acids and/or unnatural fatty acid isomers present in hydrogenated coconut oil (b) development of essential fatty acid deficiency in the coconut oil group (c) the relatively high amount of beta-sitosterol in safflower oil (0.40%)¶ as compared to hydrogenated coconut oil (0.13%)¶ (d) a hypercholesteremic effect of saturated fatty acids in coconut oil or a hypocholesteremic effect of unsaturated fatty acids in safflower oil (e) unknown substances in the fats, or (f) a combination of these possibilities. Two groups have reported that, in absence of dietary cholesterol, hydrogenated fat feeding did not produce atheroma in rabbits. Kritchevsky, *et al.*(10) found negligible atheroma production with either hydrogenated vegetable shortening (Primex) or corn oil when both were fed at 9% of the diet. Van Handel, *et al.*(16) reported that isocaloric substitution of hydrogenated vegetable fat (linoleate 1.5%) for liquid cottonseed or corn oil had no cholesteremic effect. Shortenings of the type used by Kritchevsky still contain up to 12% linoleic acid after hydrogenation(17) and differ considerably in this respect from hydrogenated coconut oil. Also both of these groups of investigators used a commercial rabbit diet and undoubtedly these preparations

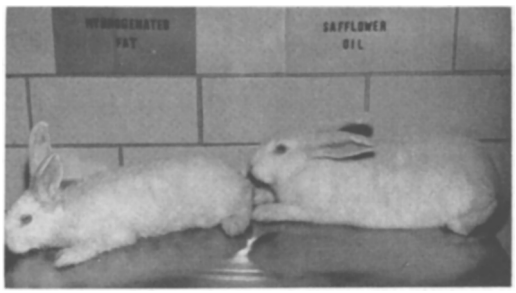


FIG. 3. Appearance of rabbits after 12 wk on purified rations consisting of either 20% hydrogenated coconut oil or safflower oil.

¶ Total Lieberman-Burchard positive substances estimated using a beta-sitosterol standard.

contain appreciable amounts of unsaturated fat. The relatively high content of short chain fatty acids in coconut oil as compared to hydrogenated vegetable shortening or hydrogenated cottonseed is also apparent. Obviously the hydrogenated coconut oil as a sole source of fat leads to an aberrant cholesterol metabolism. Whether this is due to greater synthesis, to a failure in excretion, or other factors is open to further investigation.

Holman and Peifer(18) produced essential fatty acid deficiency in rabbits by feeding diets containing 2% hydrogenated coconut oil and 1% cholesterol. Using higher levels of hydrogenated fat we have produced a similar deficiency either with or without exogenous cholesterol. This latter finding concurs with the observation of Deuel, *et al.*(19) that development of an essential fatty acid deficiency can be hastened in the rat by addition of hydrogenated coconut oil to an otherwise fat-free diet.

Recently we found that the time period necessary to produce atheroma in rabbits fed hydrogenated coconut oil may vary. It appears likely that both blood cholesterol levels and time are important factors in determining extent of atheroma production.

*Summary.* Rabbits fed cholesterol-supplemented purified diets containing 20% of hydrogenated shortening or safflower oil showed lower plasma cholesterol levels on safflower oil diets, but only negligible differences between oils with respect to aortic atheroma production. Similar studies with cholesterol-free diets showed that rabbits on hydrogenated coconut oil are much more prone to hypercholesteremia than rabbits on safflower oil diets. In these latter studies aortic atheromatous lesions developed in all rabbits in 16 weeks on 20% of saturated fat as contrasted to negligible lesion production on 20% safflower oil.

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