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By Erwin T. Janssen

The relationship of dietary fatty acids and the cholesterol levels found in serum and liver is, and has been, under study by many investigators. The relationships involved are undoubtedly intricate and more complex than most investigators seem to imply. Although Jones *et al.*[1] imply a close relationship between rats and man in dietary requirements and habits, it is good to keep in mind differences that are intrinsic to both man and experimental animals. Effects produced on experimental animals and those produced upon man in dietary studies in some cases may be vastly different. With this in mind, an attempt will be made throughout the following paper to limit discussion to results found through work with experimental animals.

Aftergood and her co-workers state that many investigators believe that serum cholesterol levels are controlled by fat intake regardless of the source of the fat.[2] However, she points out that evidence exists to show that the source of fat may be vitally concerned with the resulting cholesterol level.

In a majority of cases, fats have been fed in the form of triglycerides. These have been arbitrarily classified into two groups: animal fats and vegetable fats. In both the animal and vegetable fats the majority of fatty acids are found as the triglycerides, and only a small fraction are found as the free acid.[3] Recently, Malmros and Wigand have shown evidence that indicates a better classification might be based on the degree of saturation, rather than on the source of the fat.[4]

Various investigators have shown that the feeding of vegetable fat gives a lower serum cholesterol level than that which is found if animal fat is administered. This might indicate that the unsaturation of the vegetable fats plays a part in the lower cholesterol levels. However, other components present in the fats cannot be disregarded. In a study performed on rabbits, Swell *et al.*[5] show that the blood cholesterol level is greatest with soybean oils that have the highest

iodine number. Also, if a 2% cholesterol diet containing free acids was fed, the free acids were more effective in raising the blood cholesterol level than were the neutral fats. Of the free fats tested, oleic acid was the most effective in raising the cholesterol values, followed by stearic and linoleic acids.

The role of choline and methionine as methyl donors and their relationship to plasma and liver cholesterol were deemed worthy of investigation. It was thought possible that some effect upon the cholesterol content of plasma and liver might be manifested by dietary addition of choline or methionine. Jones *et al.*[1] found that in rats fed a supplement of 0.5% DL-methionine, a rise in the serum cholesterol value took place. However, they point out that Mann and co-workers noted just the opposite effect in the serum of methionine deficient monkeys made deficient by feeding soybean protein when a methionine supplement was given.

Since in a majority of studies the fats have been administered as triglycerides, the following study was designed to investigate the effects of dietary fats as free fatty acids on both plasma and liver cholesterol. In addition, diets containing supplements of DL-methionine and choline chloride were fed to determine if there was any effect on plasma and liver cholesterol.

Experimental

A group of twenty-five male albino rats of the Holtzman strain was divided into five groups of four animals each and one group of five animals, this group to be used as the control group. The animals were housed in screen bottom animal cages of the Army Medical School type and maintained on food and water *ad libitum*. The room in which they were kept was held at a temperature of around 25° C., but fluctuations of 3° C. did occur. The animals were maintained on Rockland Rat Diet for a period of approximately seven weeks before dietary studies were begun. Records of daily diet consumption were kept, and body weights were recorded at two day intervals.

The basic experimental diet constituent was Rockland Rat pellets. At first, all animals were maintained on the diet in pellet form. When the experimental diets were made up, the pellets were ground to powder form and the various dietary supplements were then blended in with an electric mixer. The dietary constituents and their relative amounts are given in Table 1. The diets containing the unsaturated linoleic acid were stored under refrigeration. All diet supplements used were obtained from the Matheson, Coleman & Bell Division of the Matheson Company. The choline chloride was added at the level of 0.350 g. per 100 g. of diet. This supplemented the 0.152 g. stated by the manufacturer to be present in the diet to a level of 0.502 g. per 100 g. of the stock diet. The DL-methionine was added in the

Table 1

Experimental Diets used. The DL-methionine was added in amounts of 0.5 g. per 100 g. of diet. The choline chloride was added in the amount of 0.350 g. per 100 g. of diet. This gave a concentration of 0.502 g. per 100 g. of diet of choline chloride. (0.152 g. per 100 g. diet present as stated by manufacturer.) The combined total gave approximately a 1.0% supplement.

Group	Stearic Acid	Linoleic Acid	Stock Diet	DL-methionine Choline Chloride
A	15 g.	———	85 g.	———
B	15 g.	———	85 g.	1.0 g.
C	———	15 g.	85 g.	———
D	———	15 g.	85 g.	1.0 g.
E	———	———	100 g.	———
F	———	———	100 g.	1.0 g.

amount of 0.5 g. per 100 g. of stock diet. To make the 15% free fatty acid diet, 15 g. of the acid was added to 85 g. of the stock diet. The diets were mixed in quantities sufficient for approximately ten day periods. Frequent mixing was done in an effort to prevent any change in the diet composition. It should be noted that the 15% fatty acid amounts were added to the stock diet which already contained about 5% fat.

The six cages of animals were divided into pairs—Group A and B, Group C and D, Group E and F. Both Groups A and B were fed 15% stearic acid, Group B in addition receiving the methionine-choline supplement. Groups C and D were fed the 15% linoleic acid, and Group D received the supplement. In Groups E and F, the stock diet was fed, Group F receiving the added methionine-choline supplement.

The thirteen-week-old animals were started on the diets, and at two week intervals 1.5 to 2.0 ml. of blood were collected from each animal under ether anesthesia by bleeding the tail vein. One to two drops of a sodium oxalate solution were added as an anticoagulant, and the formed elements were separated from the plasma by centrifugation. Separate samples were taken from the supernatant plasma for the determination of free and total cholesterol. In every analysis except the final one, 0.3 ml. of the plasma was used for the determination of the free cholesterol. For the last analysis 0.4 ml. was used. Throughout the entire analysis, 0.2 ml. of plasma was used for the determination of total cholesterol. Both free and total cholesterol were analyzed by the method of Sperry and Webb.[6]

After eight weeks on the diets, the animals were sacrificed by heart puncture under ether anesthesia. The livers were removed, blotted dry, and weighed to the nearest 0.1 gram. Approximately 0.5 g. of liver was taken for analysis and this portion was weighed accurately to the nearest milligram. The liver sample was homogenized in 4 ml. of 1:1 solution of ethyl alcohol and acetone, and this heated to

boiling. The homogenate was extracted twice, and the combined extracts were made up to a volume of 10 ml. Both free and total liver cholesterol were each determined in 2 ml. aliquots by the method of Sperry and Webb. Standards containing 0.1 mg. of cholesterol were used with each group of determinations.

Discussion of Results

It is evident from Table 2 that the gain in weight for all of the groups is nearly identical. Table 3 shows the daily average diet intake per rat. A possible explanation for the higher values seen in the data for the stearic acid diets is that this solid acid is not as readily absorbed into the intestine as is the liquid linoleic acid.

Table 2

Animal Weight Gain During Experiment. The Results Are the Average Per Animal in Each Cage

Group	Initial weight (g.)	Final weight (g.)	Gain (g.)
A	356	425	69
B	365	434	69
C	380	460	80
D	375	455	80
E	343	419	76
F	362	424	62

Table 3

Daily Average Intake Per Rat During Experimental Period

Group		Daily average per rat (g.)
15% Stearic	(A)	24.5
15% Stearic +M-C*	(B)	25.0
15% Linoleic	(C)	20.6
15% Linoleic +M-C*	(D)	19.6
Stock Diet	(E)	22.6
Stock Diet +M-C*	(F)	22.5

*Signifies the DL-methionine and chlorine chloride supplement.

The overall plasma cholesterol levels for the groups on stearic acid, linoleic acid, and stock diet are shown in Table 4. It is to be noted that there is no significant difference between the animals fed the 15% stearic acid diets and the animals fed the regular stock diets. However, a significant difference ($p=0.02$) was seen in the animals fed the 15% linoleic acid. Thus, the cholesterol level of the rat is raised by feeding of the unsaturated free fatty acid. This is in agreement with the work done by Klein on acids fed as the triglycerides.[7]

Table 4

Overall Total Cholesterol Levels of Plasma. Results Are Expressed in mg. per 100 ml. Plasma (mg.%)

Groups		Total Cholesterol of Plasma (mg.%)	(p) different from stock*
15% Stearic	(A & B)	42.1	>0.05
15% Linoleic	(C & D)	52.9	0.02
Stock Diet	(E & F)	39.7	———

*Probability greater than 0.05 is taken to be non-significant.

Table 5

Total Cholesterol of Plasma for Individual Groups. The Free Cholesterol Values Were Not Significant

Group		Total Cholesterol of Plasma (mg.%)	(p) of difference between groups*
15% Stearic	(A)	41.7	
15% Stearic +M-C	(B)	42.4	>0.05
15% Linoleic	(C)	48.1	
15% Linoleic +M-C	(D)	57.7	0.05>p>0.02
Stock Diet	(E)	36.5	
Stock Diet +M-C	(F)	42.4	>0.05

*Probability greater than 0.05 is taken to be non-significant.

Table 5 shows the results of feeding the methionine-choline supplement. No significant difference was noted in the free cholesterol in any of the groups. Likewise, the total cholesterol values of Groups A and B and Groups E and F showed no significant difference. However, in the groups receiving the 15% linoleic acid (Groups C and D) the methionine-choline supplement produced a significantly higher total plasma cholesterol level than was produced if only 15% linoleic acid was fed. It will also be noted that the lowest level in the 15% linoleic acid groups (Group C) still remained above the values of the 15% stearic or the stock diet groups. The higher values of the methionine-choline supplemented animals are in agreement with the findings of Jones *et al.*[1]

Table 6 shows the liver cholesterol values. In comparing the ester values of the groups with the stock diet group it is seen that the only significant difference is to be found in the animals on the 15% linoleic acid. If the plain 15% fat and stock diets are to be compared to their corresponding methionine-choline supplemented groups, only the 15% linoleic acid groups differ significantly. The 15% linoleic diet with the supplement showed a lower liver cholesterol ester level than its plain 15% linoleic acid counterpart ($p=0.02$).

Friedman and Byers have cited that the liver is the chief organ

of regulation of ester cholesterol.[8] Thus, above results might tend to indicate that the livers of the animals on the 15% linoleic acid diet in the absence of a methionine-choline supplement (Group C) were more effective in controlling the plasma cholesterol. More work would be needed in order to arrive at a definite conclusion about this relationship.

Table 6

Liver Cholesterol After Eight Weeks. The Comparison Is Made Using the Cholesterol Ester Values

Group	Free Chol. (mg.%)	Total Chol. (mg.%)	Ester Chol. (mg.%)	(p) diff. than stock*	(p) diff. between groups*
A	165	194	29	>0.05	>0.05
B	162	197	35	>0.05	
C	190	313	123	0.02	0.02
D	170	234	64	0.02	
E	197	226	29	———	>0.05
F	182	213	31	>0.05	

*Probability greater than 0.05 is taken to be non-significant.

Summary

Five groups of four animals per group were maintained on separate diets composed of 15% stearic acid, 15% linoleic acid; and three groups had diets supplemented with approximately a 1.0% combined mixture of DL-methionine and choline chloride. Blood was drawn at two week intervals and analyzed for both total and free cholesterol. At the completion of the eight week period, the animals were sacrificed by heart puncture. The livers as well as the plasma were analyzed for the cholesterol content. The results indicated that the feeding of 15% free acids caused a rise in the plasma cholesterol levels, the significant rise being in animals on the 15% linoleic acid diet. When a supplement of DL-methionine and choline chloride was fed, it produced a greater rise in the cholesterol levels than was produced by only the 15% linoleic acid. The liver cholesterol level showed only slight variations from the values of the control group, except in the case of the animals fed the 15% linoleic acid. The unsupplemented 15% acid diet showed a higher liver cholesterol ester content than the diet containing added methionine-choline. Indications were that on a 15% linoleic acid diet the addition of methonine-choline decreases the ester content of the liver while raising the plasma cholesterol ester content.

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