Interrelations Between the Kind and Amount of Dietary Fat and Dietary Cholesterol in Experimental Hypercholesterolemia

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THE TRADITIONAL experimental approach in biologic research has been to isolate the experimental variable of interest and to study this variable independent of other factors which might influence the result. In nutrition research the use of selected inbred animals, purified diets which allow the variation of a single nutrient, constant environmental temperature control, etc., are considered as nearly a *sine qua non* of an adequate experimental design. The value of such control is clearly evident from the advances which have been made during the past.

The emphasis upon holding all variables constant except that one under study may lead, however, to the unwarranted conclusion that the variable studied has a function or action which is universal and independent of other variables. So many dietary interrelations are already known that it is obvious such assumptions can seldom be made. The point can be made, nevertheless, that often there is so much emphasis upon the nutrient under study that little consideration is given to the conditions under which the nutrient was studied.

Studies upon hypercholesterolemia and atherosclerosis, both in experimental animals and in human subjects, provide a case in point of wide interest at the present time. With human beings as subjects, it has been shown that the effects of different dietary fats upon serum cholesterol levels are (a) best correlated with iodine number or the over-all degree of unsaturation,[1] (b) explained by the amounts of saturated and polyunsaturated fatty acids in the diet, the monounsaturated fatty acids having no effect,[2] and (c) largely a reflection of the essential fatty acid or linoleic acid content.[3] Further, (d) hydrogenation of corn oil, thus decreasing both the iodine number and essential fatty acid content, has little or no influence upon its ability to lower serum cholesterol levels,[4] and (e) adding a highly saturated fat to a highly unsaturated fat does not increase the lowered serum level caused by the latter.[5] It is apparent that some of the findings are contradictory.

In experimental studies upon atherosclerosis and hypercholesterolemia the number of factors now known to be of some consequence continues to grow. In cholesterol and/or cholic acid-fed animals the following are reported to be of some importance: (a) kind and amount of fat; (b) amount of cholesterol and cholic acid; (c) kind and amount of protein; (d) kind and presumably the amount of different carbohydrates; (e) magnesium intake and possibly the amount of calcium or the calcium

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magnesium ratio; (f) various vitamins including pyridoxine, vitamin D, ascorbic acid, and vitamin E; (g) purine and pyrimidines in the diet; (h) thyroid function; (i) sex and sex hormones; and (j) bacterial flora. This list is not all-inclusive and, of course, such variables as the length of time the experiment is run and the species used are of great importance. Certain interrelationships between some of these variables are obvious, others less so. The point which requires emphasis, however, is the distinct possibility that many and perhaps *most of these variables may have definable effects only in relation to many or all of the others*. If this should be true, then the most profitable search might not be to define the effect of fat composition on hypercholesterolemia and atherosclerosis, for example, but the interrelationships of dietary fats with other environmental and inherited factors which influence the serum cholesterol level. It is possible, perhaps likely, that the apparently contradictory studies discussed heretofore with regard to the effects of different fats on serum cholesterol levels are not contradictory but each may be true relative to the particular experimental conditions. If we assume that some or all of the variables operative in experimental animals are or might be of importance in human subjects, it is perhaps not unlikely that the formula-feeding technic using a homogeneous diet composed of fat and skim milk powder may give results quite different from those obtained with a low-fat diet of mixed foodstuffs.

The large number of variables of interest, if tested at various levels, would give an almost infinite number of combinations, outside the possibilities of a single experiment or probably of a single laboratory. Still, efforts should be made toward experimental designs which will yield the maximum amount of information with the minimum expenditure of animals, time, and effort. The present study represents a preliminary effort in this direction.

EXPERIMENTAL

The experimental design included the study of five different fats—coconut oil, olive oil, safflower oil, corn oil, and an equal mixture of coconut and safflower oil. Each of these was fed at three different levels in the diet, at 5, 10, and 20 per cent of the diet. All of these variations in kind and amount of oil were fed with 0.45 per cent cholesterol and with 1.35 per cent cholesterol in the diet. Thirty groups were thus required. Two animals per group, the minimum number necessary to obtain an estimate of variation between animals, were used. Young adult male rats weighing 250 to 300 g were housed in pairs in cages approximately 12 × 12 × 15 in. with raised screen bottoms.

The diets were similar to those previously employed.[6,7] They contained 10% casein, 5% salt mixture, 5% celluflour, 0.3% choline, 0.45% cholic acid, and vitamin supplements as previously described. The remainder of the diet consisted of the appropriate kind and amount of fat, 5, 10, or 20%, either 0.45 or 1.35% cholesterol, and sufficient glucose to complete the diet. It is recognized that the protein/calorie ratio varies in these diets depending upon the amount of fat added and that the protein intake is thus not constant. It should be noted that all diets contained cholic acid in order to produce a reasonably high serum cholesterol level.[6]

In order to minimize the time required for feeding and preparation of diets, a sufficient amount was prepared to last approximately one month and was stored in the refrigerator. The animals were fed either two or three times a week but were inspected daily to be sure that food was available. Uneaten food was discarded weekly. Such a feeding schedule results in a great saving in time as compared to the usual practice in this laboratory of feeding daily and the preparation of diets upon a weekly basis. However, it allows more exposure of the diet to the relatively high temperatures in the animal room and more opportunity for the development of rancidity or destruction of labile constituents.

Animals were bled every two weeks during the eight-week experimental period. The end of the tail was clipped and 0.2–0.3 ml of blood collected in a small centrifuge tube. This was centrifuged after clotting and 0.02 ml

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TABLE I
The Experimental Design and Mean Serum Cholesterol Values (in mg/100 ml) Obtained in Each Group of Two Animals

Dietary fat		Dietary cholesterol level										
		0.45%					1.35%					
		Weeks					Weeks					
Kind	Amount %	2	4	6	8	Mean	2	4	6	8	Mean	Grand means
Olive	5	228	288	300	372	297	622	892	634	572	680	489
	10	474	544	628	472	529	492	864	804	872	758	644
	20	352	572	488	444	464	708	994	800	820	831	647
	Means	351	468	472	429	430	607	917	746	755	756	593
Coconut	5	207	402	504	445	390	328	362	422	439	388	389
	10	188	292	420	378	320	278	526	582	605	498	409
	20	148	165	286	293	223	186	190	204	191	193	208
	Means	181	286	403	372	311	264	359	403	412	359	335
Safflower	5	98	154	150	173	144	110	117	150	150	132	138
	10	148	159	190	206	176	248	198	288	290	256	216
	20	138	130	292	157	179	162	196	198	197	188	184
	Means	128	148	211	178	166	173	170	212	212	192	179
Corn	5	308	310	290	340	312	192	202	389	220	251	281
	10	260	234	304	239	259	282	208	276	324	273	266
	20	153	162	224	191	183	282	282	287	221	268	225
	Means	240	235	273	257	251	252	231	317	255	364	257
Safflower and coconut	5	273	344	302	249	292	292	216	198	206	228	260
	10	220	124	174	189	177	252	234	322	187	249	213
	20	113	180	196	166	164	184	194	208	235	205	184
	Means	202	216	224	201	211	243	215	243	209	227	219
Grand means		221	271	317	288	274	308	378	384	369	360	317

of serum taken for cholesterol analysis by a fluorimetric method.[8] A total of 240 values were thus obtained, four from each animal.

RESULTS

The mean values of each group of two animals at different times are shown in Table I. An inspection of these values will reveal some apparent effects, such as differences in the action of oils, the cholesterol level, and changes with time. Whereas some of the groups reached maximum serum cholesterol levels rather early in the experiment, others had apparently not reached maximum by the end of the eight-week period. There is an apparent difference in action of fats at different levels. For example, as the amount of olive oil or safflower oil in the diet was increased, the serum cholesterol levels rose considerably between 5 and 10 per cent fat. The further addition of olive oil had no effect, but the higher level of safflower oil may have caused a decrease in the serum cholesterol. With the safflower-coconut mixture and with corn oil, the serum cholesterol level fell with increasing amounts, and there was a considerable drop as the coconut oil was raised from 10 to 20 per cent.

The complete analysis of variance (logarithms of the serum cholesterol values were used throughout*) presented in Table II

* The general use of logarithms of the serum cholesterol values rather than the actual serum levels were preferred for several reasons. In the previously published paper[6] the serum cholesterol response was not proportional to the log of the cholesterol content of the

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TABLE II
Analysis of Variance

	Degrees of freedom	Sum of squares	Mean squares	F*
Total	239	12.936896	—	—
Within groups of two	120	0.933722	—	—
Between rats	30	0.377870	0.01260	—
Within rat (time-rat interaction)	90	0.555852	0.00618	—
Treatments (between groups of two)	119	12.003174	—	—
Main effects				
Cholesterol	1	0.480932	0.48093	38.2†
Level	2	0.353932	0.17697	14.0†
Oil	4	7.267266	1.81682	144.2†
Time	3	0.506012	0.16867	27.3†
Interactions				
Chol-level	2	0.111466	0.05573	4.4‡
Chol-oil	4	0.395346	0.09884	7.8†
Chol-time	3	0.032637	0.01088	1.7
Level-oil	8	1.229425	0.15368	12.2†
Level-time	6	0.022826	0.00380	0.6
Oil-time	12	0.371844	0.03094	5.0†
Chol-level-oil	8	0.470824	0.05885	4.7†
Chol-level-time	6	0.133324	0.02222	3.6†
Chol-oil-time	12	0.086734	0.00723	1.2
Level-oil-time	24	0.245223	0.01022	1.6
Chol-level-oil-time	24	0.295383	0.01231	2.0‡

* 0.01260 was used in the denominations of those F ratios where time was not involved; 0.00618 was used in the others.
† $P < 0.01$.
‡ $0.01 < P < 0.05$.

adequately demonstrates the complexity of the situation. All of the major variables, i.e., dietary cholesterol level, level of oil, kind of oil, and time, have significant effects upon the serum cholesterol level. Many of the interactions are also of significance, indicating the difficulty of defining clearly the specific effects of any of these variables without consideration of the other variables in the experiment. It may be noted that the interactions which include time as a variable have, in general, relatively small mean squares and several were insignificant in this experiment. Thus, time was of relatively less importance than the other variables. Obviously, had two weeks not been allowed prior to the determination of the first serum cholesterol determinations, this would not have been true.

Clearly the kind of oil used was the major variable, having by far the largest mean square. The interaction of level of oil and kind of oil has the largest mean square among the interactions and would appear to be of considerable importance. Thus, it may be impossible to define an effect of an oil without consideration of the level fed. This has generally been ignored in experiments with human beings. The next largest mean square in the interactions is that of cholesterol and oil. Thus, as previously noted,[6,7] the response to cholesterol feeding is not constant when the kind of oil in the diet is varied.

While the variance analysis should provide adequate caution against undue conclusions based upon limited experiments, it is not particularly useful in attempting to define the over-all effects of oils of different kinds since it merely demonstrates that significant differences exist.

A more complete description of the experiment was attempted by computing the regression equations relating the over-all composition of the dietary oils* to the mean of the logarithms of the serum cholesterol values. For this purpose the mean of the log serum cholesterol values for each animal during the experiment (thus ignoring the effects of time and level of dietary cholesterol) were used. The amounts of saturated, monounsaturated acids, and linoleic acid provided in the different diets and the mean log serum cholesterol values are shown in Table III.

The data in Table III yield the following regression equation:

$$\text{I.}\quad \log \text{serum cholesterol} = -0.00648S + 0.02105M - 0.02415P + 2.4836$$

where S, M, and P equal grams of saturated,

diet when different oils were included in the diet. It has since been shown that the response curves with different oils are essentially parallel if log dose–log serum cholesterol is plotted. The comparison at different levels of cholesterol feeding is thus greatly facilitated. It is also known that the technical error in cholesterol measurement is increased as the serum cholesterol level rises (Watkins *et al.*, *J. Clin. Investigation* 33:874, 1954). Statistical comparison is more appropriately made when this variation is minimized by the use of logarithms.

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TABLE III
Diet Composition and the Mean of the Logs of the Serum Cholesterol Values

Kind of oil	Amount %	Fatty acid content, %			Mean serum log cholesterol	
		Sat'd	Mono	Poly	Found	Calculated*
Olive	5	0.56	4.20	0.25	2.6874 2.6088	2.5623
	10	1.22	8.40	0.49	2.7917 2.7879	2.6407
	20	2.44	16.80	0.98	2.7754 2.7970	2.7977
Coconut	5	4.52	0.38	0.10	2.5587 2.5909	2.4599
	10	9.03	0.76	0.20	2.5703 2.5652	2.4362
	20	18.10	1.52	0.40	2.3252 2.2839	2.3885
Safflower	5	0.59	0.31	4.05	2.1088 2.1312	2.3885
	10	1.17	0.61	8.10	2.2709 2.3626	2.2932
	20	2.34	1.22	16.20	2.2462 2.2532	2.1029
Corn	5	0.59	2.32	2.10	2.4561 2.4134	2.4779
	10	1.17	4.63	4.20	2.4503 2.3860	2.4720
	20	2.35	9.26	8.40	2.3028 2.3645	2.4604
Coconut and safflower	5	2.55	0.35	2.08	2.4126 2.3972	2.4242
	10	5.10	0.69	4.16	2.2867 2.3296	2.3646
	20	10.20	1.38	8.32	2.2756 2.2384	2.2455

* According to equation I (see text).

monounsaturated, and polyunsaturated acid per 100 g of diet, respectively. The coefficient of multiple correlation, R, is 0.811. According to this equation, the polyunsaturated acid (in this case linoleic acid, since the oils contained insignificant amounts of other polyunsaturated acids) is primarily responsible for the lowering of the serum cholesterol, having a coefficient of -0.02415. The saturated acids contribute in the same direction but appear to have only about one-fourth the activity. The monounsaturated acids, on the other

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hand, gave a positive coefficient of 0.02105. As the amount of oleic acid increased the serum cholesterol value rose. Thus, linoleic and oleic acids apparently had about equal and opposite effects.

In the above analysis the influence of time and cholesterol level in the diet were ignored. In order to examine partially the effects of time and cholesterol level, the regression equations were calculated for the two cholesterol levels separately over the entire experiment and also for the data on the fourth week alone. The regression coefficients and multiple correlation coefficients are shown in Table IV. The

TABLE IV

Regression Coefficients Relating Oil Composition to Log Serum Cholesterol Level at Different Times and Cholesterol Intake

Data included	Regression coefficients			R
	S	M	P	
Both cholesterol, all weeks	−0.00648	+0.02105	−0.02415	0.807
1.35% cholesterol, all weeks	−0.00668	+0.02855	−0.02365	0.774
0.45% cholesterol, all weeks	−0.00618	+0.01428	−0.02443	0.772
1.35% cholesterol, 4th week only	−0.0045	+0.0352	−0.0946	0.753
0.45% cholesterol, 4th week only	−0.0091	+0.0286	−0.0210	0.644

magnitude of the regression coefficients varies considerably with the selected data, as might be expected from the previously demonstrated interactions of time, oil, level of oil, and level of cholesterol. The sign of the regression coefficients remains the same, however, and, as might be expected, the coefficient of multiple regression decreases with more limited data.

Plots of the calculated log serum cholesterol versus the actual values found are of some interest. In Figure 1, the actual values and those calculated from equation I are shown. It is apparent that the fit is reasonably good but that the equation underestimates the effect of the 5 per cent safflower oil diet and overestimates the effect of the 20 per cent safflower diet. All the equations in Table II fail to adequately place some of the oils,

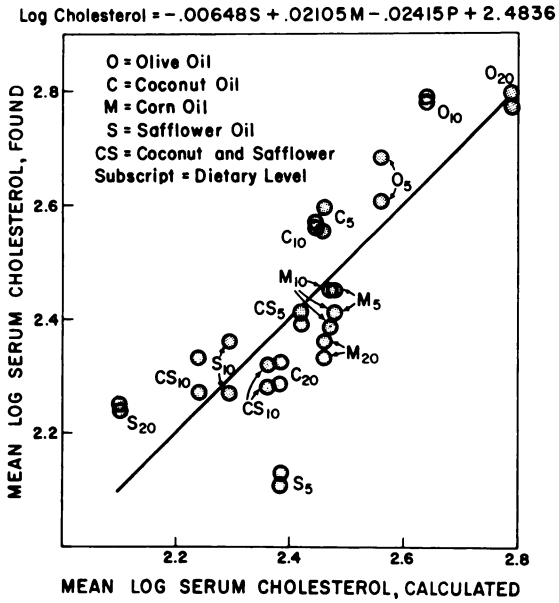

Fig. 1. The relationship between the calculated mean log serum cholesterol values according to the equation and the values from which the equation was derived.

usually the 5 or 20 per cent safflower group or both.

This suggested that the inclusion of the total level of oil in the equation might improve the fit. Thus, the following equation was derived:

II. mean log chol. = +0.00531S + 0.03268M − 0.01128P − 0.01160% fat + 2.4778

This equation fails to improve the fit to any appreciable degree over that obtained with equation I. It is instructive to note the changes in the coefficients of S, M, and P when the fourth variable is included in the equation. The coefficient of S is of approximately the same size as in equation I but opposite in sign. The coefficient of M is about 50 per cent larger while the coefficient of P is only half as large. As Snedecor[9] has pointed out, a general feature of multiple regression is that the various coefficients are intercorrelated and the introduction of a new variable changes all of the other coefficients. Thus, it is difficult to evaluate the absolute meaning of any of the coefficients, since in any equation the size and perhaps even the sign may be changed if another variable is included or omitted.

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In a previous publication[7] we concluded that when diets were fed containing a constant amount of fat of different kinds, the product of the saturated and linoleic acid content of the diet was a parameter with a very high negative correlation with the log of serum cholesterol level ($r = -0.94$). Hence, for these data the equation relating the product and amount of oil was calculated. This yields:

III. log mean serum chol. = 0.0171% fat − 0.274 log product + 2.6745

The coefficient of multiple regression is 0.80. As seen from the plot of the calculated versus actual values (Fig. 2) the fit is about the

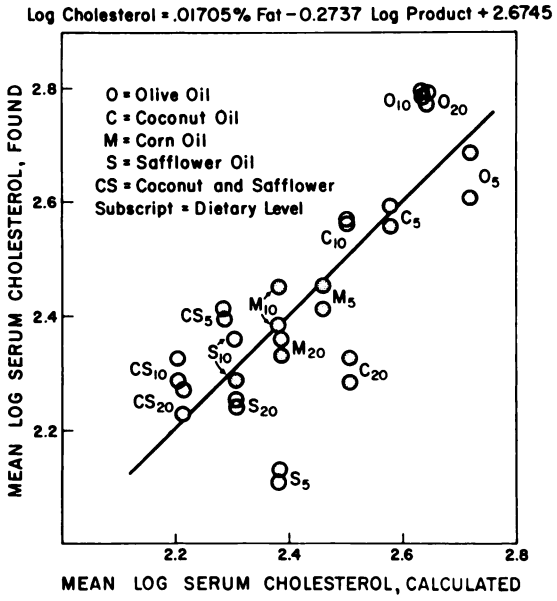

Fig. 2. The calculated mean log serum cholesterol values obtained from the equation are plotted against the actually determined values.

same as in the previous plots of equation I Here the 5 per cent safflower diet and the 20 per cent coconut diet are the most seriously misrepresented by the equation. This equation has a positive coefficient for the percentage of fat in the diet whereas in equation II including this variable gave a negative coefficient. Again the danger of attaching too much physical significance to the regression coefficient is apparent.

SUMMARY

An extremely large number of variables are reported to influence hypercholesterolemia and atherosclerosis in experimental studies, and there is need for experimental designs adequate to evaluate the relative importance of these variables and their interrelation to each other. A study is reported in which five different oils, each at three levels, and two different levels of dietary cholesterol were investigated. Only two animals per groups were used. All of the major variables (time, kinds of oil, level of oil, and amount of dietary cholesterol) have significant effects upon the hypercholesterolemia and many of the numerous interactions are also significant. It would appear, therefore, that the action of any of these variables can only be stated at the present time in terms relative to the others. The same situation is probably true with other variables not specifically included in this study. Such interrelations may well account for the apparently contradictory results in the literature as to the effects of various fats upon hypercholesterolemia in human subjects.

Regression equations relating the amount of fatty acid (saturated, monounsaturated, and polyunsaturated) in the diet to the serum cholesterol level were calculated. With these three variables in the equation, the coefficient for the monounsaturated acid is positive. The coefficients for the saturated and polyunsaturated acids are negative.

The equation suggests that the monounsaturated acid raises the serum cholesterol level while the saturated and polyunsaturated acids reduce it, the saturated acids being about one-fourth as active as the polyunsaturated acid. However, other equations can be derived which give as good a "fit" as this equation, and the magnitude and even the sign of the coefficients vary depending upon the variables included in the equation. Caution should be used in assigning too much significance to the calculated coefficients.

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