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Effect of dietary lipids on rat alveolar macrophage function

Sangeeta R. Mehta

Rochester Institute of Technology
The College of Science

EFFECT OF DIETARY LIPIDS ON RAT ALVEOLAR MACROPHAGE FUNCTION

A Research Report in
Clinical Chemistry

by

Sangeeta R. Mehta

submitted in partial fulfillment
of the requirements
for the degree of

Master of Science

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Sangeeta R. Mehta

Form 9
M.S. Clinical Chemistry

ROCHESTER INSTITUTE OF TECHNOLOGY
Rochester, New York 14623
Department of Clinical Sciences

TO: Registrar

This is to certify that Sangeeta Mehta _____
Candidate's Name Social Security Number

has satisfactorily completed all requirements for the degree of Master of Science in Clinical Chemistry.

Thesis Title: Effect of Dietary Lipids on Rat Alveolar Macrophage Function

7/26/91 John M. Wandge
Date Head, Department of Clinical Sciences

John M. Wandge
Director, Clinical Chemistry Program

Approved: _____
Assistant Dean, College of Science

Departmental Student Record File - original

cc: Student
Dean's Office, College of Science

ABSTRACT

An *in vitro* assay has been developed to measure bacterial uptake by rat alveolar macrophages. The assay is used to study the effect of a diet rich in high omega-6 linoleic acid on the phagocytic response of the macrophages obtained from young and old rats. Within the range of diets studied, no significant difference ($p<0.05$) is observed in the phagocytic capability of macrophages from young rats. Significant difference is observed between diet groups for the old rats ($p<0.05$), with the group on a fat-rich diet showing suppressed phagocytic response. Significant difference is also found between age groups for the same diet ($p<0.05$), older animals showing suppressed response.

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INTRODUCTION

Total parenteral nutrition of debilitated and malnourished patients may help normalize certain host defenses. Lipid emulsions are commonly used in such a dietary regimen where lipids provide both calories and essential fatty acids, for reducing the respiratory quotient, and for replacing reduced carbohydrate calories in presence of glucose intolerance. However, numerous reports have suggested possible impairment of immune function with fat emulsions (1-5). Other studies, both *in vitro* and *in vivo*, have shown that essential fatty acids can alter cell-mediated immune function (6,7). On the other hand, it is argued that many of the controversies surrounding fat emulsions and the host immune response have been the result of the animal models, *in vitro* experimental methods, or excessive infusion rates. Under properly controlled conditions of administration of fat emulsions to humans, no demonstrable deleterious effect may occur (8).

Many hospitals provide Pulmocare™, a diet rich in fat and poor in carbohydrates, to their pulmonary patients. The fat-rich diet is primiarily used to reduce the respiratory quotient of the patient. Unlike total parenteral nutritional diets, Pulmocare™ is fed orally. Nevertheless, the question remains whether such a diet has any adverse effect on lung's defense against bacterial infection. Phagocytic cells are thought to constitute the principle defense of the lungs against bacteria that escape more proximal mechanical barriers and ciliary action. Previous studies have shown that lung

alveolar macrophages from rats have bactericidal activity (9,10). Other studies have suggested that crude rat and human lung lavage fluid enhance intracellular killing of *S. aureus* by rat alveolar macrophages (11,12). Subsequently, lung lavage surfactant has been suggested as the active principle that enhances intracellular killing(13). Again, a different report (14) finds no demonstrable effect of alveolar lining material from healthy humans on host defense against bacteria. Surprisingly, very little information is available on the effect of high dietary fat on pulmonary macrophage function.

We have studied, *in vitro*, the effect of an omega-6 linoleic acid rich diet on rat alveolar macrophages challenged by *S. aureus*. The diet mimics Pulmocare™ in terms of fat content, although it was fed as a powder. In our test system, phagocytic capability of macrophages was significantly suppressed for macrophages obtained from old rats but was unaffected for macrophages obtained from young rats. Significant differences were also found between the two age groups, regardless of the diet.

METHODS

ANIMALS

Male Sprague-Dowley rats were used in all experiments. Two age groups were used, young and old. The young rats weighed 200 to 250 gms and their age ranged from 46 to 51 days. The old rats

(called retired breeders) weighed 350 - 400 gms and were 6 to 8 months old.

BACTERIA

Tryptic soy broth or blood agar plates were used to culture Staphylococcus aureus (ATCC 25932). The overnight growth of bacteria was centrifuged at 4000 RPM for 20 minutes. The pellet was washed and centrifuged twice with irrigation saline. The bacterial suspension was adjusted to 10^8 cells/ml.

DIET

Three types of diets were used with young rats. Only two types of diets were used with old rats.

- Meritene powder, a low fat diet containing 3% fat.

- A mixture of Meritene powder and corn oil having an overall fat content of 9.2%. It mimiced Pulmocare™ in terms of its fat content. Corn oil is 100% omega-6 linoleic acid.

- Impact™, a commercially available liquid diet, having 8% omega-6 and 6% omega-3 fatty acids with 24% medium chain triglycerides. It is claimed to be immunonutritionally balanced.

PHAGOCYTIC ASSAY

Development of the assay occurred in three stages. The first stage was an *in vivo* technique.

STAGE I

An attempt was made to infect rat lungs with suspension of known number of bacteria after keeping the animals on a prescribed diet for two weeks. After the infection, lungs were removed surgically at different time intervals, ground, and the number of viable bacteria determined. Thus, a measure of phagocytic activity of alveolar macrophages was obtained.

In the past, aerosolized supsension was used unsuccessfully to infect the animals. Therefore, bacterial suspension was introduced into the lungs with a surgical procedure. Animals were anesthetized with sodium pentobarbital 50 mg/kg intraperitonially. The trachea was exposed slightly. Bacterial suspension was introduced in the trachea through the needle. At different time intervals, the lungs were removed surgically, ground, and washed in a tissue grinder. A sample from that was serially diluted with saline and appropriate dilutions were plated on blood agar plates. After the overnight incubation, colonies were counted and the number of bacteria/ml were determined.

The tracheal introduction of bacterial suspension was to be made very slowly, otherwise animals could not survive due to 'drowning'. The animals could tolerate up to 0.2 ml of fluid, introduced over 1 min. Since the trachea was exposed for injecting, contamination was inevitable. Some droplets of suspension were found near animals' noses, indicating mucociliary clearance. During the period of the experiment, some animals recovered from anesthesia. They showed less number of colonies in their tissue suspension than others. It was thought that anesthesia could have caused suppression of bacterial clearance. This led to search for an alternative *in vitro* technique.

STAGE II

The second stage of development of the assay involved use of lung washings (lavage) in tissue culture plates to study bacterial uptake *in vitro*. After two weeks on the desired dietary regimen, the rats were anesthetized and cardiac puncture was done. The blood was collected in a syringe coated with a drop of Heparine (1000 units/ml). The blood was centrifuged at 8000 RPM for 8 minutes and serum was seperated. For each group, equal volumes of serum were pooled into a test tube. This was used for opsonizing the bacteria as well as for the analysis of serum fat. Next, the trachea was exposed and a silk thread was passed under it. While holding the trachea with a thread, a small slit was made in it. A half cut tubing from a butterfly infusion set was attached to a 3 ml. syringe, filled with 1.5 ml. of 0.9% sterile irrigation saline. This volume was necessary

to get a large enough concentration of macrophages. The tubing was inserted into tracheal opening and saline was slowly introduced. Care was taken to allow the animal to breathe until the lungs were fully washed. This was important to prevent the lungs from collapsing. Lungs were washed by back and forth movement of the syringe piston. The lavage fluid was kept on ice. The lavage fluid was also pooled for the assay. The number of macrophages were counted in 4 WBC squares of a hemocytometer slide with a high power (40X) optical microscope. In a separate experiment, lavage fluid was centrifuged and cell pellet was used to make the slide. By histological staining, it was determined that 80 -90% of the cells in the pellets were macrophages.

A two hundred microliter portion of the lavage fluid was dispensed into each of the tissue culture wells. This was followed by addition of 100 µl of serum into the wells. The serum was to supply the nutrients as well act as an opsonin. Next, 100 µl of bacterial suspension was added into each well. An attempt was made to keep the macrophage:bacteria ratio between 1:100 to 1:10 so as not to overwhelm the cells. A sample of 100 µl was taken immediately from the first well into a test tube containing 0.9 ml. saline and was serially diluted. Appropriate dilutions were plated out on blood agar plates and incubated overnight at 37^{o} C. The tissue culture plate was incubated as well. At various time intervals, a 100 µl aliquot was taken out and analysed similarly. It was found that the resultant data had very high variability. It was felt that the fluid volume in each well might have been excessive,

preventing sufficient contact between macrophages and bacteria. This led to the final stage of the development of the assay.

STAGE III

First, the lavage volume was halved to 100 µl per well. Next, the pooled serum was mixed with equal volume of minimum essential medium (MEM; without antibiotics) as a nutrient. Fifty µl of serum mixture was added to each of the wells. This was followed by addition of 50 µl of bacterial suspension. The final concentration of serum in each well was 12.5 % , which was similar to that used in the published literature(13). The entire first well was washed immediately with 1.8 ml saline and then serially diluted to determine initial number of bacteria. At 15 minute intervals, the above procedure was repeated. All the readings were run in duplicate. Each experiment lasted for 3 hours. The bacterial count was not expected to increase during that period according to a previous study (15). Also, tissue culture wells showed evaporation of fluid if incubated for longer times.

Viability of macrophages was measured by trypan blue exclusion (Vital stain, CI. 23850). Four hundred mg of dye was added to 90 ml of distilled water, containing 810 mg NaCl, 60 mg K_2HPO_4, and 50 mg methyl p-Hydroxybenzoate. The mixture was heated to boiling point, cooled, and its pH adjusted to 7.4 with approximately 8 drops of 1 N NaOH. The final volume of the solution was adjusted to 100 ml. A drop of this solution was mixed with lavage fluid and a

slide was prepared. Approximately 100 marophages were counted under the high power optical microscope and percentage viable cells were determined. The living cells excluded the dye while the dead ones were stained blue. The viabilty of macrophages was more than 90 % for the duration of the experiment.

CONTROLS

Two controls were used in each phagocytic assay.

1) *Macrophage Control*: 100 µl of lavage fluid was mixed with 50 µl of saline and 50 µl of bacterial suspension in a well and incubated for 3 hours. This was treated the same way as other readings for actual assay. This control helped in evaluating the effect of bacterial uptake by macrophages alone.

2). *Serum Control*: 100 µl of saline was mixed with 50 µl of serum + MEM solution and 50 µl of bacterial suspension in a well and incubated at 37^{o} C for 3 hours. The bacterial analysis was carried out the same way as the other readings. This control helped in evaluating the effect of serum killing in absence of macrophages.

Initially, only young rats were used. A group of 4 animals was used for each of the three diets. The animals were weighed at the beginning and the end of the 15 day period during which they were housed in seperate cages. Each day, weighed amount of dietary powder, along with tap water in a separate bottle, was put in the

cages and the daily consumption by each animal was recorded. Since Impact™ was a liquid diet, it was given in a bottle. Some of the animals had diarrhea with Impact™. Care was taken to change cages every 2 - 3 days to avoid risk of contamination with these rats. The left over Impact™ was thrown away every day and bottles were cleaned thoroughly. The average weight of each of the comparative groups was found to be the same at the end of the 15 day period. The experiment was replicated thrice for each diet, yielding a total of 9 experiments.

Subsequently, older animals were used. A group of 3 rats was used for two of the diets. Impact™ was not used as it had degraded during the storage. The experiment on old rats was replicated twice for each diet, yielding 4 additional experiments.

The cardiac puncture was done on each of these groups. The blood was collected with a drop of Heparin(1000 units/ ml). Serum was separated and analysed for cholesterol and triglyceride on an American Monitor Parallel. An attempt was also made initially to determine lecithin : sphingomyelin ratio of the cell free lavage fluid. No difference was found between the two diet groups for the lecithin : sphingomyelin ratio.

ANALYSIS

For each experimental group, the number of colony forming units (CFU) per ml were determined as a function of time. The time interval was typically 15 minutes and the experiment lasted for about 180 minutes. In general, the observed rate of decrease in viable count can be characterized by two rate processes: bacterial multiplication and bacterial inactivation. The duplication time of *S. aureus,* however, is reportedly very large (15) and can be neglected in our experiments. Then, the rate of decrease can be attributed directly to the rate of bacterial inactivation by the concerted action of serum and macrophages. It is customary to assume this rate as a first order process (15) and hence one can write

$$N(t) = N(0) \exp(-k t) \qquad [1]$$

where N is the number of bacteria in the incubation well at time t and k is the inactivation rate constant.

Ln (N/N0) v/s time was plotted for every diet and age group. A least-square linear regression was used to find the best fit straight line whose slope represented the inactivation rate constant ($-\bar{k}$). The intercept of the line represented uncertainty in the determination of initial number of bacteria. Even though the data showed a non-linear pattern in many cases, R-squared (r^2) values of the best fit lines ranged from 0.766 to 0.976. The results of the

regression analysis for all 13 sets are shown in Table 1. The inactivation rate constants ranged from 0.01124/min to 0.05038/min.

The inactivation rate constants were analysed using analysis of variance (general linear model procedure) for a cross design (Diet x Age). Thus, differences due to diet and age, and set to set variability within a diet group were considered in the analysis.

For macrophage control, the uptake rate constant k_m was determined directly from eqn.[1] where N(0) and N(180) were the macrophage control readings at 0 and 180 min. Serum killing constant, k_s, was similarly determined from the serum control data. It was also possible to calculate inactivation rate constants k_{sm}, based only on the initial and final bacterial counts, which were then compared with $\bar{k}$. The resultant values are listed in Table 2. The serum lipid levels are also reported in Table 2.

RESULTS

Duncan grouping at alpha = 0.05 for k_m (obtained from macrophage control data) showed no statistical difference among different diet and age groups (see Appendix - I). Similarly, k_s (obtained from serum control data) showed no statistical difference among different diet and age groups. Therefore, any observed statistical difference in average inactivation rate constant, $\bar{k}$, had

to be attributed to macrophages and serum acting in concert, not independently.

Duncan grouping at alpha = 0.05 for $\bar{k}$ and k_{sm} (see Appendix - I) indicated the following: 1) No significant difference existed among different diet groups for young animals. 2) Significant differences existed among the two diet groups for the old rats. 3) Significant differences also existed between the response of young and old animals to the same diet. A better appreciation of the results is obtained by considering figures 1-4. For the purpose of illustration only, all the replicate data were averaged and semi-log plots made. Figure 1 shows comparison of data for young rats for all three diets. Best fit line, with 95% confidence band, is shown only for the meritene powder group. For the most part, data for all diets fall within the band. If similar best fit lines were drawn for each diet group, the corresponding 95% confidence bands would encompass all data points, suggesting lack of statistical difference among all three diet groups for the young rats. On the other hand, the longer time data for one diet group for old rats do fall outside the 95 % confidence band of the other diet group as shown in figure 2. The data of figure 1 and 2 are re-plotted in figures 3 and 4, to show the existence of significant differences between the two age groups for both the diets very clearly.

Duncan grouping of the triglyceride and cholesterol data at alpha=0.05 suggested that differences in values were not statitistically significant (see Appendix -II). Thus, serum

cholesterol and triglyceride are not useful lipid markers for the diets used in the present study.

DISCUSSION

In principle, the phagocytic assay used in the present study is similar to other more sophisticated assays using radio-labelled micro-organisms. On the other hand, assays like the present one suffer from three major difficulties: inability to separate bacteria that are attached to the cell membrane from those that have actually been ingested, reproducibility , and inability to correct for killing by serum. It is felt, however, that the present assay , in conjunction with the present method of analysis, is adequate for comparative purposes. This is based on the fact that even with a relatively small number of animals used in the experiments, statistically significant differences are found. Though correction for serum killing is not done, statistical analysis suggests that differences in phagocytic responses can not be attributed to serum killing alone. For a preliminary investigation like the present one, very useful, albeit only suggestive, information is obtained by the current approach.

The effect of dietary fat on various immune systems has been extensively studied. However, there is little information available on this effect for pulmonary immune system. The present study is the first to report the effect of high dietary fat on bacterial uptake by alveolar macrophages of young and old rats. The experiments on

young rats showed no difference between high and low fat diet groups ($p<0.05$, $n=3$). On the one hand, this is similar to a previous findings on phagocytic ability of peritoneal macrophages of young rats (16). On the other hand, high fat diet reportedly does suppress phagocytosis by the reticulo-endothelial macrophages of young mice (4). Interestingly, significant differences were observed between the two diet groups for the old animals ($p<0.05$, $n=2$). Also, for the same diet, the response was significantly suppressed for old animals as compared to young animals ($p<0.05$, $n=2$). Overall, the phagocytic repsonse was influenced both by the age and the diet of the rats.

Dietary fatty acids can influence the host immunity in two ways (17): structural alterations, and chemical mediation. Structural alterations in fatty acid components of membrane phospholipids may alter receptor binding sites which could significantly affect the bacterial uptake by the cell. Similarly, surfactants in alveolar lining could coat the bacterial surface and act as ligands that bind with macrophage surface (13). It is possible that opsonization by serum components- *eg*., free fatty acids, immunoglobulins- may overwhelm many of these alterations. On the other hand, alveolar macrophages are believed to reside in a relatively opsonin deficient environment and macrophages may have to adapt their requirements for bacterial ingestion to accomodate this environment. Chemical mediation may affect synthesis, release, and/or binding of immune factors. For example, overproduction of

eicosanoids, such as prostaglandins, may lead to significant cell function modification.

The potential clinical relevance of our findings is the suggestion that a diet rich in omega-6 linoleic acid may predispose older patients to staphylococcal pneumonia, but it may not predispose younger patients. In experimental terms, the method of analysis used in this report may be adapted for a clinical study although the phagocytic assay would need some extensions and refinements.

SUMMARY

An *in vitro* assay has been developed to measure bacterial uptake by rat alveolar macrophages. The assay is used to study the effect of a diet rich in high omega-6 linoleic acid on the phagocytic response of the macrophages obtained from young and old rats. Within the range of diets studied, no significant difference ($p<0.05$) is observed in the phagocytic capability of macrophages from young rats. Significant difference is observed between diet groups for the old rats ($p<0.05$), with the fat-rich diet group showing suppressed phagocytic response. Significant difference is also found between age groups for the same diet ($p<0.05$), older animals showing suppressed response. It is speculated that dietary fat may alter phagocytosis by a number of structural or chemical modifications of cellular or lavage fluid components.

SUGGESTED FUTURE PATH

The present assay does not provide any information on intracellular killing of bacteria, nor does it differentiate between bacteria attached to the wall from those actually ingested. Direct observation of ingested bacteria under a microscope would be helpful in overcoming a major limitation of the present assay. It may be advantageous to get information on intracellular killing. It would also be necessary to find a suitable marker for dietary fat and measure its level. An earlier study (14) suggests free fatty acid in bronchoalveolar lavage fluid as a potential marker. It may also be necessary to measure prostaglandin levels in the lavage fluid and/or serum as omega-6 linoleic acid is known to affect PG synthesis (16). While the present study considered the effect of dietary fat intake for 2 weeks, longer periods of fat-rich intake may show adverse effect for young animals as well (18). Similarly, it may be more useful to compare two liquid diets, Pulmocare™ and Impact™, unlike the present study where Meritene powder based solid diets are compared with a liquid diet (Impact™).

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Table 1: Inactivation Rate Constants

Experiment	Diet	$\bar{k}$ min^{-1}	r^2	σ ($\bar{k}$)	Intercept
1.1	M	0.05038	0.862	0.007	-1.518
1.2	MC	0.03752	0.870	0.005	-1.611
1.3	IM	0.04006	0.914	0.004	-0.902
2.1	M	0.01918	0.965	0.001	-0.085
2.2	MC	0.01254	0.766	0.002	-0.688
2.3	IM	0.01466	0.886	0.002	-0.646
3.1	M	0.02936	0.976	0.002	-0.020
3.2	MC	0.02204	0.844	0.001	-0.231
3.3	IM	0.02202	0.871	0.003	-0.883
4.1	M	0.02777	0.916	0.003	-0.437
4.2	MC	0.01124	0.821	0.002	-0.345
5.1	M	0.02104	0.921	0.002	0.101
5.2	MC	0.01759	0.844	0.003	-0.270

M- Meritene Powder
MC- Meritene Powder + Corn Oil
IM - Impact

Expt 1.1-3.3 : Young Rats

4.1-5.2 : Old Rats

Table 2: Inactivation Rate Constants and Serum Lipids

Experiment	$\bar{k}$ min^{-1}	k_{sm} min^{-1}	k_s min^{-1}	k_m min^{-1}	Chol (mg/dL)	TriGly (mg/dL)
1.1	0.050	0.050	0.032	0.017	86	86
1.2	0.038	0.041	0.030	0.014	72	80
1.3	0.040	0.041	0.025	0.017	63	23
2.1	0.019	0.018	0.010	0.002	89	45
2.2	0.013	0.015	0.015	0.004	77	41
2.3	0.015	0.016	0.015	0.015	55	24
3.1	0.029	0.032	0.014	0.013	70	62
3.2	0.022	0.022	0.013	0.012	96	149
3.3	0.022	0.024	0.016	0.007	63	23
4.1	0.028	0.027	0.021	0.024	84	92
4.2	0.011	0.011	0.006	0.007	82	75
5.1	0.021	0.017	0.011	0.013	98	86
5.2	0.018	0.015	0.015	0.014	74	78

Inactivation rate constant.....

$\bar{k}$: ....based on all data points
k_{sm} : ....based on initial and final data points
k_m : ....for macrophage control
k_s : ....for serum control

Chol : Serum Cholesterol
TriGly : Serum Triglycerides

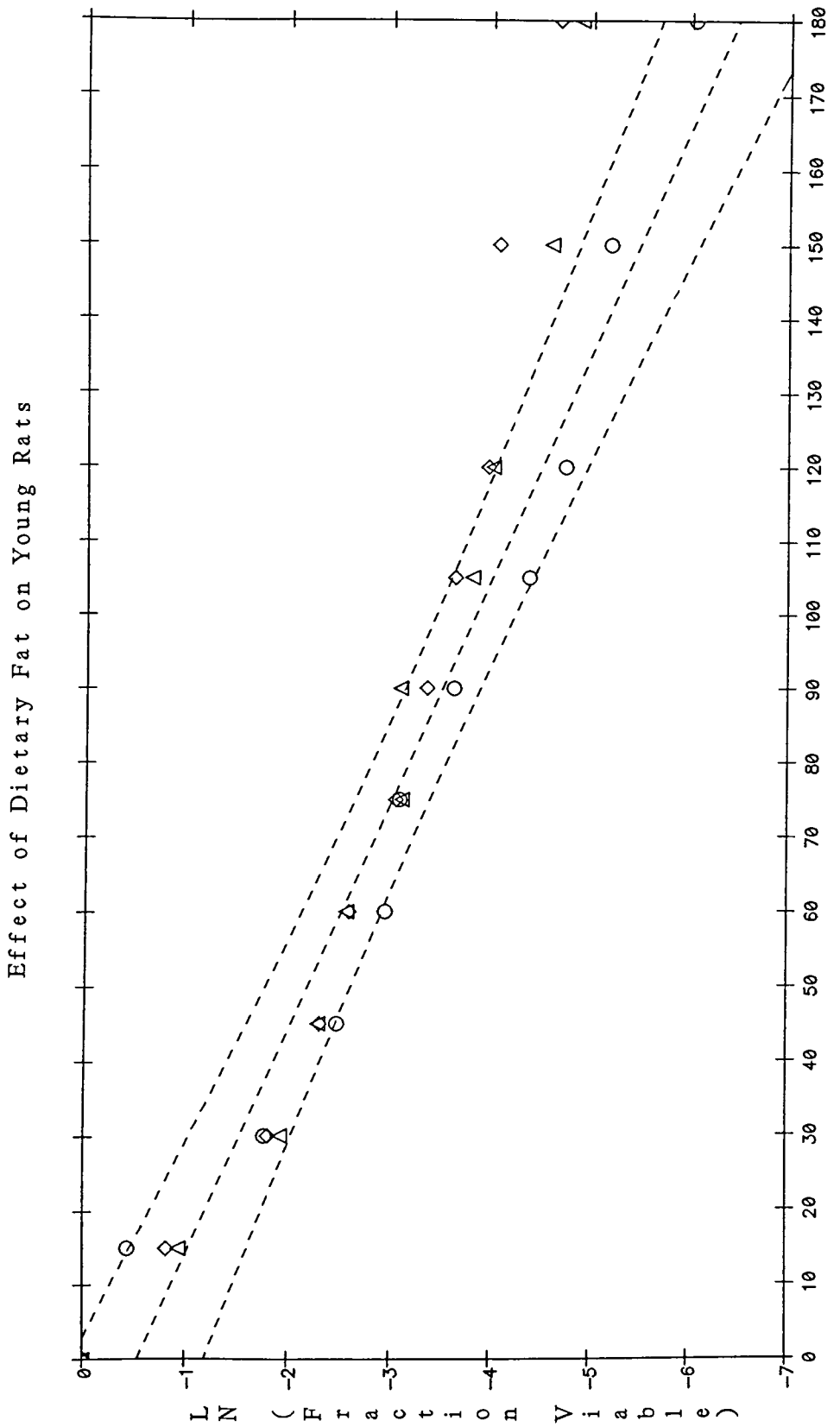
Effect of Dietary Fat on Young Rats
LN (Fraction Viable)
0
-1
-2
-3
-4
-5
-6
-7
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
Incubation Time (minutes)
○ Meritene Powder
◇ Meritene Powder + 10% Corn Oil
△ Impact

Figure 1

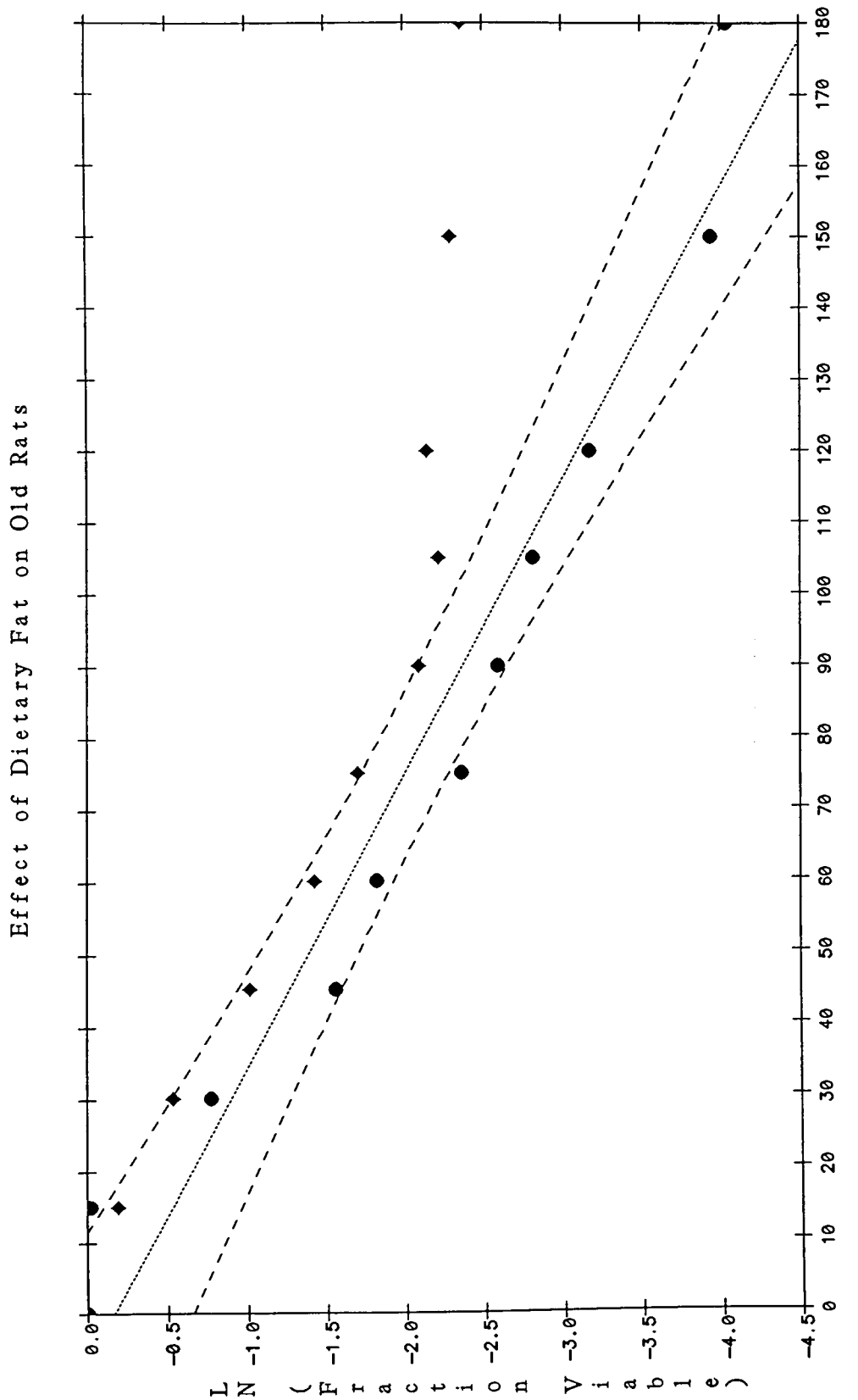
Effect of Dietary Fat on Old Rats
LN (Fraction Viable)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
Incubation Time (minutes)
Meritene Powder
Meritene Powder + 10% Corn Oil

Figure 2

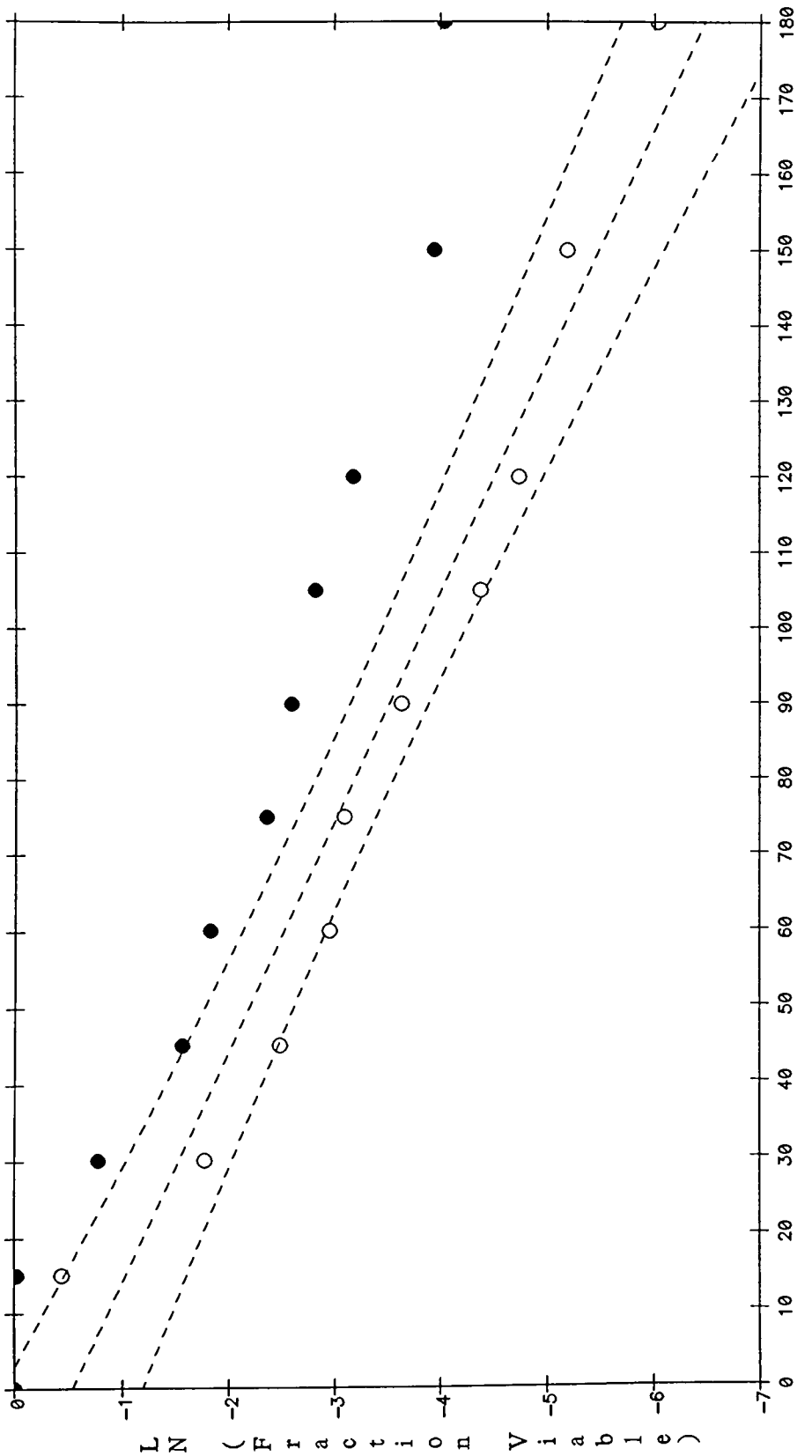
Effect of Meritene Powder
LN (Fraction Viable)
0
-1
-2
-3
-4
-5
-6
-7
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
Incubation Time (minutes)
Young Rats
Old Rats

Figure 3

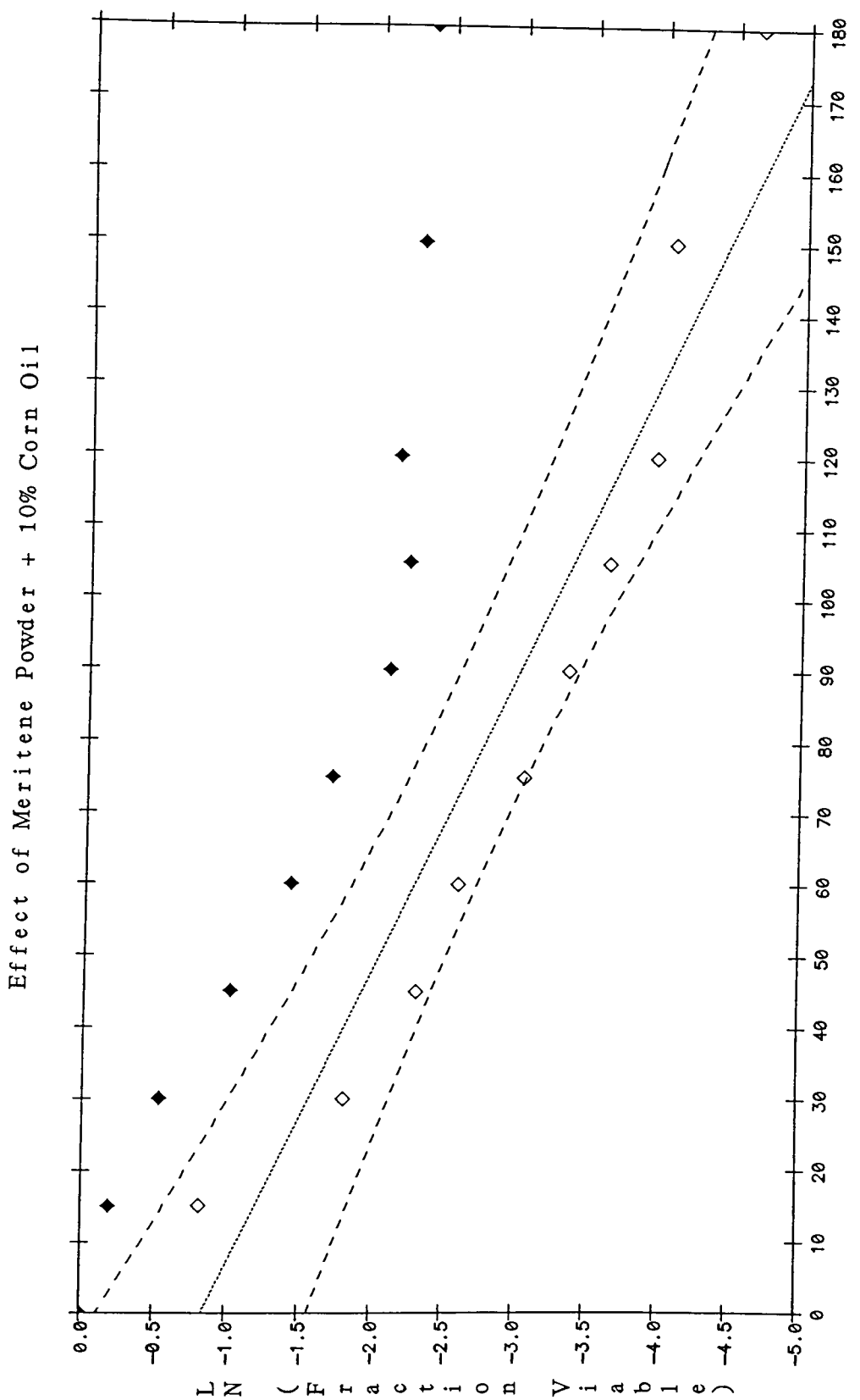
Effect of Meritene Powder + 10% Corn Oil
LN (Fraction Viable)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
Incubation Time (minutes)
Young Rats
Old Rats

Figure 4

Appendix I

```
)ptions nocenter;
;options device=ibm32784;

lata design;
.nput Group $ Age $ Expt     kbar      ks          km       ksm ;
)ards;
{      Y       1          0.05038  0.032374  0.016819 0.050359
{      Y       2          0.01918  0.010344  0.001974 0.018307
{      Y       3          0.02936  0.013622  0.012639 0.031876
{C     Y       1          0.03752  0.029667  0.014352 0.041109
{C     Y       2          0.01254  0.015561  0.003815 0.014936
{C     Y       3          0.02204  0.012818  0.011882 0.021597
:M     Y       1          0.04006  0.024934  0.017349 0.041026
:M     Y       2          0.01466  0.015214  0.014591 0.016134
:M     Y       3          0.02202  0.015667  0.006547 0.024070
{A     O       4          0.02777  0.020763  0.024390 0.027426
{A     O       5          0.02104  0.011378  0.013123 0.017419
{CA    O       4          0.01124  0.005743  0.007002 0.011059
{CA    O       5          0.01759  0.014722  0.014077 0.015166

)roc glm data=design;
     class Group Expt;
     model kbar ks km ksm= Group Expt;
     means Group/duncan alpha=.05;

'un;cms x sang listing;run;
```

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: KBAR

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	7	0.00157058	0.00022437	19.55	0.0024	0.964744	13.5358
ERROR	5	0.00005740	0.00001148		ROOT MSE		KBAR MEAN
CORRECTED TOTAL	12	0.00162798			0.00338811		0.02503077

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
GROUP	4	0.00041931	9.13	0.0161	3	0.00023678	6.88	0.0318
EXPT	3	0.00115127	33.43	0.0010	3	0.00115127	33.43	0.0010

SAS

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: KBAR
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=0.05 DF=5 MSE=1.1E-05

WARNING: CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=2.5

NUMBER OF MEANS	2	3	4	5
CRITICAL RANGE	.00779819	.00803764	.00812147	.00816645

MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

DUNCAN GROUPING	MEAN	N	GROUP
A A	0.032973	3	M
B A	0.025580	3	IM
B B	0.024405	2	MA
B B	0.024033	3	MC
C	0.014415	2	MCA

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: KSM

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	7	0.00172629	0.00024661	17.71	0.0030	0.961228	14.6795
ERROR	5	0.00006963	0.00001393		ROOT MSE		KSM MEAN
CORRECTED TOTAL	12	0.00179593			0.00373179		0.02542185

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
GROUP	4	0.00052633	9.45	0.0150	3	0.00018781	4.50	0.0696
EXPT	3	0.00119997	28.72	0.0014	3	0.00119997	28.72	0.0014

SAS

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: KSM
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=0.05 DF=5 MSE=1.4E-05

WARNING: CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=2.5

NUMBER OF MEANS	2	3	4	5
CRITICAL RANGE	0.0085892	.00885294	.00894527	.00899481

MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

DUNCAN GROUPING	MEAN	N	GROUP
A	0.033514	3	M
A			
B A	0.027077	3	IM
B A			
B A	0.025881	3	MC
B			
B	0.022422	2	MA
C	0.013112	2	MCA

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: KS

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	7	0.00058433	0.00008348	3.14	0.1133	0.814481	30.1032
ERROR	5	0.00013310	0.00002662		ROOT MSE		KS MEAN
CORRECTED TOTAL	12	0.00071743			0.00515938		0.01713900

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
GROUP	4	0.00012686	1.19	0.4164	3	0.00003499	0.44	0.7356
EXPT	3	0.00045747	5.73	0.0450	3	0.00045747	5.73	0.0450

SAS

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: KS
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=0.05 DF=5 MSE=2.7E-05

WARNING: CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=2.5

NUMBER OF MEANS	2	3	4	5
CRITICAL RANGE	0.011875	0.0122396	0.0123673	0.0124358

MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

DUNCAN GROUPING	MEAN	N	GROUP
A	0.019349	3	MC
A			
A	0.018780	3	M
A			
A	0.018605	3	IM
A			
A	0.016070	2	MA
A			
A	0.010232	2	MCA

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: KM

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	7	0.00025480	0.00003640	0.96	0.5398	0.572171	50.6097
ERROR	5	0.00019052	0.00003810		ROOT MSE		KM MEAN
CORRECTED TOTAL	12	0.00044532			0.00617283		0.01219692

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
GROUP	4	0.00011588	0.76	0.5931	3	0.00008117	0.71	0.5863
EXPT	3	0.00013891	1.22	0.3949	3	0.00013891	1.22	0.3949

SAS

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: KM
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=0.05 DF=5 MSE=3.8E-05

WARNING: CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=2.5

NUMBER OF MEANS	2	3	4	5
CRITICAL RANGE	0.0142076	0.0146438	0.0147966	0.0148785

MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

DUNCAN GROUPING	MEAN	N	GROUP
A	0.018756	2	MA
A			
A	0.012829	3	IM
A			
A	0.010539	2	MCA
A			
A	0.010477	3	M
A			
A	0.010016	3	MC

Appendix II

```
options nocenter;
goptions device=ibm32784;

data design;
input Group $ Expt        Chol TriGly;
cards;
M             1             86    86
M             2             89    45
M             3             70    62
MC            1             72    80
MC            2             77    41
MC            3             96    149
IM            1             63    23
IM            2             55    24
IM            3             63    23
MA            4             84    92
MA            5             98    86
MCA           4             82    75
MCA           5             74    78
;
proc glm data=design;
     class group expt;
     model chol trigly= Expt group;
     means group/duncan alpha= 0.05;

run;cms x fat listing;run;
```

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CHOL

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	7	1376.29914530	196.61416361	1.45	0.3532	0.669707	15.0117
ERROR	5	678.77777778	135.75555556		ROOT MSE		CHOL MEAN
CORRECTED TOTAL	12	2055.07692308			11.65141861		77.61538462

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
EXPT	4	297.07692308	0.55	0.7106	3	23.22222222	0.06	0.9802
GROUP	3	1079.22222222	2.65	0.1603	3	1079.22222222	2.65	0.1603

SAS

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: CHOL
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=0.05 DF=5 MSE=135.756

WARNING: CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=2.5

NUMBER OF MEANS	2	3	4	5
CRITICAL RANGE	26.8173	27.6407	27.929	28.0837

MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

DUNCAN GROUPING	MEAN	N	GROUP
A A	91.00	2	MA
B A B A	81.67	3	MC
B A B A	81.67	3	M
B A B A	78.00	2	MCA
B B	60.33	3	IM

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: TRIGLY

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	7	11102.53632479	1586.07661783	1.88	0.2530	0.724367	43.7363
ERROR	5	4224.69444444	844.93888889		ROOT MSE		TRIGLY MEAN
CORRECTED TOTAL	12	15327.23076923			29.06783255		66.46153846

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE III SS	F VALUE	PR > F
EXPT	4	4162.06410256	1.23	0.4035	3	2629.13888889	1.04	0.4517
GROUP	3	6940.47222222	2.74	0.1530	3	6940.47222222	2.74	0.1530

SAS

GENERAL LINEAR MODELS PROCEDURE

DUNCAN'S MULTIPLE RANGE TEST FOR VARIABLE: TRIGLY
NOTE: THIS TEST CONTROLS THE TYPE I COMPARISONWISE ERROR RATE,
NOT THE EXPERIMENTWISE ERROR RATE

ALPHA=0.05 DF=5 MSE=844.939

WARNING: CELL SIZES ARE NOT EQUAL.
HARMONIC MEAN OF CELL SIZES=2.5

NUMBER OF MEANS	2	3	4	5
CRITICAL RANGE	66.9034	68.9578	69.6769	70.0629

MEANS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT.

DUNCAN GROUPING	MEAN	N	GROUP
A	90.00	3	MC
A			
A	89.00	2	MA
A			
A	76.50	2	MCA
A			
A	64.33	3	M
A			
A	23.33	3	IM