Variability of Serum Cholesterol Values in Baboons

By Jack P. Strong, M.D., Paul B. Radelat, M.D., Marion A. Guidry, Ph.D., and C. A. McMahan, Ph.D.

In order to plan efficiently a long term experiment on the effect of dietary factors on serum lipids and arterial lesions in baboons, studies of biological and technical (measurement) variation of serum cholesterol values were conducted. In the major experiment, treatment effects of dietary fat, protein, and cholesterol will be investigated. The limited number of animals that can be maintained and the expense involved require careful planning so that the experiment will stand a good chance of providing answers to the questions posed. This report describes the results of these pilot studies of variability of serum cholesterol values and their implications in planning such an experiment.

Variability of serum cholesterol levels in man as well as in monkeys and other experimental animals is generally recognized. A report of cholesterol and other serum lipid levels in South African baboons, *Papio ursinus*, estimated variability among and within animals over a specified period of time; normal ranges, by age of animal, were also given. For adult baboons, mean serum cholesterol values were $139 \pm 12$ (mean $\pm 2$ SE) milligrams per 100 milliliters of serum (based upon 206 observations on 19 animals). From cholesterol determinations on 108 baboons trapped and necropsied in their native habitat in Kenya, the mean cholesterol level was 78 milligrams per 100 milliliters. Animals of both sexes and all ages were the subject of the latter study; there were no striking differences in cholesterol values by sex or "relative age."

**Methods**

Eight young adult male baboons, *Papio anubis*, ranging in weight from 6.6 to 14.2 kilograms were used in this experiment. The animals were trapped in the same area of Kenya as those in the report by McGill et al., and were shipped to the United States by air freight. Three of the animals were in captivity for approximately one year prior to the experiment and five for about two months. All of the animals were on a standard monkey chow diet prior to and during the experiment. The animals were not fed for 22 hours before blood was drawn.

In the present study, the serum cholesterol values were determined in each of two separate laboratories on blind duplicate samples of serum from blood drawn on four successive days from the eight baboons. Two randomly numbered Vacutainer tubes for collecting blood were assigned to each animal for each of four days. After sedation of the animal with intramuscular injection of Sernylan,† 1 milligram per kilogram of body weight, two tubes of blood, 17 milliliters each, were drawn from the femoral vein of each animal (without a tourniquet or even temporary vein obstruction) daily from March 27 to March 30, 1962. Blood was refrigerated, allowed to clot, and the serum separated after 24 hours by centrifuging at 2500 rev/min for 15 minutes. Aliquots of serum from each tube of clotted blood were placed in each of two similarly numbered tubes,
one for each of the two laboratories. Sixty-four samples of serum (eight animals \times four days \times two duplicate samples), identified only by randomly selected code numbers, were sent to each laboratory for total serum cholesterol determinations. This portion of the design of the experiment was unknown to the director of either laboratory.

In our own laboratory, Laboratory Number One (Biochemistry Laboratory of the Department of Pathology, Louisiana State University), total serum cholesterol was determined by the method of Sperry with the following modifications. Saponification was allowed to proceed for two hours at 37°C; the alkali was neutralized to phenolphthalein end point with 10% acetic acid; and all solutions were made slightly acid by the addition of one drop of acetic acid. The solutions of cholesterol standards, made from cp cholesterol obtained from Pfanstiehl Laboratories, Inc., were introduced into the procedure at this stage and these were also made acid by the addition of one drop of acetic acid. The digitonin solution was made by dissolving five grams of digitonin in one liter of 1:1 (v/v) water-absolute ethanol. All of these determinations were performed by the same technologist.

In Laboratory Number Two, a ferric chloride method for cholesterol determination was used.

Results

FIRST IMPRESSIONS

The data from the experiment are shown in Table 1. Preliminary inspection of the table indicates the following:

1. Substantial measurement variation is present, as demonstrated by differences in duplicate determinations by a specific laboratory on serum from one drawing of blood from a single animal. (For example, observe the data on baboon number 269 for 3/27/62.)

2. Laboratory Two obtained substantially lower values than Laboratory One.

3. Differences among animals are larger than any of the other differences. Further-

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Day and laboratory</th>
<th>Estimated animal mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-27-62 Laboratory</td>
<td>123.4</td>
</tr>
<tr>
<td></td>
<td>3-28-62 Laboratory</td>
<td>119.3</td>
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<tr>
<td></td>
<td>3-29-62 Laboratory</td>
<td>130.0</td>
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<tr>
<td></td>
<td>3-30-62 Laboratory</td>
<td>118.0</td>
</tr>
</tbody>
</table>

Note. See text for selected measures of variability; other appropriate measures could be computed.
CHOLESTEROL IN BABOONS

TABLE 2
Analysis of Variance of Total Serum Cholesterol in Baboons

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main effects:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animals</td>
<td>7</td>
<td>49,517.94</td>
<td>7,073.99</td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td>3</td>
<td>2,117.91</td>
<td>705.97</td>
<td>2.25</td>
</tr>
<tr>
<td>Laboratories</td>
<td>1</td>
<td>6,888.44</td>
<td>6,888.44</td>
<td>5.14**</td>
</tr>
<tr>
<td>Interaction effects:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animals with days</td>
<td>21</td>
<td>6,592.65</td>
<td>313.94</td>
<td>9.35**</td>
</tr>
<tr>
<td>Days with laboratories</td>
<td>3</td>
<td>2,740.09</td>
<td>913.36</td>
<td>10.69**</td>
</tr>
<tr>
<td>Animals with laboratories</td>
<td>7</td>
<td>964.99</td>
<td>137.86</td>
<td>2.26*</td>
</tr>
<tr>
<td>Second order interaction</td>
<td>21</td>
<td>1,794.73</td>
<td>85.46</td>
<td>1.40</td>
</tr>
<tr>
<td>Technical variation</td>
<td>64</td>
<td>3,907.50</td>
<td>61.05</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>137</td>
<td>74,524.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Denotes significance at 0.05 level.
** Denotes significance at 0.01 level.

more, there is considerable consistency for each animal throughout the four-day period.

THREE FACTOR FACTORIAL

This experiment included all combinations of the three factors, animals, laboratories, and days, and was designed to be analyzed as a three-factor factorial arrangement of treatments with days and laboratories as fixed factors and the eight animals regarded tentatively as a random sample representing the supply of experimental animals available.

ANALYSIS OF VARIANCE (ANOVA)

The results of the analysis of variance computations are shown in table 2. These results corroborate first impressions. The mean square for error (MSE), technical variation, is 61.05; since the square root of 61.05 is about 8, this indicates a precision of approximately ±16 (i.e., ±2 \( \sqrt{\text{MSE}} \)) for a single laboratory determination. Entries in the table emphasize the large animal differences, large differences between the two laboratories, and little tendency for determinations on serum from blood drawn on different days over a short period (diet held constant) to differ. In addition, the analysis of variance table indicates that the overall average differences, among animals, laboratories, and days do not explain all of the variation in the data. There are significant interactions among the factors.

Significant Interaction

It is difficult to avoid criticism of the summary of results when interactions are large. In view of significant interaction probably the next step should be to make a transformation of the data or examine differences among the means of the two-factor treatment combinations. One could start by observing, for example, that laboratory differences are consistent from day to day, or from animal to animal, but animal differences are not completely stable over days. However, in the remaining part of this ANOVA, these large interactions will be considered merely as warning that results for a specific animal-day-laboratory cannot be predicted reliably from the main effects.

Animals

Animal means ranged from a low of 108.3 to a high of 169.2. When the variance among animal means is computed directly from the eight means, the value of 442.58 is obtained. However, when variation due to measurement is excluded, the estimated variance of animal means is 438.31. This implies a range of biological variation of about ±42 (±2 \( \text{sd} \) or ±2 \( \sqrt{\text{MSE}} \)) for the central 95% of animals.

Laboratories

In examining the differences between laboratories it is of interest to estimate the technical variation for each laboratory separately. The technical mean squares for the two laboratories are 50.73 and 71.38, indicating precisions for individual determinations.
of ± 14 (± 2 \times \text{square root of the mean square for error, } \text{MSE}) \) and ±17 ( ± 2 \sqrt{\text{MSE}}) for Laboratory One and Laboratory Two respectively. Even though the difference between laboratories depends significantly on the day that the blood was drawn as well as upon the particular animal, these differences are usually in the direction of lower values for Laboratory Two. The difference in overall average is 14.7 (137.0 — 122.3).

**Days**

The variation between determinations on serum from blood samples taken on different days is puzzling because the relationship among overall averages for the days (main effects) is not consistently found in the two laboratories when viewed separately; the interaction effects are as large as the overall day effects. This suggests that the differences are due to measurement variation in the laboratories rather than to fluctuation from day to day in the serum cholesterol levels of the animals themselves. The laboratories were not instructed to process the samples in any particular order; and since Laboratory Two received all of the samples of serum in a single lot while Laboratory One processed the samples in small groups, this finding is not surprising "after the fact." Nevertheless, a second experiment was conducted to obtain an estimate of the magnitude of variation due to “laboratory processing day” or “run.”

**TEST OF DAY TO DAY LABORATORY VARIABILITY**

Serum samples from four different baboons were divided into twelve aliquots each, and each of the 48 aliquots was coded with a randomly assigned two-digit number. Duplicate samples, identified only by a code number, from the serum of the four animals were submitted to each of the two laboratories on three different days. The eight samples submitted on a given day (duplicate samples from the four animals) were analyzed on the same day by the methods for cholesterol previously described. Thus the experiment was a test of interlaboratory and intralaboratory variability of measurement of serum cholesterol, determinations being made on the identical serum from each animal over a period of three laboratory work days (runs). In other words, variability of serum cholesterol with time within the animals was eliminated by using the same serum. Performing the laboratory determinations on the same serum on different days yielded information on laboratory variability with time.

Data from the second experiment are presented in table 3. Again, estimates of technical variation for the two laboratories were computed. The previous estimates were reduced

**TABLE 3**

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Day 1 Laboratory</th>
<th>Day 2 Laboratory</th>
<th>Day 3 Laboratory</th>
<th>Estimated animal mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>184</td>
<td>113</td>
<td>118</td>
<td>109</td>
<td>105</td>
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<td>115</td>
<td>108</td>
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<td>185</td>
<td>113</td>
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<td>117</td>
<td>114</td>
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<td>112</td>
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<td>584</td>
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<td>586</td>
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<tr>
<td>136</td>
<td>135</td>
<td>134</td>
<td>131</td>
<td>136</td>
</tr>
<tr>
<td>Estimated mean</td>
<td>122.0</td>
<td>120.2</td>
<td>119.8</td>
<td>120.8</td>
</tr>
</tbody>
</table>

Note. See text for selected measures of variability; other appropriate measures could be computed.
by a factor greater than 2; the estimates of precision for individual determinations were
± 7 (± 2 √MSE) and ± 6 for Laboratories One and Two respectively. The day differences were found again, being statistically significant for Laboratory Two but not for Laboratory One. For this experiment, however, these day differences represent laboratory measurement variation only, since the samples of serum processed on the separate days are sample aliquots from the same animal pools of serum.

Discussion
This discussion is limited to implications of this study (and other published results) for conducting experiments in which serum cholesterol in baboons is measured. Perhaps many of the points would also apply to experiments involving the measurement of serum cholesterol in other animals.

1. The greatest variability seems to be associated with individual animals. It would seem wise to make a preliminary study of each group of animals to determine a baseline total serum cholesterol value for each animal.

2. Serum from each animal should be taken at the start of an experiment and stored so that simultaneous determinations could be made for comparison with samples taken midway through the experiment and at the termination of the experiment. This procedure should detect differences due to laboratory changes during the course of the experiment.

3. In order to increase precision in the estimates of individual cholesterol levels, determinations should be made on serum from blood collected at regular intervals throughout the experiment.

4. Animals should be blocked into groups that are relatively homogeneous in serum cholesterol level before they are assigned to the experiment. If possible, animals should be homogeneous as to sex and age. Obviously, the practice of assigning animals within homogeneous blocks to treatment using a randomizing device is necessary.

5. Blood should be drawn from all animals in a block on the same day and at approximately the same time of day. Time since last meal and emotional disturbance should be kept constant.

6. There is strong evidence that one laboratory obtains higher values than the other. Determinations should be made in a single laboratory, or else special care must be exercised to avoid confounding laboratory differences with experimental differences. In comparing results with other laboratories it will be necessary to investigate differences between laboratories.

7. An investigation of the sources of technical variation within the laboratory used for the experiment seems worthwhile. In most of the data gathered thus far, decreases both in between-day variation and in the within-day technical variation would be desirable. Until the technical variation can be materially reduced, blind duplicate determinations on each sample of serum should be obtained.

8. In view of the between-day variation in the laboratories, serum from blood drawn from a block at a specified time should be processed in a single laboratory run. Control specimens from a stored pool should be submitted with each batch of specimens processed; identification regarding control or experimental specimens should not be available to the laboratory.

9. The number of animals per treatment should be determined using the estimates of biological and technical variation available in combination with the specifications of the magnitudes of dietary effects of interest. If three factors (for example, cholesterol level, fat saturation, and protein level) are to be investigated, eight treatment combinations (each factor at high and low levels) could be studied simultaneously. Using four animals to a treatment group, a total of 32 animals, any treatment that changed the serum cholesterol level by as much as 20 milligrams or more per 100 milliliters of serum would almost certainly be detected, if the biological variation and technical variation were of the order observed in the pilot experiments described above.
Summary

Variability of serum cholesterol in baboons was studied in order to obtain estimates of biological and technical variation for use in planning a long term dietary experiment. Variability due to animals, to laboratories, to the day on which blood was drawn, and to laboratory work day was estimated. Implications for the design of experiments involving serum cholesterol in baboons are discussed.

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