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STUDIES OF EFFECTS OF CLOSED MICROBIAL ECOLOGY

Report of 180-Day Test Period

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Submitted by

Alan J. Kenyon, D.V.M., Ph.D.
Professor of Biochemistry
Department of Pathobiology
University of Connecticut,
Storrs, Connecticut 06268

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I. INTRODUCTION

The concept of microbial simplification or shock resulting from restricted environmental exchange of flora has been considered a potential risk to crew members during extended space flight. To investigate and establish whether, in fact, microbial modification could be induced by placing carnivores in a closed microbial system, the following study was conducted with ferrets (<u>Mustela putorius</u>).

II. EXPERIMENTAL DESIGN

A. Selection of Ferrets

1. General Parameters

The rationale for selection, as described below, was intended to provide small, healthy, young ferrets which are immunologically responsive and free from latent lymphoproliferative diseases or processes which will complicate appraisal of host responsiveness to microbial environment.

The ferrets used in the Open (O Group) and Closed (C Group) environmental systems were dark color phase females, 4 to 6 months old, and weighing between 400 and 600 grams. Each animal was examined for clinical signs of disease or conformational abnormalities.

2. Immunologic Criteria

Ferrets selected shall have <u>gamma</u> globulin levels of 3 to 15% of total serum protein as determined by immunoelectrophoresis on cellulose acetate (Microzone Systems, Beckman).

3. Clinical Chemistry and Hematology

Clinical chemistry and hematology was performed initially to select ferrets and at each of the three test period kill days.

B. Equipment Used

1. Open Environment Control

The 36 ferrets in the O Group were housed in a 20' x 24' room. The air flow will be maintained by forced air ventilation.

2. Test Chambers

The C Group ferrets were housed in three, 6-feet (6 MP) and one, 4-feet (4 MP) rigid isolators modified with inside glove-port lids to prevent damage to gloves in the event of an escape of caged ferrets. Each isolator had a germicidal entry port (peracetic acid) and a submersion (iodine) dip tank. Both air intake and exit ports were equipped with absolute filters. The isolators were constructed of 1/4 inch mold acrylic Plexiglas with 2 sets of glove ports on the "working side" and one set near the gas entry port on the back side. Air flow was 6 chamber volumes per hour (velometer). Ferrets were housed singly in soft plastic isolators.

3. Anaerobic Culture Chamber

The anaerobic chamber, shown in Preliminary Protocol 1 (manufactured by Lab-Line Instruments, Chicago, Illinois) was used. Anaerobic culture conditions are produced with Gaspak disposable hydrogen-carbon dioxide generators (manufactured by Baltimore Biological Laboratory, Baltimore, Maryland).

4. Cages

The ferrets in the O and C Groups were contained separately in cages 14" x 12" x 11", constructed of 10-gauge galvanized hardware cloth. Urine and feces collecting trays were enamel pans from which samples were routinely removed for analyses and which could be cleaned by swabbing. At weekly intervals, pans were exchanged for sterilized containers brought through the gas entry port. Soiled pans were placed in plastic bags and removed through the iodine dip tank.

C. Diets

Until the ferrets were distributed into the study groups they were maintained on the test diet. The test diet was a commercially prepared cat ration with the following analysis:[1]

Crude protein	10.0%
Crude fat	3.0%
Carbohydrate	6.8%
Crude fiber	1.5%
Ash	3.5%
Calcium	0.4%
Phosphorus	0.3%
Salt	0.5%
Moisture	74.0%

Each animal was fed sterile water *ad libitum*. The solid ration was tested for sterility within each lot, and then introduced into the test

[1] Calo cat food, Borden, Inc., Oakland, California 94623.

chamber with peracetic acid treatment of the container's exterior in the gas entry port. The amount fed to each ferret was in part based on amount readily consumed at an estimated level of 50 to 100 grams per day.

D. Test System

1. Experimental Design

The general experimental plan, showing the scheme for culturing saliva, axillary skin, and feces, in described in Table 1. At days 60, 120, and 180, 12 ferrets were selected from each Group for intensive study. At days 20, 80, and 140, saliva was cultured; on days 40, 100, and 160, axillary skin was cultured.

2. Evaluation of Microbiota

a. Feces

The procedure for quantitation of fecal flora is that described by Floch et al. (Methods for the Quantitative Study of the Aerobic and Anaerobic Intestinal Bacterial Flora of Man. Yale J. Biol. Med., 1968, 41: 50). The method, as applied to ferrets was performed as follows so as to detect a trend relative to the exposure of the study groups.

(1) Fecal collection: On day 60, 36 ferrets were to be observed for the passage of stools. On day 120, 24 fecal samples were to be collected, and on day 180, 12 fecal samples were to be taken. The freshly passed fecal material was placed in a dish within the isolator for the Closed Group and with both groups cultured within 15 to 30 minutes after collection.

(2) Dilution of fecal sample: The entire voided fecal material was weighed and a 1% (weight-volume) fecal suspension was made with distilled water, purified on norite A charcoal. The contents were mixed by vigorous shaking (mechanical shaker) for 15 minutes, then serially diluted by adding 1.0 ml. of dispersed stool to 9.0 ml. of charcoal water and so on until at least 9 tubes are prepared. The first tube prior to serial dilution was a 1:100 (10^{-2}) and the last tube represented a 1:10,000,000,000 (10^{-10}).

(3) Procedure for streaking: A loopful of each diluted specimen was streaked on solid media (loop volume is approximately 0.01 ml. of fluid). The number of organisms was based on number of colonies per dilution and was that dilution multiplied times 10^{-2}, i.e., if the last tube had 5 colonies it was expressed as $5 \times 10^{-2} \times 10^{-10}$ or 5×10^{-12} or its common logarithmic number which represents the number of bacteria per gram of feces.

(4) Media: Seven selected media were used to determine ecologic trends relative to each environment. Formulas are given in Appendix I.

Medium A. Streaked for total counts (dilutions 10^{-6} to 10^{-10}) with 2 plates per dilution (1 incubated anaerobically and the other aerobically). (Schaedler *et al.*, J. Exptl. Med., 1965, 122: 59.)

Medium C. Streaked and incubated anaerobically for *Bacteroides* spp. and *Clostridium* spp.

Rogosa medium. Streaked and incubated anaerobically for *Lactobacillus* spp. (one plate/dilution in candle jar for microaerophilic species).

Medium M. Streaked and incubated anaerobically for gram-positive cocci (Slanetz and Bartley, J. Bacteriol., 1957, 74: 591).

Medium E (Tergitol 7). Streaked and incubated aerobically for coliforms, *Shigella*, and *Salmonella* spp.

Medium PEA. Streaked and incubated aerobically for *Streptococcus* and *Staphylococcus* spp.

Medium XLD. Streaked and incubated aerobically for *Shigella* and *Salmonella* spp.

(5) Incubation: Incubation was conducted in a standard 37 C. incubator. For microaerophilic organisms a candle jar was used and for strict anaerobic organisms the BBL Gaspak was used in the anaerobic chamber.

(6) Interpretation. The interpretations of colony growth were based on gram staining and microscopic appearance of the morphology.

b. Saliva

Saliva was aspirated from ferrets' mouths by allowing the animals to bite on a stainless steel canula equipped with bulb syringe. The culture system described above was applied to saliva (0.1 ml. saliva instead of 0.1 gm. feces) and diluted through 10^{-6} with charcoal distilled water.

c. Axillary Skin

The axillary region of ferrets was swabbed after the guard hairs had been clipped off with a scissors. Swabs were placed in thioglycollate broth cultures and smears were made on Medium A and incubated both aerobically and anaerobically.

d. Intestinal Tract

Upon postmortem, the major segments of the intestinal tract were clamped with hemostats and a portion of the contents of each segment were removed for quantitative study of the flora. The procedure for culturing was similar to that described for feces.

3. Evaluation of Physiologic Status of Gut

a. Intestinal Motility

Intestinal motility was determined by administering 2.0 ml. of India ink at time intervals to allow 1, 2, 3, and 4 hours to elapse before each of the test ferrets were killed. The distribution of dye in the isolated portions of the gut indicated the motility.

b. Lumen Volume

The lumen volume was measured from the pyloric valve to the rectum. This was done by draining the fluid content, then reverse flushing so as to exclude trapped gas. The amount of fluid was measured and recorded.

c. Assimilation Rates

Assimilation rates were established by the absorption of labeled amino acid. For this purpose the ileo-cecal junction and colon were ligated to produce a sac from which tritiated ^{14}C UL glycine (20 μc/ferret) was absorbed. The duration of the experiment was 60 minutes. The ferrets were bled by caudal amputation at 15-minute intervals to determine the relative amount of isotope (level determined by liquid scintillation counter) absorbed by both groups.

d. Intestinal Weights and Contents

Total weight of contents, dry weight of contents, weight of duodenum, ileum, and colon wall were made at appropriate times to evaluate the physiologic status of the intestinal tract.

4. Quantitation of Serum Enzymes

At days 60, 120, and 180, blood was collected by caudal amputation and by cardiac puncture. Serum from caudally obtained blood was used for enzyme analyses. The values obtained at these periods were compared with pre-test values obtained on each ferret.

D. Analyses of Data

Where possible, statistical analyses were made of data collected with the assistance of the University of Connecticut's biostatistician. Interpretation of histologic data was made withassistance of veterinary anatomists and veterinary pathologists.

The experimental design was constructed so that analyses promptly made permitted close observation in the event of a progressive change in the microflora of the test group.

III. RESULTS

A. Microbiology

Quantitative microbial analyses were made on 7 selective media previously described. On days 0, 60, 120, and 180, 4 dilutions of fecal samples from individual ferrets were cultured. Also on days 0 and 180, 4 dilutions of samples from the duodenum, ileum, and colon were also cultured on selective media. Saliva and axillary skin were cultured at intervals to determine the types and distribution of flora.

1. Fecal Analyses

a. Total Aerobes

The bacterial counts of fecal samples of ferrets in the Open and

Closed Groups fluctuated from 1.1×10^{9} total aerobes at day 0 to 4.5×10^{10} (Open) and 6.8×10^{10} for the Closed Group at 60 days. At 120 days the average count dropped to 3.4×10^{9} for the Open Group and 2.3×10^{9} for the Closed Group. By 180 days the average count increased to 1.5×10^{10} for the Open Group and 1.8×10^{10} for the Closed Group. See Tables 2-9; Figure 1. Tables 3-9 contain the range and averages for day 0, 60, 120, and 180 days for Open and Closed Groups.

b. Total Anaerobes

The average number of total anaerobes showed a slight increase from day 0 to day 60 in both groups, a 3 log decrease from day 60 to day 120 in both groups, and a 2 log increase from day 120 to day 180 in both groups. These counts were 2.1×10^{10} at day 0, 1.1×10^{11} at day 60, 9.0×10^{7} at day 120, and 3.2 $\times 10^{9}$ at day 180 for the Open Group. The value for the Closed Group at day 60 was also 1.1×10^{11} while at day 120 it was 1.8×10^{8}, and at day 180 it was 8.1×10^{9}. See Tables 2-9; Figure 2.

c. Bacteroides

In some instances a few ferrets did not have sufficient flora to fall within the dilution range (dilutions selected on the basis of the previous chamber experiment), therefore each class of organism will be described on the basis of percentage of animals possessing detectable levels at the lowest dilution tested.

The percentage of animals with detectable Bacteroides increased from 25% on day 0 to 90.5% on day 60 in the Open Group and 87.5% in the Closed Group. By day 120, 100% of the animals in both groups showed Bacteroides. On day 180, 100% of the animals in the Open Group showed Bacteroides, while 85.7% in the Closed Group did.

The average number of Bacteroides increased 3 logs from day 0 to day 60 in the Open Group and 1.5 logs in the Closed Group. At day 120 the Open Group showed an additional 0.5 log increase and the Closed Group showed an additional increase of 1.5 logs. By day 180 the Open Group showed another 0.5 log increase and the Closed Group showed an increase of 1 log. See Tables 2-7; Figure 3.

d. Coliforms

The percentage of animals with detectable coliforms increased from 91.7% at day 0 to 100% at day 60 and remained at 100% at day 120 and day 180 in both groups.

The average number of coliforms dropped 0.5 logs in the Open Group from day 0 to day 60 and dropped 1 log in the Closed Group. From day 60 to day 120 both groups decreased 1 log. From day 120 to day 180 the Open Group increased 1 log while the Closed Group increased 1.5 logs. See Tables 2-9.

e. Aerobic Lactobacillus

The percentage of animals with detectable aerobic Lactobacillus dropped from 75% at day 0 to 0% at day 60 in both groups and remained at this level at day 120. Due to testing at a lower dilution at day 180 both groups showed 100% aerobic Lactobacillus.

The average number of aerobic Lactobacillus dropped at least 3 logs from day 0 to day 60 in both groups. At day 120, although a still lower dilution was tested (10^{-5} dilution with a range of 10^{-8}), there wereno detectable colonies in either group. At day 180 there was at least a 2 log increase in both groups (lowest dilution tested was 10^{-3}). See Tables 2-9; Figure 5.

f. Anaerobic Lactobacillus

The anaerobic Lactobacillus clearly reflected the change in the gastrointestinal physiology induced by the hemorrhagic gastritis (HG) agent. An alteration in gastric secretion resulted in a change in pH in the intestinal tract which is reflected by this population of organisms. The apparent leveling off between day 120 and 180 is no doubt due to the loss of virulence of the HG agent.

The percentage of animals with detectable Lactobacillus decreased from 91.7% at day 0 to 85% at day 60 in the Open Group and 62.5% in the Closed Group. By day 120 the percentage of animals with anaerobic Lactobacillus had decreased further to 63% in the Open Group and 13% in the Closed Group. By day 180 both groups showed 100% because a 2 log lower dilution was tested.

The average number of anaerobic Lactobacillus decreased 0.5 logs in both groups from day 0 to day 60. By day 120 both groups had decreased an additional 3 logs with a slightly greater decrease in the Closed Group. By day 180 the Open Group increased 0.5 logs while the Closed Group decreased 1 log. See Tables 2-9; Figure 6.

g. Aerobic Streptococcus

The percentage of animals with detectable aerobic Streptococcus decreased from 100% at day 0 to 95.2% at day 60 in the Open Group and 93.8% in the Closed Group. On day 120, 100% of the animals in the Open Group again showed aerobic Streptococcus while in the Closed Group there was a slight decrease to 88%. By day 180 both groups showed 100%.

The average number of aerobic Streptococcus remained constant in the Open Group from day 0 to day 60 while the Closed Group showed a 0.5 log increase. By day 120 both groups showed a 2 log decrease. By day 180 the Open Group showed a 1 log increase while the Closed Group showed a 0.5 log increase. See Tables 2-9; Figure 7.

h. Anaerobic Streptococcus

The percentage of animals with detectable anaerobic Streptococcus decreased from 100% at day 0 to 95.2% at day 60 in the Open Group and 93.8% in the Closed Group. On day 120 both groups had decreased to 75%. By day 180 the Open Group increased to 87.5% while the Closed Group increased to 100%.

The average number of anaerobic Streptococcus showed a slight increase from day 0 to day 60 in the Open Group and a 1 log decrease in the Closed Group. On day 120 the Open Group showed a 3 log decrease and the Closed Group a 2 log decrease. On day 180 the Open Group remained constant while the Closed Group showed a 1 log increase. See Tables 2-9; Figure 8.

i. Shigella

The percentage of animals with detectable Shigella increased from 58.3% at day 0 to 95.2% at day 60 in the Open Group and 93.8% in the Closed Group. By day 120 only 38% of the animals in the Open Group showed Shigella while 100% of the animals in the Closed Group demonstrated Shigella. By day 180, 100% of the animals in both groups showed Shigella.

The average number of Shigella increased 2 logs from day 0 to day 60 in the Open Group and 3 logs in the Closed Group. By day 120 the Open Group decreased 1.5 logs and the Closed Group decreased 2.5 logs. By day 180 the Open Group increased 0.5 logs and the Closed Group increased 1.5 logs. See Tables 2-9; Figure 9.

j. Salmonella

The percentage of animals with detectable Salmonella increased from 0% at day 0 to 42.8% in the Open Group at day 60 and 6.3% in the Closed Group. By day 120 the Open Group decreased to 13% in the Closed Group to 0%. By day 180 the Open Group increased to 50% and the Closed Group to 14.3%.

The average number of Salmonella increased at least 3 logs in the Open Group from day 0 to day 60 and at least 1 log in the Closed Group. At day 120 the Open Group showed a 2 log decrease and the Closed Group at least a 1 log decrease. By day 180 the Open Group showed a 0.5 log increase and the Closed Group showed at least a 3 log increase. See Tables 2-9; Figure 10.

k. Individual Ferrets

Ferrets in both the Open and Closed Groups were assayed for fecal microbiota on day 60, day 120, and day 180. There were 8 animals in the Open Group and 7 in the Closed Group.

Total aerobes in the Open Group throughout the test period showed an increase in 3 animals and a decrease in 5 animals, while in the Closed Group 2 showed an increase and 5 animals showed a decrease. See Table 10.

Total anaerobes in the Open Group throughout the test period showed an increase in 1 animal and a decrease in 7, while in the Closed Group 2 animals showed an increase and 5 showed a decrease. See Table 11.

Bacteroides in the Open Group throughout the test period showed an increase in 6 animals and a decrease in 2, while in the Closed Group 6 showed an increase and 1 showed a decrease. See Table 12.

Coliforms in the Open Group throughout the test period showed an increase in 3 animals and a decrease in 5, while in the Closed Group 3 animals showed an increase and 4 showed a decrease. See Table 13.

Aerobic Lactobacillus in both the Open and Closed Groups were undetectable at the lowest dilution tested at days 60 and 120, but were demonstrable at day 180. See Table 14.

Anaerobic Lactobacillus in the Open Group throughout the test period showed an increase in 3 animals and a decrease in 5, while in the Closed Group 3 showed an increase and 4 a decrease. See Table 15.

Aerobic Streptococcus in the Open Group throughout the test period showed an increase in 5 animals and a decrease in 3, while in the Closed Group 1 showed an increase and 6 showed a decrease. See Table 16.

Anaerobic Streptococcus in the Open Group throughout the test period showed an increase in 1 animal and a decrease in 7, while in the Closed Group 1 showed an increase and 6 a decrease. See Table 17.

Shigella in the Open Group throughout the test period showed an increase in 1 animal and a decrease in 7, while in the Closed Group 5 animals showed an increase, 1 a decrease, and 1 remained constant. See Table 18.

Salmonella in the Open Group throughout the test period showed an increase in 2 animals, a decrease in 4 animals, and remained undetectable in 2 animals at the lowest dilution tested, while in the Closed Group 1 animal showed an increase and 6 remained undetectable at the lowest dilution tested. See Table 19.

2. Intestinal Analyses

a. Duodenum

Total aerobes in the Open Group increased from 5.1×10^6 at day 0 to 5.6×10^7 at day 180 and decreased in the Closed Group to 2.7×10^5.

Total anaerobes in the Open Group increased from 8×10^6 at day 0 to 2.1×10^7 at day 180 and decreased in the Closed Group to 5.3×10^4.

Bacteroides in the Open Group increased from 16×10^1 at day day 0 to 2.5×10^2 at day 180 and increased in the Closed Group to 1.3×10^3.

Coliforms in the Open Group decreased from 8×10^7 at day 0 to 3.9×10^5 at day 180 and decreased in the Closed Group to 1.1×10^4.

Aerobic Lactobacillus in the Open Group increased from 21×10^2 at day 0 to 6.8×10^3 at day 180 and increased in the Closed Group to 5.8×10^3.

Anaerobic Lactobacillus in the Open Group increased from 72×10^4 at day 0 to 5.2×10^7 at day 180 and decreased in the Closed Group to 3.3×10^3.

Aerobic Streptococcus in the Open Group increased from 7.8×10^6 at day 0 to 1.9×10^7 at day 180 and decreased in the Closed Group to 1.6×10^5.

Anaerobic Streptococcus in the Open Group increased from 29×10^4 at day 0 to 2.8×10^7 at day 180 and decreased in the Closed Group to 6.4×10^4.

Shigella in the Open Group increased from 0 at day 0 to 8.6×10^4 at day 180 and increased in the Closed Group to 3.4×10^5.

Salmonella in the Open Group decreased from 5×10^2 at day 0 to 0 at day 180 and decreased in the Closed Group to 0.

See Tables 20-23.

b. Ileum

Total aerobes in the Open Group increased from 12 x 10^6 at day 0 to 8.7 x 10^7 at day 180 and increased in the Closed Group to 4.8 x 10^7.

Total anaerobes in the Open Group increased from 53 x 10^5 at day 0 to 1.1 x 10^8 at day 180 and increased in the Closed Group to 9.3 x 10^6.

Bacteroides in the Open Group increased from 15 x 10^2 at day 0 to 2.3 x 10^5 at day 180 and increased in the Closed Group to 5.6 x 10^6.

Coliforms in the Open Group decreased from 5.6 x 10^7 at day 0 to 2.5 x 10^7 at day 180 and decreased in the Closed Group to 1.9 x 10^6.

Aerobic Lactobacillus in the Open Group increased from 36 x 10^2 at day 0 to 5.4 x 10^4 at day 180 and increased in the Closed Group to 4.3 x 10^4.

Anaerobic Lactobacillus in the Open Group increased from 58 x 10^5 at day 0 to 1.4 x 10^8 at day 180 and decreased in the Closed Group to 4.8 x 10^4.

Aerobic Streptococcus in the Open Group decreased from 4.2 x 10^7 at day 0 to 5.1 x 10^6 at day 180 and decreased in the Closed Group to 4.3 x 10^5.

Anaerobic Streptococcus in the Open Group increased from 6.6 x 10^6 at day 0 to 1.0 x 10^8 at day 180 and decreased in the Closed Group to 3.8 x 10^5.

Shigella in the Open Group increased from 73 x 10^3 at day 0 to 3.3 x 10^5 at day 180 and increased in the Closed Group to 1.7 x 10^5.

Salmonella in the Open Group decreased from 13 x 10^2 at day 0 to 0 at day 180 and decreased in the Closed Group to 0.

See Tables 24-27.

c. Colon

Total aerobes in the Open Group increased from 2.5 x 10^8 at day 0 to 9.9×10^8 at day 180 and increased in the Closed Group to 6.5×10^8.

Total anaerobes in the Open Group increased from 19×10^7 at day 0 to 1.2×10^9 at day 180 and increased in the Closed Group to 2.8×10^8.

Bacteroides in the Open Group increased from 12×10^5 at day 0 to 6.5×10^6 at day 180 and increased in the Closed Group to 3.6×10^7.

Coliforms in the Open Group decreased from 4.5×10^8 at day 0 to 2.6×10^8 at day 180 and decreased in the Closed Group to 1.1×10^8.

Aerobic Lactobacillus in the Open Group decreased from 4.5×10^4 at day 0 to 4.0×10^4 at day 180 and increased in the Closed Group to 9.5×10^4.

Anaerobic Lactobacillus in the Open Group increased from 5.3×10^7 at day 0 to 1.8×10^9 at day 180 and decreased in the Closed Group to 4.2×10^7.

Aerobic Streptococcus in the Open Group decreased from 3.2×10^8 at day 0 to 2.7×10^7 at day 180 and decreased in the Closed Group to 2.0×10^8.

Anaerobic Streptococcus in the Open Group increased from 8.4×10^8 at day 0 to 1.3×10^9 at day 180 and decreased in the Closed Group to 6.1×10^7.

Shigella in the Open Group increased from 2.0×10^6 at day 0 to 3.7×10^6 at day 180 and increased in the Closed Group to 7.5×10^6.

Salmonella in the Open Group decreased from 16×10^2 at day 0 to 0 at day 180 and decreased in the Closed Group to 0.

See Tables 28-31.

3. Saliva Analyses

As previously described, saliva analyses were directed at general identification of organisms rather than enumeration.

a. Day 0

Thioglycollate: slightly pungent odor.

Aerobic: 10 had coliforms; 2 had bacillus.

Anaerobic: 11 had Lactobacillus; 1 had Bacteroides

b. Day 20

Open Group: Thioglycollate: pungent odor.

Aerobic: 9 had Bacillus sp. and coliforms; 6 had coliforms; 2 had Bacillus sp.; and 1 had staphylococcus and coliforms.

Anaerobic: 14 had bacillus; and 4 had bacillus and coliforms.

Closed Group: Thioglycollate: pungent odor.

Aerobic: 4 had Bacillus sp. and coliforms; 2 had coliforms; 11 had Bacillus sp.; and 1 had staphylococcus and Bacillus sp.

Anaerobic: 15 had bacillus; 2 had coliforms; and 1 had staphylococcus and Bacillus sp.

c. Day 80

Open Group: Thioglycollate: very pungent odor.

Aerobic: 12 had coliforms; and 3 had Bacillus sp. and coliforms.

Anaerobic: 11 had coliforms; and 3 had Lactobacillus sp.

Closed Group: Thioglycollate: very pungent odor

Aerobic: 13 had coliforms; and 2 had Bacillus sp. and coliforms.

Anaerobic: 10 had coliforms; 1 had Lactobacillus sp. and coliforms; and 1 had Lactobacillus sp.

4. Axillary Skin

a. Day 0

Thioglycollate: slightly pungent odor.

Aerobic: 11 had coliforms; 1 had bacteroides.

Anaerobic: 12 had lactobacillus

b. Day 40

Open Group: Thioglycollate: pungent odor

Aerobic: 8 had Bacillus sp. and coliforms; and 1 had Bacillus sp.

Closed Group: Thioglycollate: pungent odor.

Aerobic: 9 had *Bacillus* sp. and coliforms; and 1 had *Bacillus* sp., coliforms, and *Staphylococcus* sp.

Anaerobic: 10 had *Lactobacillus* sp.

c. Day 100

Open Group: Thioglycollate: very pungent odor.

Aerobic: 1 had coliforms; and 8 had *Staphylococcus* sp. and coliforms.

Closed Group: Thioglycollate: very pungent odor.

Aerobic: 2 had coliforms; 6 had *Staphylococcus* sp. and coliforms; 2 had *Staphylococcus* sp. and *Bacillus* sp.; and 1 had *Bacillus* sp.

Anaerobic: 2 had *Lactobacillus* sp.; and 9 had *Lactobacillus* sp. and *Staphylococcus* sp.

B. Physiology

On days 0 and 180, the following physiologic parameters were observed: Body temperature, intestinal motility, intestinal lumen volume, content weight, wall weight, assimilation rate, hematocrit, red and white blood cell counts, differential blood count, and protein balance and distribution.

1. Body Temperature

On day 0 the average body temperature was 102.6 F. On day 60 the average temperature was 102.3 F. in the Open Group and 103.0 F. in the Closed Group. By day 120 the average body temperature in the Open Group was 102.7 F., while in the Closed Group it was 103.5 F. On day 180 the average temperature in the Open Group was 103.5 F. and 103.6 F. in the Closed Group. See Figure 11.

2. Gut Motility

While the gut motility was 0.418 inches per minute at day 0, by day 180 it had increased to 0.695 inches per minute in the Open Group and 0.549 inches per minute in the Closed Group. See Figure 12.

3. Gut Volume

a. Duodenum

The volume of the duodenum increased from 2.3 ml. at day 0 to 2.8 ml. in the Open Group at day 180 and 2.9 ml. in the Closed Group.

b. Ileum

The volume of the ileum increased from 23.7 ml. at day 0 to 27.5 ml. in the Open Group at day 180 and decreased to 22.7 ml. in the Closed Group.

c. Colon

The volume of the colon remained at 7.2 ml. from day 0 to day 180 in the Open Group but decreased to 6.7 ml. in the Closed Group.

d. Total Volume

The average total volume of the gut increased from 33.2 ml. at day 0 to 37.5 ml. in the Open Group at day 180 and decreased slightly to 32.3 ml. in the Closed Group. See Table 32.

4. Weight of Gut Contents

a. Duodenum

At day 0 the wet weight was 1.21 gm., the dry weight 0.17 gm., which calculated to 77% moisture. In the Open Group at day 180 the wet weight was 0.61 gm., the dry weight 0.11 gm., with 82% moisture. In the Closed Group at day 180 the wet weight was 0.55 gm., the dry weight was 0.11 gm. with 80% moisture. See Figures 13 and 14.

b. Ileum

At day 0 the wet weight was 7.38 gm., the dry weight 1.28 gm., which calculated to 81% moisture. In the Open Group the wet weight was 3.84 gm., the dry weight was 0.63 gm., with 83% moisture. In the Closed Group at day 180 the wet weight was 1.00 gm., the dry weight 0.21 gm., with 78% moisture. See Figures 15 and 16.

c. Colon

At day 0 the wet weight was 3.00 gm., the dry weight was 0.53 gm., which calculated to 79% moisture. In the Open Group at day 180 the wet weight was 3.51 gm., the dry weight 0.64 gm., with 82% moisture. In the Closed Group at day 180 the wet weight was 0.35 gm., the dry weight 0.08 gm. with 77% moisture. See Figures 17 and 18

5. Weight of Gut Wall

a. Duodenum

On day 0 the average standard unit of measurement was 12.3 mg. At day 180 the Open Group had decreased to 9.5 mg., while the Closed Group had decreased to 9.0 mg. See Figure 19.

b. Ileum

On day 0 the wall weight of the ileum was 12.6 mg. At day 180 the Open Group had decreased to 7.7 mg., while the Closed Group had decreased to 9.5 mg. See Figure 20.

c. Colon

On day 0 the wall weight of the colon was 11.0 mg. At day 180 there was only a slight decrease to 10.7 mg. in the Open Group and 10.9 mg. in the Closed Group. See Figure 21.

6. Assimilation

The assimilation of C^{14} glycine from the colon at day 0 reached a maximum of 2,300 cpm at 60 minutes postadministration. At day 180 a peak was reached at 10 minutes postadministration, however, the level for the Open Group was 1,800 cpm while that for the Closed Group was only 600 cpm. See Figures 22 and 23.

C. Blood

1. Hematology

a. Hematocrit

The average hematocrit was 40% at day 0. By day 60 the hematocrit had increased to 52% in the Open Group and 49% in the Closed Group. At day 120 it was 53% in the Open Group and 50% in the Closed Group. By day 180 the hematocrit in the Open Group was 48% and in the Closed Group 49%. See Figure 24.

b. White Blood Cell Count

The average white blood cell count was 5,600 cells per mm^3. at day 0. At day 180 the Open Group was 12,000 while the Closed Group was 6,900.

c. Red Blood Cell Count

The average red blood cell count was 6.6×10^6 cells per mm^3. at day 0. At day 180 the Open Group was 8.9×10^6, while the Closed Group was 8.5×10^6 cells.

d. Differential Count

Differential blood counts showed a 20% increase in lymphocytes from day 0 to day 180 in both groups, and a concomitant 20% decrease in neutrophils. See Table 33.

2. Serology

a. Gamma Globulin

While the gamma globulin level was only 5% of total serum protein at day 0, it had increased to 11.3% in the Open Group and 13.8% in the Closed Group by day 60. By day 120 the Open Group had changed very little, with an average of 10.9%, while the Closed Group had increased to 21.3% with one individual having a gamma globulin level of 47.9%. By day 180 the Open Group averaged 10.2% and the Closed Group 20.7%. Animal No. 46 was 47.9% at day 120 and had decreased to 26.8% at day 180, while Animal No. 43 was 22.1% at day 120 and had increased to 43.7% at day 180. See Figure 25.

b. Total Serum Protein

The total serum protein concentration at day 0 was 4.7%. By day 60 the Open Group had increased to 7.7% and the Closed Group to 7.6%. By day 120 the Open Group had a average percent protein of 6.8 while the Closed Group was 8.1%. On day 180 the Open Group had an average percent protein of 7.7 while the Closed Group was 8.1. Although the 4.7% day 0 value is a low normal, 8.1% is above the upper normal and may indicate an uncompensated dehydration. See Figure 26.

3. Serum Enzymology

a. Serum Lactate Dehydrogenase (LDH)

On day 0 the average serum LDH was 1,128 spectrophotometric units. The Open Group had an average LDH of 837 and the Closed Group 1,862 at day 60. The 2-fold increase in LDH of the Closed Group over than of the Open Group may reflect a greater morbidity due to the hemorrhagic gastritis (HG) agent or the early induction phase of a lymphoproliferative response as indicated by the hypergammaglobulinemia described in the previous section. By day 120 the average serum LDH was 1,471 spectrophotometric units in the Open Group and 1,543 in the Closed Group. On day 180 the average serum LDH was 2,522 spectrophotometric units in the Open Group and 2,681 in the Closed Group. See Figure 27.

b. Serum Alkaline Phosphatase

The average serum alkaline phosphatase was 92 spectrophotometric units at day 0. The day 60 values were 68 for the Open Group and 89 for the Closed Group. By day 120 the Open Group had an average alkaline phosphatase of 73 and the Closed Group had an average of 75. By day 180 the Open Group had decreased to an average of 32 and the Closed Group to 42. See Figure 28.

c. Serum Glutamic Oxalo-acetic Transaminase (SGOT)

At day 0 the average SGOT was 75 spectrophotometric units. By day 60 the Open Group had an average SGOT of 159, while the Closed Group was 271. At day 120 the Open Group average SGOT was 125 and the Closed Group was 177. By day 180 the average SGOT for the Open Group was 204 and the Closed Group 229 spectrophotometric units. See Figure 29.

D. Organs

1. Weights

a. Body Weight

The average body weight increased from 450 gm. at day 0 to 777 gm. at day 180 in the Open Group and 696 gm. in the Closed Group. The 180 day values are comparable to the final values of the 90-day Chamber Study. The day 0 values are lower because the animals were somewhat younger at the onset of the 180-day Chamber Study.

b. Liver

Average liver weight, expressed as percent of body weight, decreased only slightly in both groups from 3.93% at day 0 to 3.67% at day 180 in the Open Group and 3.42% in the Closed Group.

c. Spleen

The spleen increased from 0.37% of body weight at day 0 to 0.54% in the Open Group at day 180 and 0.60% in the Closed Group.

d. Kidney

The kidney decreased slightly from 0.35% at day 0 to 0.24% at day 180 in the Open Group and 0.28% in the Closed Group.

e. Thymus

The thymus showed a 2- to 3-fold increase in both groups from 0.22% at day 0 to 0.64% at day 180 in the Open Group and 0.60% in the Closed Group.

See Table 34.

E. Hemorrhagic Gastritis (HG)

With the onset of death due to hemorrhagic gastritis (HG) in the 180 day Chamber Study, the possibility of a dietary deficiency being the cause was considered. It was postulated that autoclaving of the canned Calo cat food for 15 minutes at 15 pounds pressure to guarantee 100% sterility might have destroyed some of the more heat labile nutritive vitamins. However, this possibility did not seem likely since the animals in the previous 90-day Chamber Study had been fed autoclaved cat food for the duration of the experiment without showing signs of HG. Nevertheless, randonly selected can were sent out to an independent laboratory for analysis.

Two cans of autoclaved cat food and two unautoclaved cans of cat food were assayed for gross composition by the Connecticut Agricultural Experiment Station (123 Huntington Street, Box 1106, New Haven, Conn. 06504). The determined amounts conformed to the label description except for the crude fat which was somewhat lower than indicated by the manufacturer in both the autoclaved and unautoclaved samples (Table 35).

Two other cans, one autoclaved and one unautoclaved, were sent to Food and Drug Research Laboratories, Inc. (Maurice Avenue at 58th Street, Maspeth 78, New York City) for thiamine assay. There was an insignificant difference of 0.014 mg. thiamine per 100 gm. cat food for the autoclaved cat food as compared to 0.017 mg. for the unautoclaved sample (Appendix II).

Early in the course of the epidemic all animals were given an injection of B-vitamin complex which appeared to have no effect on the disease progression. Since a dietary deficiency appeared not to be an etiologic factor, investigations were initiated to detect a possible bacterial or viral agent.

1. Clinical Disease

On day 22 ferrets began to die of what we have described as HG. The animals initially were listless and emaciated and showed marked anorexia 3 to 4 days prior to death with tarry stools apparent 1 to 2 days before death. At the time of death there was a 50% weight loss with extreme dehydration. Gross postmortem revealed blood in the lumen with petechial hemorrhages of the gastric mucosa as well as marked thymic and splenic atrophy (Table 36).

2. Mortality

The first animal to die was in the Closed Group at day 22. Three days later the first animal in the Open Group died. The animals in the Closed Group reached 50% mortality by day 60 while the animals in the Open Group did not reach 50% until day 80. From day 80 to day 120 the mortality in both groups steadily increased to 80% and leveled off at that time. The last death which occurred due to HG was on day 126 (Figure 30).

3. Organ Weight

a. Body Weight

Those animals dying of HG throughout the course of the 180 day Chamber Study showed at least a 50% weight loss at death. The Closed Group ferrets which died weighed slightly less than the Open Group (Figure 31).

b. Liver

The average liver weight, expressed as percent of body weight, remained higher in the diseased animals than in the normal ferrets in both groups. See Figure 32. Notice the peaks (increased weights) at 5, 11, and 16 weeks which may be an indicator of disease progression.

c. Spleen

The spleen weights of diseased ferrets increased sharply at week 5 and then leveled off at the normal weight range until week 13. At this time an extreme divergence of spleen weights for both groups took place. However, these levels may not be significant since each point represents only single animals (Figure 33).

d. Kidney

The average weight of both kidneys was markedly higher than the normal in both groups. Initially the Open Group was higher than the Closed Group, while later the Closed Group was higher. See Figure 34.

e. Thymus

In all the animals dying of HG the thymus was so minute that reliable weight estimates could not be made.

4. Histology

Complete necropsies revealed a consistent spectrum of lesions. In all animals, including ferrets which were the recipients of inocula (blood) from Chamber Test animals, the following lesions were observed:

Thymus: Complete absence of lymphoid cells — only epithelial stroma remained.
Lymph node: Medullary cords were empty of lymphocytes — regions corresponding to bursal dependent areas were acellular and contained an eosinophilic, hyaline-like substance.

Intestinal tract: All regions contained blood — capillaries of villi were distended with infiltrates of lymphocytes and other cellular components of blood.

Adrenal gland: Slight enlargement of cortical region.

Liver: Complete vacuolization of hepatocytes — no retention of glycogen and normal cytoplasmic constituents.

5. Disease Transmission

Several types of inocula from ferrets showing clinical signs of HG, as well as the gross postmortem changes previously described, have been inoculated into several different hosts using various routes. Ferrets were inoculated orally and intraperitoneally to determine the transmissibility of the disease from ferret to ferret. Mice were inoculated intraperitoneally and subcutaneously, and chicken embryos were inoculated via the chorioallantoic sac and yolk sac in order to isolate a possible viral etiologic agent.

a. Ferret Inoculation

(1) Whole blood inoculum

(a) Whole blood from Open Group ferret Q8032 that died 56 days after the onset of the experiment was inoculated intraperitoneally into ferret R876. This animal died approximately 60 days postinoculation showing the typical clinical signs of HG. Gross postmortem examination revealed congestion of the lower small intestine, extensive pulmonary congestion, and hepatic solidification.

(b) Whole blood from Closed Group ferret Q8052 that died 56 days after the onset of the experiment was inoculated intraperitoneally into ferret R231. The body weight of this inoculated ferret was not recorded at the time of inoculation, but at 26 days postinoculation it was 605 gm., while at day 33, when the animal died, it was 445 gm. The liver was 3.52% of the body weight, the spleen 0.31%, and the kidney 0.36%. Although these percentages fall within normal ranges, gross postmortem examination revealed bloody fluid and hemorrhagic regions in the stomach, hemorrhagic regions in the upper intestine, and an atrophied thymus. At death ferret R231 had a white blood cell (WBC) count of 18.7×10^3, a red blood cell (RBC) count of 9.6×10^6, and an hematocrit of 74. Both the WBC and the hematocrit were above normal range.

(c) Whole blood from Open Group ferret Q8003 that died on day 56 was inoculated intraperitoneally into 2 ferrets (R1137 and Q9529).

The body weight of ferret R1137 was not recorded at the time of inoculation, but at 26 days postinoculation was 760 gm., while at 73 days postinoculation the body weight was 378 gm. The liver was 3.77% of the body weight, the spleen 0.53%, and the kidney 0.56%. The kidney percentage was high, the liver slightly high, and the spleen within the normal range. On postmortem examination there were no grossly visible lesions. At 14 days postinoculation ferret Q9529 became comatose. Gross postmortem examination revealed a 2.0 mm. cyst in the cortico-medullary junction of the right kidney. The stomach was without apparent lesions but contained 7.0 ml. of a clear, slightly greenish fluid.

Whole blood from ferret Q9529 was inoculated into the yolk sac of six 7-day-old chicken embryos. One embryo died at 1 day postinoculation, another at 7 days, and a third at 8 days postinoculation. Yolk sac material from the embryo that died at 7 days postinoculation was frozen for future isolation work.

Whole blood from ferret Q9529 also was inoculated onto the chorioallantoic sac of six 9-day-old chicken embryos. Five embryos died at 1 day postinoculation. Since these deaths were considered nonspecific no material was saved.

Whole blood from ferret Q9529 was inoculated into 2 ferrets (#14 and #15). The body weights of the recipients were recorded at various times postinoculation.

Postinoculation Day	Ferret #14	Ferret #15
11	665	790
77	915	981
91	849	820
105	865	817
160	789	706

On day 85 postinoculation the following blood work was performed:

Assay	Ferret #14	Ferret #15
Hematocrit	51	56
WBC x 10^3	10.0	12.3
RBC x 10^6	8.9	9.5
Gamma globulin (%)	13.8	5.8
Protein (%)	6.7	6.4
LDH	1300	1120
Alkaline phosphatase	40	32
SGOT	132	108

All of these figures fall within normal ranges.

At approximately 180 days postinoculation these two animals are still surviving.

At 85 days postinoculation ferrets #14 and #15 were bled. Eight suckling mice were inoculated intraperitoneally with 0.1 ml. of a 1/10 dilution of whole blood from each ferret. No mice inoculated with blood from ferret #14 died and one mouse inoculated with blood from ferret #15 died at 8 days postinoculation. There were no deaths in the group of control mice. All remaining mice were killed at 21 days postinoculation.

(d) Whole blood from Open Group ferret Q8009 that died 70 days after the onset of the experiment was inoculated intraperitoneally into 2 ferrets (#17 and #21). The body weights of the recipients were recorded at various times postinoculation.

Postinoculation day	Ferret #17	Ferret #21
11	830	905
77	1083	1125
91	987	1050
105	980	1062
160	820	1042

On day 85 postinoculation the following blood work was performed:

Assay	Ferret #17	Ferret #21
Hematocrit	54	53
WBC x 10^3	15.4	11.7
RBC x 10^6	9.3	9.0
Gamma globulin (%)	11.5	9.2
Protein (%)	6.9	8.8
LDH	1120	1810
Alkaline phosphatase	24	16
SGOT	312	200

Only the WBC of #17 was abnormal, being somewhat high. All other values were within normal ranges.

At approximately 180 days postinoculation these two animals are still surviving.

At 85 days postinoculation ferrets #17 and #21 were bled. Eight suckling mice were inoculated intraperitoneally with 0.1 ml. of a 1/10 dilution of whole blood from each ferret. One mouse inoculated with blood from ferret #17 died at 9 days postinoculation. Three mice inoculated with blood from ferret #21 died at 6 days postinoculation and another died at 7 days postinoculation. All remaining mice were killed at 21 days postinoculation. There were no deaths in the group of control mice.

(e) Whole blood from Closed Group ferret Q8068 that had been housed in a 4-unit isolator and died 126 days after the onset of the experiment was inoculated intraperitoneally into four, 2-month-old ferrets. The body weights of the recipients were recorded at various times postinoculation.

Day	Ferret				
Postinoculation	R1254	R1254	R1255	R1256	Average
0	390	408	410	378	396
13	513	516	532	503	516
27	600	640	584	651	619
39	725	723	787	734	742
90	888	827	904	785	851

Uninoculated ferrets (controls) showed a 100 gm. greater weight increase between day 39 and 90 than did these inoculated. Otherwise, they showed no clinical signs of HG and at day 120 are still surviving.

(2) Stomach contents inoculum

(a) Stomach contents from Open Group ferret Q8009 which died 37 days after the onset of the experiment was inoculated orally into 2 ferrets (#27 and #28). The body weights of the recipients were recorded at various times postinoculation.

Postinoculation day	Ferret #27	Ferret #28
11	790	783
77	924	1160
91	795	985
105	846	1038
160	749	958

On day 85 postinoculation the following blood analyses were performed:

Assay	Ferret #27	Ferret #28
Hematocrit	50	53
WBC x 10^3	9.1	11.2
RBC x 10^6	8.6	8.8
Gamma globulin (%)	25.0	6.9
Protein (%)	8.3	7.4
LDH	1140	1300
Alkaline phosphatase	40	48
SGOT	108	142

Only the percent gamma globulin of ferret #27 was abnormal, being somewhat elevated. All other values were within normal ranges.

At approximately 180 days postinoculation these two animals are still surviving.

At 85 days postinoculation ferrets #27 and #28 were bled. Eight sukling mice were inoculated intraperitoneally with 0.1 ml. of a 1/10 dilution of whole blood from each ferret. No mice inoculated with blood from either ferret died. There were no deaths in the group of control mice. All mice were killed at 21 days postinoculation.

(3) Organ homogenate inoculum

(a) An organ homogenate from Open Group ferret Q8013 that died 62 days after the onset of the experiment was inoculated intraperitoneally into three 2-month-old ferrets. The organ homogenate consisted of a 10% (weight/volume) suspension of liver and kidney that had been freeze-thawed three times. The body weights of the recipients were recorded at various times postinoculation.

	Ferret			
Postinoculation day	R1267	R1268	R1269	Average
0	380	415	410	402
13	503	564	565	544
27	626	687	656	656
39	722	911	849	824
90	902	1109	1017	1009

These figures were within the same ranges as the uninoculated control ferrets.

(b) An organ homogenate from Closed Group ferret Q8070 that died 111 days after the onset of the experiment was inoculated intraperitoneally into three, 2-month-old ferrets. The organ homogenate consisted of a 10% (weight/volume) suspension of liver, spleen, and kidney that had been freeze-thawed three times. The body weights of the recipients were recorded at various times postinoculation.

	Ferret			
Postinoculation day	R1270	R1271	R1272	Average
0	440	415	358	404
13	533	514	472	506
27	626	634	664	641
39	755	754	789	766
90	909	895	900	901

These figures were within the same range as uninoculated control ferrets.

b. Mouse Inoculation

(1) Plasma inoculum

(a) Plasma from Open Group ferret Q8003 which died 56 days after the onset of the experiment was inoculated into mice. Six suckling mice each received 0.1 ml. intraperitoneally. Five mice died 1 day postinoculation. Remaining mice were killed 35 days postinoculation.

(b) Plasma from Open Group ferret Q8032 which died 56 days after the onset of the experiment was inoculated into mice. Six suckling mice each received 0.1 ml. intraperitoneally. Four mice died 1 day postinoculation and 1 each on days 2 and 3.

(c) Plasma from Closed Group ferret Q8052 which died 56 days after the onset of the experiment was inoculated into mice. Six suckling mice each received 0.1 ml. intraperitoneally. Two mice died 1 day postinoculation, 2 on day 2, and 2 on day 3.

(2) Whole blood inoculum

(a) Whole blood from Q8003 was inoculated into six suckling mice. All died on day 1 postinoculation. A 1/10 dilution of blood from Q8003 was inoculated into six suckling mice. Two died on day 4 postinoculation. Remaining mice were killed at 35 days postinoculation.

(b) Whole blood from Q8032 was inoculated into six suckling mice. Four died on day 1 postinoculation and 1 died on day 27. Remaining mice were killed at 35 days postinoculation.

(c) Whole blood from Q8052 was inoculated into six suckling mice. One died on day 1 and 5 died on day 6 postinoculation. A 1/10 dilution of blood from Q8052 was inoculated into six suckling mice. One died on day 2, 1 on day 6, 1 on day 8, and one on day 9. Remaining mice were killed on day 35 postinoculation.

(3) Stomach contents inoculum

(a) Stomach contents from Open Group ferret Q8013 which died 62 days after the onset of the experiment was inoculated into 27-day-old mice. One group of 6 received 0.1 ml. intraperitoneally, and the other group of 6 received 0.1 ml. subcutaneously. At death, Q8013 had a WBC of 8.6×10^3, an RBC of 9.0×10^6, and a percent protein of 8.1, all of which were within normal ranges. The hemorrhagic stomach contents were diluted with sterile saline and then centrifuged. The supernatant fluid served as the inoculum. In the group that received the inoculum intraperitoneally, 2 mice died 8 day postinoculation. In the group inoculated subcutaneously there were no deaths. All remaining mice were killed at 30 days postinoculation.

(4) Spleen homogenate inoculum

(a) A spleen homogenate from Open Group ferret Q8018 that died 84 days after the onset of the experiment was inoculated into mice. The spleen was homogenized as a 10% (weight/volume) suspension in sterile saline. It was centrifuged and the supernatant fluid was put through a 200 mμ Millipore filter. Three groups of 10 mice were inoculated: Group 1, 2-day-old mice, were inoculated with 0.05 ml. of the filtrate intraperitoneally; Group 2, 8-day-old mice, were likewise inoculated with 0.1 ml.; and Group 3, 21-day-old mice, were inoculated with 0.1 ml. None of the 2- or 8-day-old mice and only 1 of the 21-day-old mice died on day 10 postinoculation. Remaining mice were killed 35 days postinoculation.

(5) Organ homogenate inoculum

(a) An organ homogenate from Open Group ferret Q8013 that died 62 days after the onset of the experiment was inoculated into mice. Ten 2-day-old mice were inoculated with 0.1 ml. intraperitoneally. Two mice died on day 1 postinoculation. These were recorded as incidental deaths. Remaining mice were killed on day 21 postinoculation.

(b) An organ homogenate from Closed Group ferret Q8070 that died 111 days after the onset of the experiment was inoculated into mice. Ten 2-day-old mice were inoculated with 0.1 ml. intraperitoneally. Six mice died on day 1 postinoculation and 1 on day 2. These were recorded as incidental deaths. Remaining mice were killed at 21 days postinoculation.

c. Chicken Embryo Chorioallantoic Sac and Yolk Sac Inoculation

(1) Plasma inoculum

(a) Plasma from Closed Group ferret Q8038 that died 47 days after the onset of the experiment was inoculated into chicken embryos for possible virus isolation. At death ferret Q8038 had a hematocrit of 34%, an RBC of 6.4×10^6, a WBC of 5.6×10^3, a percent protein of 4.8, and a differential blood count that showed 11% lymphocytes, 80% segmented neutrophils, 3% band neutrophils, and 6% monocytes. This extremely high percentage of neutrophils may indicate a bacterial infection which may or may not be related to the etiology of HG.

Plasma was inoculated onto the chorioallantoic sac of four 9-day-old chicken embryos. All 4 died 1 day post-inoculation. These were considered nonspecific deaths.

Plasma from Q8038 also was inoculated into the yolk sac of four 6-day-old chicken embryos. All 4 were killed on day 8 postinoculation and yolk sac material was passed by inoculation into six 7-day-old embryos. These all died on days 1, 4, and 11. The yolk sac material was pooled from those that died on day 4 and was passed by inoculation into the yolk sac of six 7-day-old chicken embryos. Three died on day 4 and 3 on day 6. Two separate pools were made of these.

In the pooled yolk sac material of the embryos that died at 4 days postinoculation a small, gram negative bacillus was identified, while in the yolk sac material of those embryos that died at day 6 postinoculation no bacteria were identified. Each of these aliquots was further divided into two fractions, one of which was freeze-thawed 4 times and the other not. Two 2-month-old ferrets each were inoculated intraperitoneally with 0.1 ml. of these 4 aliquots. The body weights of the recipients were recorded at various time postinoculation.

Group 1: Embryos died day 4; bacteria identified; freeze thawed.

Postinoculation day	Ferret R1257	Ferret R1258	Average
0	420	422	421
13	558	512	535
27	614	632	623
39	750	838	794
90	932	923	927

Group 2: Embryos died day 4; bacteria identified; not freeze-thawed.

Postinoculation day	Ferret R1259	Ferret R1260	Average
0	375	407	391
13	500	497	499
27	588	646	617
39	670	766	718
90	936	983	970

Group 3: Embryos died day 6; no bacteria identified; freeze-thawed.

Postinoculation day	Ferret R1261	Ferret R1262	Average
0	398	462	425
13	518	553	535
27	573	600	617
39	715	877	796
90	866	1137	1002

Group 4: Embryos died day 6; no bacteria identified; not freeze-thawed.

Postinoculation day	Ferret R1263	Ferret R1264	Average
0	465	432	448
13	532	557	545
27	593	598	596
39	654	764	709
90	884	931	908

These 8 ferrets showed no clinical signs of HG and at 120 days postinoculation are still surviving.

(2) Stomach contents inoculum

(a) Stomach contents from Open Group ferret Q8009 that died 70 days after the onset of the experiment was inoculated into chicken embryos.

Stomach contents were inoculated onto the chorioallantoic sac of six 9-day-old embryos, but none died.

Stomach contents were also inoculated into the yolk sac of six 7-day-old embryos. Four embryos died on days 4 and 7 postinoculation. The yolk sac material of those embryos that died on day 7 was pooled and frozen for future virus isolation work.

(3) Spleen homogenate inoculum

(a) A spleen homogenate from Closed Group ferret Q8038 that died 47 days after the onset of the experiment was inoculated into chicken embryos.

Spleen homogenate was inoculated onto the chorioallantoic sac of four 9-day-old chicken embryos. All 4 were killed on day 8 postinoculation and chorioallantoic fluid was passed by inoculation onto the chorioallantoic sac of six 9-day-old chicken embryos. One embryo died on day 4 and the chorioallantoic fluid was passed by inoculation onto the chorioallantoic sac of six 9-day-old chicken embryos. However, none died and this mode of passage was abandoned.

Spleen homogenate from Q8038 also was inoculated into the yolk sac of four 6-day-old embryos. Two embryos died on day 1 postinoculation, 1 on day 7, and 1 on day 8. Yolk sac material was harvested from the embryo that died on day 8 and the material was passed by inoculation into the yolk sac of six 7-day-old embryos. Five embryos died on days 1, 5, and 11. The yolk sac material from those that died on day 5 was pooled and was passed by inoculation into the yolk sac of three 7-day-old embryos. One embryo died on day 4 and 1 on day 7. Yolk sac material from the embryos that died on day 7 was harvested and frozen for future virus isolation work.

IV. SUMMARY

Experiments were performed to determine the influence closed microbial ecologies have on modification or simplification of natural intestinal flora of ferrets in a closed environmental system. On the basis of previous tests in which certain species (Salmonella and Bacteroides) were decreased at 90 days of enclosure, a second trial was constructed for 180-day tests. In this trial there was little difference in the 8 major classes of intestinal flora between animals in the Open and Closed environmental groups except for the level of Lactobacillus.

It is of extreme importance to note that when both Open and Closed groups contracted hemorrhagic gastritis (see previous Progress Report No. NAS 9-10844), the interrelationship of this agent with other intestinal flora produced a more profound effect on animals from the Closed Group, particularly with reference to Lactobacillus levels.

Manuscripts are being developed to describe the basic character of hemorrhagic gastritis and, when completed, will be submitted as supplements to the Progress Reports.

V. APPENDIX I

A. Formula for Medium A[1]

1. Make up hemin solution by dissolving 100 mg. of recrystallized hemin (Nutritional Biochemicals Co.) in 99 ml. of distilled H_2O and 1 ml. of 5N NaOH. Store in refrigerator.
2. Make up cystine solution just before using by dissolving 4 gm. L-cystine (Pfanstiehl) in 99 ml. distilled H_2O and 8 ml. of 5N NaOH. Prepare fresh each time.
3. Measure out the following ingredients and pour through a funnel into a 2-liter flask.

Pancreatic digest of casein, U.S.P. (Trypticase, Fisher Scientific)	10 gm.
Proteose peptone No. 3 (Difco)	5 gm.
Dextrose	5 gm.
Yeast extract (Difco)	5 gm.
Tris buffer, 7 to 9 (Sigma)	3 gm.
Hemin solution	10 ml.
L-cystine solution	10 ml.
Agar agar #3 (Oxoid)	15 gm.
Distilled H_2O	1000 ml

4. Bring solution in the flask to a full boil, swirling constantly.
5. Plug flask, and autoclave medium for 15 minutes at 15 pounds pressure.
6. Pour immediately into plates and flame each plate as pouring.
7. Allow to harden and store in refrigerator, upside down.

B. Formula for Medium C[1]

1. Steps 1 and 2 as in Medium A
2. Reconstitute neomycin sulfate powder by adding 5 ml. sterile saline to an ampule containing 0.5 gm. of the antibiotic. Add the saline with a sterile syringe. Shake the ampule until powder is dissolved and store in the refrigerator until needed. Reconstitute a new ampule of the antibiotic each time the medium is made, and discard the ampule after use.
3. Measure out the ingredients listed in Step 3 (Medium A). Add 2 gm. placenta powder (Nutritional Biochemical Co.).
4. Cool flask to approximately 50 C. by swirling the flask in a basin filled with cold tap water.

4\. Add 0.2 ml. of the reconstituted neomycin with a sterile 1.0 ml. stylex disposable tuberculin syringe.

5\. Mix thoroughly.

6\. Pour media into plates, flame plates, and when hard store upside down in the refrigerator.

[1]Schaedler, R. W., Dubos, R., and Costello, R.: The development of the bacterial flora in the gastrointestinal tract of mice. J. Exptl. Med., 1965, <u>122</u>: 59-66.

C. Formula for Medium E[1]

1. Prepare triphenyl tetrazolium chloride (Nutritional Biochemicals Co.) by bringing 0.4 gm. up to 100 ml. with distilled H_2O in a 100 ml. volumetric flask and mix to dissolve.
2. Filter the 4% tetrozolium chloride through a Millipore filter which has been previously sterilized by autoclaving.
3. Rehydrate Tergitol 7 agar (Difco).
4. Bring to full boil, shaking continuously, and then autoclave.
5. Cool as in Step 4 (Medium C).
6. Add 10 ml. of the tetrazolium chloride with a sterile pipette to the cooled medium.
7. Swirl thoroughly.
8. Pour, flame plates, and when hardened store upside down in the refrigerator.

D. PEA, Enterococcus MG, and Rogosa S.L. medium are available from Difco (See Difco Manual.

E. XLD Medium obtainable from Baltimore Biological Laboratory.[2]

[1]Schaedler et al., 1965. Op cit.

[2]Taylor, W. I.: Isolation of shigellae. I. Xylose lysine agars; new media for isolation of enteric pathogens. Am. J. Clin. Pathol., 1965, 44: 471-475.

VI. APPENDIX II

Food and Drug Research Laboratories
INCORPORATED

Maurice Avenue at 58th Street
Maspeth 78, New York City
Telephone: TWining 4-0800
Cable: Foodlabs, New York

REPORT

Submitted to University of Connecticut
Storrs, Connecticut 06268

Date February 15, 1972

Laboratory No. 0993

Sample Cat foods (2)

Marking "Calo Complete original flavor cat food, Net wt. 26 oz. (1 lb, 10 oz.)
Distributed by: Calo Pet Foods","Borden, Inc., Oakland, California 94623"
(a) Written on can in black marker: "Autoclaved"
(b) Written on can in black marker: "Not Autoclaved"

Examination Requested
Thiamine assay

RESULTS Thiamine (Vitamin B_1) was determined by fluorometry (AOAC method, p. 773, 11th ed., 1970)

Sample	Thiamine Found mg/100 g
(a) autoclaved	0.014
(b) not autoclaved	0.017

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Henry E. Swann, Jr.

Henry E. Swann, Jr., Ph. D.
Director Maspeth Laboratory

ab

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Form PA32b Adopted 11-1-57 ©.

VII. APPENDIX III

TABLE 1—Experimental Design.

Group	No. of animals	Group status	Day culture taken of Saliva	Day culture taken of Skin	Day culture taken of Feces	Kill day	Postmortem parameters
A	7	Control	0	0	0	0	Gut flora
B	3	Control	—	—	—	0	Wall weight,histology
C	3	Control	—	—	—	0	Assimilation,motility
D	6	Open	20	40	60	60	Gut flora
E	3	Open	—	—	—	60	Wall weight,histology
F	3	Open	—	—	—	60	Assimilation,motility
G	6	Closed	20	40	60	60	Gut flora
H	3	Closed	—	—	—	60	Wall weight,histology
I	3	Closed	—	—	—	60	Assimilation,motility
J	6	Open	20,80	40,100	60,120	120	Gut flora
K	3	Open	—	—	—	120	Wall weight,histology
L	3	Open	—	—	—	120	Assimilation,motility
M	6	Closed	20,80	40,100	60,120	120	Gut flora
N	3	Closed	—	—	—	120	Wall weight,histology
O	3	Closed	—	—	—	120	Assimilation,motility
P	6	Open	20,80,140	40,100,160	60,120,180	180	Gut flora
Q	3	Open	—	—	—	180	Wall weight,histology
R	3	Open	—	—	—	180	Assimilation,motility
S	6	Closed	20,80,140	40,100,160	60,120,180	180	Gut flora
T	3	Closed	—	—	—	180	Wall weight,histology
U	3	Closed	—	—	—	180	Assimilation,motility

Table 2 Feces: Percent of Animals with Detectable Species of Bacteria

	Day 0	Day 60		Day 120		Day 180		Lowest Dilution Tested*
		Open	Closed	Open	Closed	Open	Closed	
Total aerobes	100	100	100	100	100	100	100	10^{-7}
Total anaerobes	100	100	100	100	100	100	100	10^{-7}
Bacteroides	25	90.5	87.5	100	100	100	85.7	10^{-4}
Coliforms	91.7	100	100	100	100	100	100	10^{-5}
Lactobacillus (aerobic)	75	0	0	0	0	100	100	10^{-3}
Lactobacillus (anaerobic)	91.7	85	62.5	63	13	100	100	10^{-4}
Streptococcus (aerobic)	100	95.2	93.8	100	88	100	100	10^{-5}
Streptococcus (anaerobic)	100	95.2	93.8	75	75	87.5	100	10^{-5}
Shigella	58.3	95.2	93.8	38	100	100	100	10^{-3}
Salmonella	0	42.8	6.3	13	0	50	14.3	10^{-3}

*at day 180

Table 3

Day 0 Feces

	Average	Range
Total aerobes	1.1×10^{9}	$(2 \times 10^{7} - 8 \times 10^{9})$
Total anaerobes	2.1×10^{10}	$(8 \times 10^{7} - 2 \times 10^{11})$
Bacteroides	1.0×10^{4}	$(1 \times 10^{4} - 1 \times 10^{5})$
Coliforms	2.7×10^{9}	$(2 \times 10^{5} - 1 \times 10^{10})$
Lactobacillus (aerobic)	4.4×10^{6}	$(1 \times 10^{6} - 4 \times 10^{7})$
Lactobacillus (anaerobic)	3.5×10^{10}	$(4 \times 10^{7} - 1 \times 10^{11})$
Streptococcus (aerobic)	3.0×10^{9}	$(1 \times 10^{6} - 2.1 \times 10^{10})$
Streptococcus (anaerobic)	1.3×10^{10}	$(1 \times 10^{6} - 1 \times 10^{11})$
Shigella	4.5×10^{4}	$(1 \times 10^{4} - 1.8 \times 10^{5})$
Salmonella	0*	0*

*10^{-4} dilution (lowest dilution tested)

Table 4

Open Group - Day 60 Feces

	Average	Range
Total aerobes	4.5×10^{10}	$(4 \times 10^{7} - 3.6 \times 10^{11})$
Total anaerobes	1.1×10^{11}	$(4 \times 10^{7} - 6.2 \times 10^{11})$
Bacteroides	5.7×10^{6}	$(1 \times 10^{4} - 1 \times 10^{8})$
Coliforms	6.2×10^{8}	$(4.3 \times 10^{7} - 1 \times 10^{10})$
Lactobacillus (aerobic)	0*	0*
Lactobacillus (anaerobic)	7.4×10^{9}	$(1 \times 10^{6} - 6.5 \times 10^{10})$
Streptococcus (aerobic)	3.7×10^{9}	$(4 \times 10^{6} - 3.4 \times 10^{10})$
Streptococcus (anaerobic)	2.0×10^{10}	$(3 \times 10^{6} - 1.5 \times 10^{11})$
Shigella	2.0×10^{7}	$(4 \times 10^{4} - 3 \times 10^{8})$
Salmonella	7.8×10^{4}	$(1 \times 10^{4} - 1 \times 10^{6})$

*10^{-6} dilution (lowest dilution tested)

Table 5

Closed Group - Day 60 Feces

	Average	Range
Total aerobes	6.8×10^{10}	$(4 \times 10^{7} - 2.7 \times 10^{11})$
Total anaerobes	1.1×10^{11}	$(2 \times 10^{8} - 5 \times 10^{11})$
Bacteroides	3.6×10^{5}	$(1 \times 10^{4} - 2.5 \times 10^{6})$
Coliforms	1.5×10^{8}	$(3.8 \times 10^{7} - 2.7 \times 10^{8})$
Lactobacillus (aerobic)	0*	0*
Lactobacillus (anaerobic)	6.7×10^{9}	$(2 \times 10^{6} - 1 \times 10^{11})$
Streptococcus (aerobic)	9.7×10^{9}	$(1.9 \times 10^{7} - 6.7 \times 10^{10})$
Streptococcus (anaerobic)	1.1×10^{9}	$(4 \times 10^{6} - 4.1 \times 10^{9})$
Shigella	1.3×10^{8}	$(3 \times 10^{4} - 1.2 \times 10^{9})$
Salmonella	6.3×10^{2}	(1×10^{4})

*10^{-6} dilution (lowest dilution tested)

Table 6

Open Group - Day 120 Feces

	Average	Range
Total aerobes	3.4×10^{9}	$(1.0 \times 10^{8} - 1.0 \times 10^{10})$
Total anaerobes	9.0×10^{7}	$(2.0 \times 10^{7} - 1.2 \times 10^{8})$
Bacteroides	2.6×10^{7}	$(1.3 \times 10^{6} - 6.3 \times 10^{7})$
Coliforms	6.7×10^{7}	$(2.0 \times 10^{5} - 5.0 \times 10^{8})$
Lactobacillus (aerobic)	0*	0*
Lactobacillus (anaerobic)	3.5×10^{7}	$(1.0 \times 10^{6} - 1.3 \times 10^{8})$
Streptococcus (aerobic)	2.0×10^{7}	$(1.0 \times 10^{6} - 5.0 \times 10^{7})$
Streptococcus (anaerobic)	5.0×10^{7}	$(5.0 \times 10^{6} - 3.0 \times 10^{8})$
Shigella	3.2×10^{5}	$(1.6 \times 10^{5} - 7.0 \times 10^{5})$
Salmonella	1.3×10^{3}	(1.0×10^{4})

*10^{-5} dilution (lowest dilution tested)

Table 7

Closed Group - Day 120 Feces

	Average	Range
Total aerobes	2.3×10^9	$(2.3 \times 10^8 - 1.2 \times 10^{10})$
Total anaerobes	1.8×10^8	$(1.0 \times 10^7 - 5.0 \times 10^8)$
Bacteroides	8.0×10^6	$(9.0 \times 10^4 - 3.0 \times 10^7)$
Coliforms	1.6×10^7	$(1.0 \times 10^6 - 6.0 \times 10^7)$
Lactobacillus (aerobic)	0[a]	0[a]
Lactobacillus (anaerobic)	1.3×10^7	(1.0×10^8)
Streptococcus (aerobic)	1.4×10^8	$(1.0 \times 10^6 - 7.0 \times 10^8)$
Streptococcus (anaerobic)	2.0×10^7	$(1.0 \times 10^6 - 1.0 \times 10^8)$
Shigella	3.3×10^5	$(4.0 \times 10^4 - 2.0 \times 10^6)$
Salmonella	0[b]	0[b]

[a] 10^{-5} dilution (lowest dilution tested)

[b] 10^{-4} dilution (lowest dilution tested)

Table 8

Open Group - Day 180 Feces

	Average	Range
Total aerobes	1.5×10^{10}	$(2.5 \times 10^{9} - 3.8 \times 10^{10})$
Total anaerobes	3.2×10^{9}	$(4.0 \times 10^{7} - 2.0 \times 10^{10})$
Bacteroides	1.0×10^{8}	$(7.0 \times 10^{4} - 8.0 \times 10^{8})$
Coliforms	8.5×10^{8}	$(1.0 \times 10^{6} - 3.4 \times 10^{9})$
Lactobacillus (aerobic)	2.3×10^{5}	$(3.0 \times 10^{4} - 6.0 \times 10^{5})$
Lactobacillus (anaerobic)	5.0×10^{7}	$(4.0 \times 10^{5} - 3.0 \times 10^{8})$
Streptococcus (aerobic)	3.1×10^{8}	$(6.0 \times 10^{6} - 1.4 \times 10^{9})$
Streptococcus (anaerobic)	5.0×10^{7}	$(1.6 \times 10^{6} - 2.0 \times 10^{8})$
Shigella	6.8×10^{5}	$(2.0 \times 10^{3} - 4.2 \times 10^{6})$
Salmonella	1.8×10^{4}	$(1.0 \times 10^{3} - 1.0 \times 10^{5})$

Table 9

Closed Group - Day 180 Feces

	Average	Range
Total aerobes	1.8×10^{10}	$(2.0 \times 10^{8} - 6.6 \times 10^{10})$
Total anaerobes	8.1×10^{9}	$(1.0 \times 10^{9} - 3.1 \times 10^{10})$
Bacteroides	7.2×10^{7}	$(1.0 \times 10^{5} - 4.0 \times 10^{8})$
Coliforms	1.2×10^{9}	$(1.0 \times 10^{6} - 3.3 \times 10^{9})$
Lactobacillus (aerobic)	3.7×10^{5}	$(8.0 \times 10^{4} - 1.3 \times 10^{6})$
Lactobacillus (anaerobic)	2.2×10^{6}	$(4.0 \times 10^{5} - 4.3 \times 10^{6})$
Streptococcus (aerobic)	5.0×10^{8}	$(4.0 \times 10^{6} - 1.9 \times 10^{9})$
Streptococcus (anaerobic)	2.3×10^{8}	$(9.0 \times 10^{5} - 9.0 \times 10^{8})$
Shigella	1.1×10^{7}	$(1.5 \times 10^{5} - 4.0 \times 10^{7})$
Salmonella	5.7×10^{4}	(4.0×10^{5})

TABLE 10. Total Aerobes x 10^7*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	40	10	1700
	12	9	—**	2700
	26	7000	1000	250
	28	1200	—	380
	29	22000	—	300
	33	900	—	340
	35	1600	20	490
	36	180	—	2500
Closed	40	27000	—	1100
	43	120	24	6600
	46	20000	23	140
	55	20	—	3900
	60	1300	30	40
	62	2300	50	600
	70	9000	1200	20
Lowest dilution tested		10^7	10^7	10^7

* Reciprocal of logarithm of dilution
** Media unsatisfactory

TABLE 11. Total Anaerobes x 10^7*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	15000	2	6
	12	40	12	220
	26	20000	11	50
	28	21000	—**	70
	29	8000	—	4
	33	21000	—	230
	35	270	—	16
	36	22000	12	2000
Closed	40	50000	1	1100
	43	30	12	300
	46	15000	21	300
	55	1000	10	3100
	60	800	50	600
	62	31000	10	200
	70	5000	32	100
Lowest dilution tested		10^7	10^7	10^7

* Reciprocal of logarithm of dilution
** Media unsatisfactory

Table 12. Bacteroides x 10^4*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	10	1000	170
	12	100	800	7
	26	200	4300	10000
	28	10000	130	80000
	29	1500	2500	90
	33	150	6300	1200
	35	10	1100	10000
	36	10	4600	400
Closed	40	20	23	600
	43	10	1900	40000
	46	90	300	200
	55	0	3000	7100
	60	60	600	0
	62	9	600	50
	70	0	9	2600
Lowest dilution tested		10^4	10^4	10^4

* Reciprocal of logarithm of dilution

TABLE 13. Coliforms x 10^5*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	1230	2	14000
	12	1580	20	19000
	26	2500	200	1200
	28	1100	2	34000
	29	2000	10	20
	33	1600	5000	100
	35	1680	39	100
	36	1180	50	10
Closed	40	2200	70	33000
	43	860	80	800
	46	1710	400	1200
	55	380	20	32000
	60	1700	120	10
	62	1700	10	16000
	70	1450	10	600
Lowest dilution tested		10^5	10^5	10^5

* Reciprocal of logarithm of dilution

TABLE 14. Aerobic Lactobacillus x 10^{4}*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	0	0	13
	12	0	0	10
	26	0	0	23
	28	0	0	50
	29	0	0	3
	33	0	0	60
	35	0	0	4
	36	0	0	22
Closed	40	0	0	8
	43	0	0	10
	46	0	0	30
	55	0	0	40
	60	0	0	20
	62	0	0	22
	70	0	0	130
Lowest dilution tested		10^{6}	10^{5}	10^{3}

* Reciprocal of logarithm of dilution

TABLE 15.

Anaerobic Lactobacillus x 10^5*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	20	--**	30
	12	0	1300	3000
	26	20	400	50
	28	1700	20	600
	29	400	20	20
	33	400	0	130
	35	200	10	190
	36	20000	0	4
Closed	40	3200	0	12
	43	0	0	26
	46	0	1000	4
	55	0	0	12
	60	240	0	16
	62	900	0	41
	70	100	0	35
Lowest dilution tested		10^6	10^6	10^4

* Reciprocal of logarithm of dilution
** Not tested

C. 2

TABLE 16.

Aerobic Streptococcus x 10^6*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	90	4	200
	12	16	12	1400
	26	60	50	200
	28	700	10	100
	29	20	20	6
	33	34000	43	500
	35	9000	20	20
	36	7000	1	50
Closed	40	67000	1	1900
	43	20	200	4
	46	19000	50	20
	55	0	10	900
	60	3000	700	60
	62	28000	1	300
	70	900	1	300
Lowest dilution tested		10^6	10^6	10^5

* Reciprocal of logarithm of dilution

TABLE 17.

Anaerobic Streptococcus x 10^{5}*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	270000	1000	100
	12	700	3000	1000
	26	460000	1000	400
	28	100000	0	100
	29	2800	50	0
	33	80000	0	2000
	35	5000	100	350
	36	180000	500	16
Closed	40	41000	10	4500
	43	600	100	18
	46	5000	1000	300
	55	0	20	2800
	60	200	60	9
	62	70000	0	9000
	70	500	0	30
Lowest dilution tested		10^{6}	10^{6}	10^{5}

* Reciprocal of logarithm of dilution

TABLE 18. Shigella x 10^3*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	270	0	2
	12	500	0	5
	26	400	700	4200
	28	2800	100	80
	29	2100	160	800
	33	1000	0	40
	35	100000	0	90
	36	1000	0	40
Closed	40	120000	60	30000
	43	60	100	500
	46	60	100	700
	55	30	200	40000
	60	60	2000	150
	62	100	40	1600
	70	4000	50	4000
Lowest dilution tested		10^4	10^4	10^3

* Reciprocal of logarithm of dilution

TABLE 19. Salmonella x 10^3*

Group	Animal Number	Day 60	Day 120	Day 180
Open	02	10	0	1
	12	0	0	0
	26	100	0	0
	28	1000	0	100
	29	200	0	0
	33	0	10	0
	35	0	0	40
	36	0	0	1
Closed	40	0	0	0
	43	0	0	0
	46	0	0	0
	55	0	0	0
	60	0	0	0
	62	0	0	0
	70	0	0	400
Lowest dilution tested		10^4	10^4	10^3

* Reciprocal of logarithm of dilution

TABLE 20. Duodenum: Day 0.

Organism	Average	Range
Total aerobes	5.1×10^6	$(2 \times 10^4$ to $13 \times 10^6)$
Total anaerobes	8×10^6	$(3 \times 10^4$ to $32 \times 10^6)$
Bacteroides	16×10^1	(1×10^3)
Coliforms	8×10^7	$(39 \times 10^4$ to $150 \times 10^6)$
Lactobacillus (aerobic)	21×10^2	$(2 \times 10^3$ to $9 \times 10^3)$
Lactobacillus (anaerobic)	72×10^4	$(1 \times 10^4$ to $41 \times 10^5)$
Streptococcus (aerobic)	7.8×10^6	$(12 \times 10^3$ to $45 \times 10^6)$
Streptococcus (anaerobic)	29×10^4	$(2 \times 10^3$ to $12 \times 10^6)$
Shigella	—	—
Salmonella	5×10^2	(3×10^3)

TABLE 21. Duodenum - Day 180 - Open Group

	Average	Range
Total aerobes	5.6×10^7	$(2.0 \times 10^6 - 1.5 \times 10^8)$
Total anaerobes	2.1×10^7	$(1.4 \times 10^4 - 4.2 \times 10^7)$
Bacteroides	2.5×10^2	(1.0×10^3)
Coliforms	3.9×10^5	$(6.0 \times 10^3 - 1.2 \times 10^6)$
Lactobacillus (aerobic)	6.8×10^3	$(2.0 \times 10^3 - 2.5 \times 10^4)$
Lactobacillus (anaerobic)	5.2×10^7	$(1.0 \times 10^3 - 8.5 \times 10^7)$
Streptococcus (aerobic)	1.9×10^7	$(1.0 \times 10^7 - 5.0 \times 10^7)$
Streptococcus (anaerobic)	2.8×10^7	$(5.0 \times 10^4 - 5.0 \times 10^7)$
Shigella	8.6×10^4	$(8.0 \times 10^4 - 1.4 \times 10^5)$
Salmonella	0*	0*

* 10^{-3} dilution (lowest dilution tested)

TABLE 22. Duodenum - Day 180 - Closed Group

	Average	Range
Total aerobes	2.7×10^5	$(2.0 \times 10^5 - 4.3 \times 10^5)$
Total anaerobes	5.3×10^4	$(1.0 \times 10^4 - 1.4 \times 10^5)$
Bacteroides	1.3×10^3	$(1.0 \times 10^3 - 4.0 \times 10^3)$
Coliforms	1.1×10^4	$(3.0 \times 10^3 - 2.9 \times 10^4)$
Lactobacillus (aerobic)	5.8×10^3	$(1.0 \times 10^3 - 2.0 \times 10^4)$
Lactobacillus (anaerobic)	3.3×10^3	$(3.0 \times 10^3 - 1.0 \times 10^4)$
Streptococcus (aerobic)	1.6×10^5	$(7.0 \times 10^4 - 5.6 \times 10^5)$
Streptococcus (anaerobic)	6.4×10^4	$(1.0 \times 10^3 - 1.5 \times 10^5)$
Shigella	3.4×10^5	$(4.0 \times 10^4 - 1.2 \times 10^6)$
Salmonella	0*	0*

* 10^{-3} dilution (lowest dilution tested)

TABLE 23. Duodenum Counts x 10 [1][a]

	Day 0	Day 180 Open	Day 180 Closed
Total Aerobes	510000	5600000	27000
Total Anaerobes	800000	2100000	5300
Bacteroides	16	25	130
Coliforms	8000000	39000	1100
Lactobacillus (aerobic)	210	680	580
Lactobacillus (anaerobic)	72000	5200000	330
Streptococcus (aerobic)	780000	1900000	16000
Streptococcus (anaerobic)	29000	2800000	6400
Shigella	0[b]	8600	340000
Salmonella	50	0[b]	0[b]

[a] Reciprocal of logarithm of dilution

[b] 10^{-3} dilution (lowest dilution tested)

TABLE 24. Ileum: Day 0.

Organism	Average	Range
Total aerobes	12×10^6	(2×10^5 to 40×10^6)
Total anaerobes	53×10^5	(5×10^5 to 19×10^6)
Bacteroides	15×10^2	(1×10^3 to 8×10^3)
Coliforms	56×10^6	(70×10^4 to 100×10^6)
Lactobacillus (aerobic)	36×10^2	(2×10^3 to 10×10^3)
Lactobacillus (anaerobic)	58×10^5	(3×10^3 to 35×10^6)
Streptococcus (aerobic)	42×10^6	(10×10^4 to 150×10^6)
Streptococcus (anaerobic)	66×10^5	(1×10^3 to 32×10^6)
Shigella	73×10^3	(1×10^3 to 150×10^3)
Salmonella	13×10^2	(2×10^3 to 6×10^3)

TABLE 25. Ileum - Day 180 - Open Group

	Average	Range
Total aerobes	8.7×10^7	$(2.2 \times 10^7 - 1.5 \times 10^8)$
Total anaerobes	1.1×10^8	$(3.0 \times 10^7 - 1.5 \times 10^8)$
Bacteroides	2.3×10^5	$(1.0 \times 10^3 - 9.0 \times 10^5)$
Coliforms	2.5×10^7	$(9.0 \times 10^5 - 5.0 \times 10^7)$
Lactobacillus (aerobic)	5.4×10^4	$(3.0 \times 10^3 - 1.5 \times 10^5)$
Lactobacillus (anaerobic)	1.4×10^8	$(6.0 \times 10^5 - 2.0 \times 10^8)$
Streptococcus (aerobic)	5.1×10^6	$(5.5 \times 10^5 - 1.6 \times 10^7)$
Streptococcus (anaerobic)	1.0×10^8	$(1.0 \times 10^6 - 2.0 \times 10^8)$
Shigella	3.3×10^5	$(1.4 \times 10^5 - 8.3 \times 10^5)$
Salmonella	0*	0*

* 10^{-3} dilution (lowest dilution tested)

TABLE 26. Ileum - Day 180 - Closed Group

	Average	Range
Total aerobes	4.8×10^7	$(9.0 \times 10^6 - 9.5 \times 10^7)$
Total anaerobes	9.3×10^6	$(2.0 \times 10^4 - 2.5 \times 10^7)$
Bacteroides	5.6×10^6	$(1.0 \times 10^3 - 2.2 \times 10^7)$
Coliforms	1.9×10^6	$(9.0 \times 10^5 - 3.8 \times 10^6)$
Lactobacillus (aerobic)	4.3×10^4	$(1.3 \times 10^4 - 1.1 \times 10^5)$
Lactobacillus (anaerobic)	4.8×10^4	$(2.0 \times 10^3 - 8.0 \times 10^4)$
Streptococcus (aerobic)	4.3×10^5	$(1.6 \times 10^4 - 1.6 \times 10^6)$
Streptococcus (anaerobic)	3.8×10^5	$(1.0 \times 10^5 - 1.0 \times 10^6)$
Shigella	1.7×10^5	$(3.0 \times 10^4 - 3.3 \times 10^5)$
Salmonella	0*	0*

* 10^{-3} dilution (lowest dilution tested)

TABLE 27. Ileum Counts x 10^{2} [a]

	Day 0	Day 180 Open	Day 180 Closed
Total Aerobes	120000	870000	480000
Total Anaerobes	53000	1100000	93000
Bacteroides	15	2300	56000
Coliforms	560000	250000	19000
Lactobacillus (aerobic)	36	540	430
Lactobacillus (anaerobic)	58000	1400000	480
Streptococcus (aerobic)	420000	51000	4300
Streptococcus (anaerobic)	66000	1000000	3800
Shigella	730	3300	1700
Salmonella	13	0[b]	0[b]

[a] Reciprocal of logarithm of dilution

[b] 10^{-3} dilution (lowest dilution tested)

TABLE 28. Colon: Day 0.

Organism	Average	Range
Total aerobes	25×10^7	(5×10^6 to 85×10^7)
Total anaerobes	19×10^7	(22×10^6 to 54×10^7)
Bacteroides	12×10^5	(1×10^4 to 7×10^6)
Coliforms	45×10^7	(98×10^4 to 150×10^7)
Lactobacillus (aerobic)	45×10^3	(1×10^4 to 1×10^5)
Lactobacillus (anaerobic)	53×10^6	(2×10^5 to 32×10^7)
Streptococcus (aerobic)	32×10^7	(15×10^6 to 100×10^7)
Streptococcus (anaerobic)	84×10^7	(10×10^4 to 100×10^5)
Shigella	2×10^6	(10×10^4 to 134×10^5)
Salmonella	16×10^2	(1×10^4)

TABLE 29. Colon - Day 180 - Open Group

	Average	Range
Total aerobes	9.9×10^8	$(4.5 \times 10^8 - 1.5 \times 10^9)$
Total anaerobes	1.2×10^9	$(7.5 \times 10^8 - 1.5 \times 10^9)$
Bacteroides	6.5×10^6	$(1.0 \times 10^5 - 2.3 \times 10^7)$
Coliforms	2.6×10^8	$(5.0 \times 10^7 - 5.0 \times 10^8)$
Lactobacillus (aerobic)	4.0×10^4	$(1.0 \times 10^4 - 6.0 \times 10^4)$
Lactobacillus (anaerobic)	1.8×10^9	$(1.5 \times 10^9 - 2.0 \times 10^9)$
Streptococcus (aerobic)	2.7×10^7	$(2.7 \times 10^7 - 4.1 \times 10^7)$
Streptococcus (anaerobic)	1.3×10^9	$(1.0 \times 10^9 - 2.0 \times 10^9)$
Shigella	3.7×10^6	$(1.4 \times 10^5 - 1.2 \times 10^7)$
Salmonella	0*	0*

* 10^{-3} dilution (lowest dilution tested)

TABLE 30. Colon - Day 180 - Closed Group

	Average	Range
Total aerobes	6.5×10^{8}	$(2.1 \times 10^{8} - 1.5 \times 10^{9})$
Total anaerobes	2.8×10^{8}	$(4.0 \times 10^{7} - 3.9 \times 10^{8})$
Bacteroides	3.6×10^{7}	$(6.0 \times 10^{5} - 8.3 \times 10^{7})$
Coliforms	1.1×10^{8}	$(5.0 \times 10^{6} - 3.5 \times 10^{8})$
Lactobacillus (aerobic)	9.5×10^{4}	$(7.0 \times 10^{4} - 1.8 \times 10^{5})$
Lactobacillus (anaerobic)	4.2×10^{7}	$(9.0 \times 10^{5} - 9.0 \times 10^{7})$
Streptococcus (aerobic)	2.0×10^{8}	$(1.5 \times 10^{7} - 4.6 \times 10^{8})$
Streptococcus (anaerobic)	6.1×10^{7}	$(2.0 \times 10^{6} - 1.2 \times 10^{8})$
Shigella	7.5×10^{6}	$(2.7 \times 10^{5} - 2.6 \times 10^{7})$
Salmonella	0*	0*

* 10^{-3} dilution (lowest dilution tested)

TABLE 31. Colon Counts x 10^{2} [a]

	Day 0	Day 180 Open	Day 180 Closed
Total Aerobes	2500000	9900000	6500000
Total Anaerobes	1900000	12000000	2800000
Bacteroides	12000	65000	360000
Coliforms	4500000	2600000	1100000
Lactobacillus (aerobic)	450	400	950
Lactobacillus (anaerobic)	530000	18000000	420000
Streptococcus (aerobic)	3200000	270000	2000000
Streptococcus (anaerobic)	8400000	13000000	610000
Shigella	20000	37000	75000
Salmonella	16	0[b]	0[b]

[a] Reciprocal of logarithm of dilution

[b] 10^{-3} dilution (lowest dilution tested)

Table 32. Gut Volume (in ml)

Day	Group	Duodenum	Ileum	Colon	Total
0		2.3	23.7	7.2	33.2
180	Open	2.8	27.5	7.2	37.5
	Closed	2.9	22.7	6.7	32.3

Table 33. Hematology

	Day 0		Day 180 Open		Day 180 Closed	
	Average	Range	Average	Range	Average	Range
cells/mm^3 WBC x 10^3	5.6	(2.6 - 7.3)	12.0	(9.7 - 13.5)	6.9	(4.3 - 11.3)
cells/mm^3 RBC x 10^6	6.6	(6.3 - 7.6)	8.9	(7.7 - 10.6)	8.5	(5.7 - 9.0)
Per cent Hematocrit	40.1	(35.8 - 44.5)	48.2	(42 - 62)	48.5	(39 - 55)
Differential						
Lymphocytes	33.5	(25 - 51)	55.1	(36 - 77)	53.0	(37 - 69)
Monocytes	2.0	(0 - 4)	2.6	(1 - 4)	0.9	(0 - 2)
Basophils	0.2	(0 - 2)	0.4	(0 - 1)	0.4	(0 - 1)
Eosinophils	1.0	(0 - 5)	3.2	(2 - 4)	2.2	(0 - 10)
Neutrophils						
Segmented	59.3	(43 - 74)	37.9	(16 - 56)	43.3	(28 - 52)
Band	4.0	(1 - 11)	0.8	(0 - 3)	0.2	(0 - 1)

TABLE 34. Weights

	Day 0	Day 180	
		Open	Closed
Body[a]	450	777	696
Liver[b]	3.93	3.67	3.42
Spleen[b]	0.37	0.54	0.60
Kidney[b,c]	0.35	0.24	0.28
Thymus[b]	0.22	0.64	0.60

[a]Grams

[b]Percent of body weight

[c]One kidney

TABLE 35. Analysis of Canned Cat Food.[1,2]

Contents (%)	Not autoclaved A	Not autoclaved B	Autoclaved C	Autoclaved D	Guaranteed analysis
Protein	11.0	10.9	11.5	12.0	10.0 minimum
Crude fat	3.13	3.38	3.00	3.75	4.0 minimum
Crude fiber	0.59	0.54	0.54	0.60	1.5 maximum
Ash	2.80	2.48	2.81	3.10	3.5 maximum
Phosphorus	0.35	0.29	0.37	0.30	0.3 minimum
Salt	0.56	0.56	0.56	0.56	0.75maximum
Moisture	72.8	72.3	71.7	71.8	74.0 maximum

[1]Calo Cat Food, Borden Company, Inc., Oakland, California 94623.

[2]Analyses performed by Connecticut Agricultural Experiment Station, New Haven, Connecticut.

TABLE 36. Hemorrhagic Gastritis: Onset of Signs.

Day	Changes
0	Clinically normal
1	Anorexia
2 to 4	Marked depression
	Loss of response to stimulation
	Incoordination
	Lateral body retraction
	Comatose
5	Death
	50% weight loss
	Dehydration
	Blood in gut contents
	Thymic atrophy

FIGURE 1.

Total Aerobes

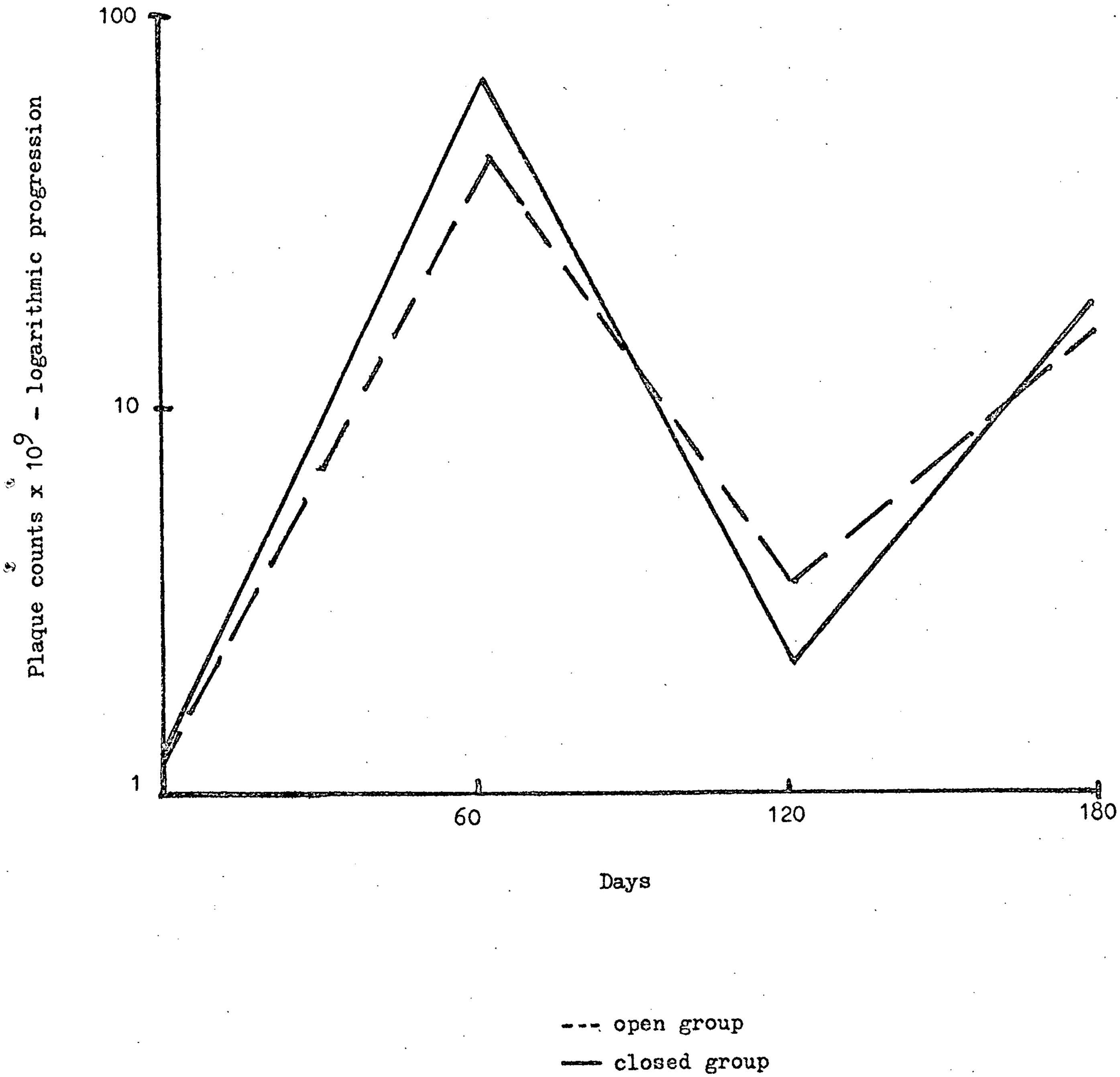


FIGURE 2. Total Anaerobes

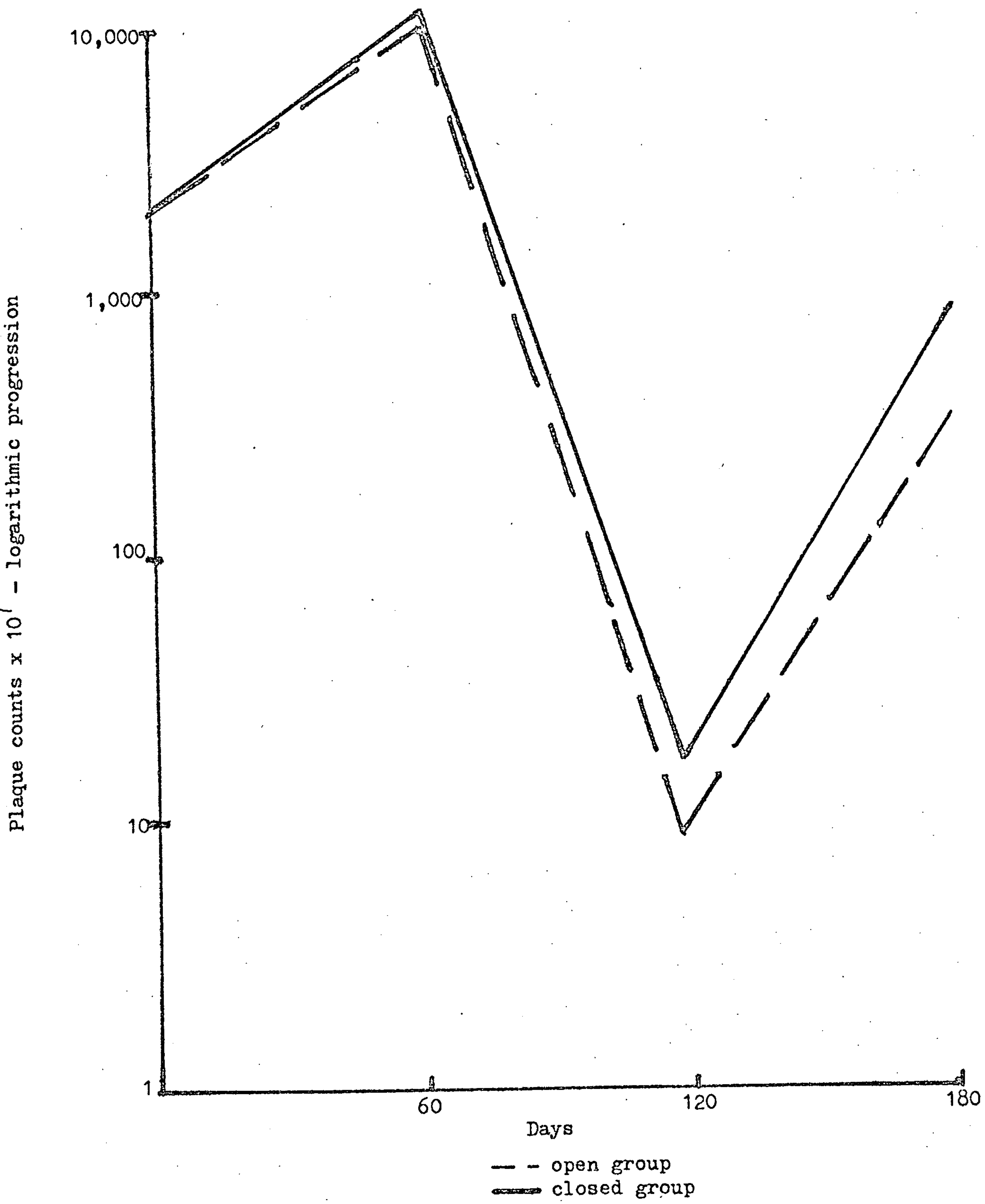


FIGURE 3. Bacteroides

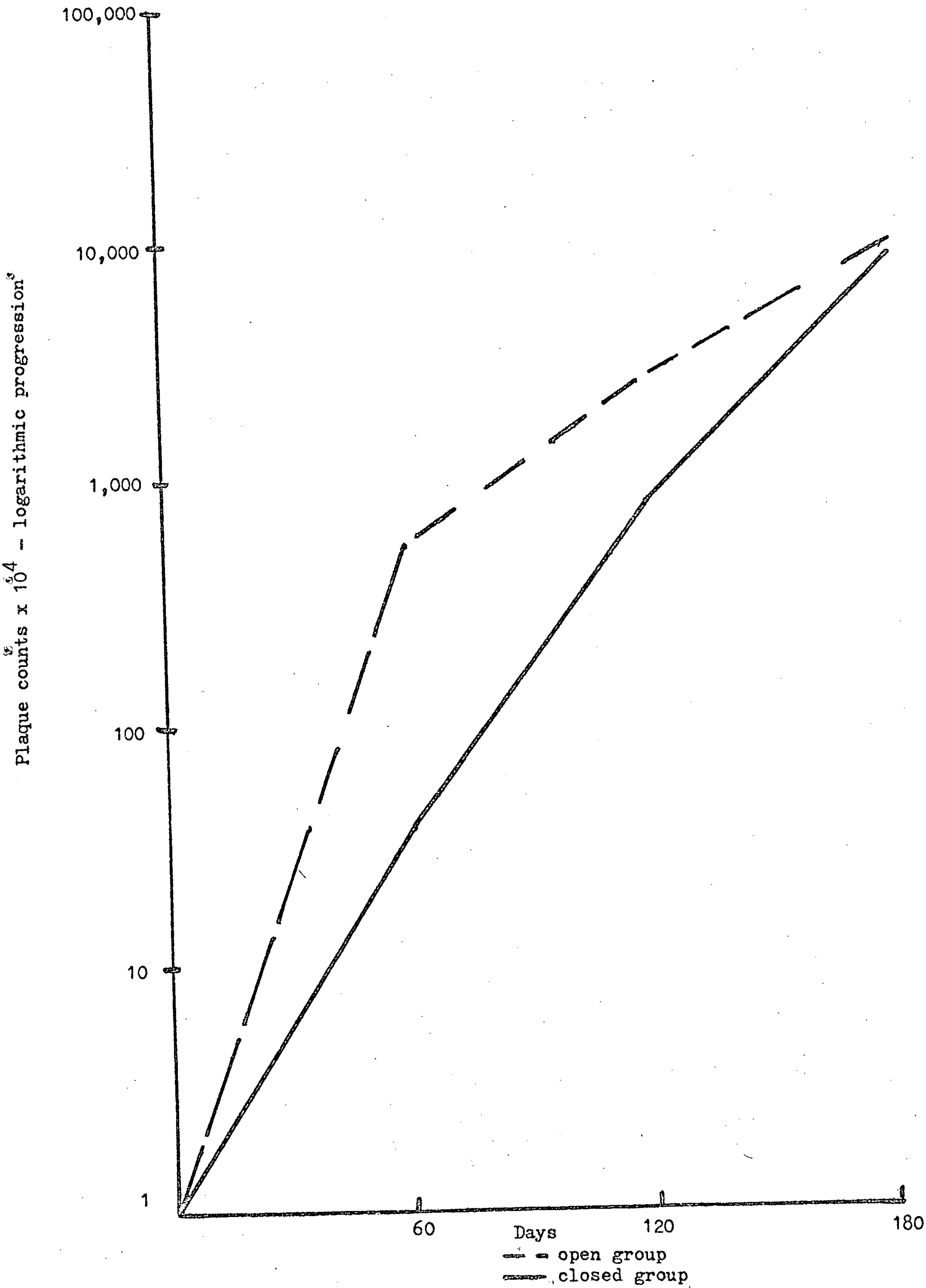


FIGURE 4. Coliforms

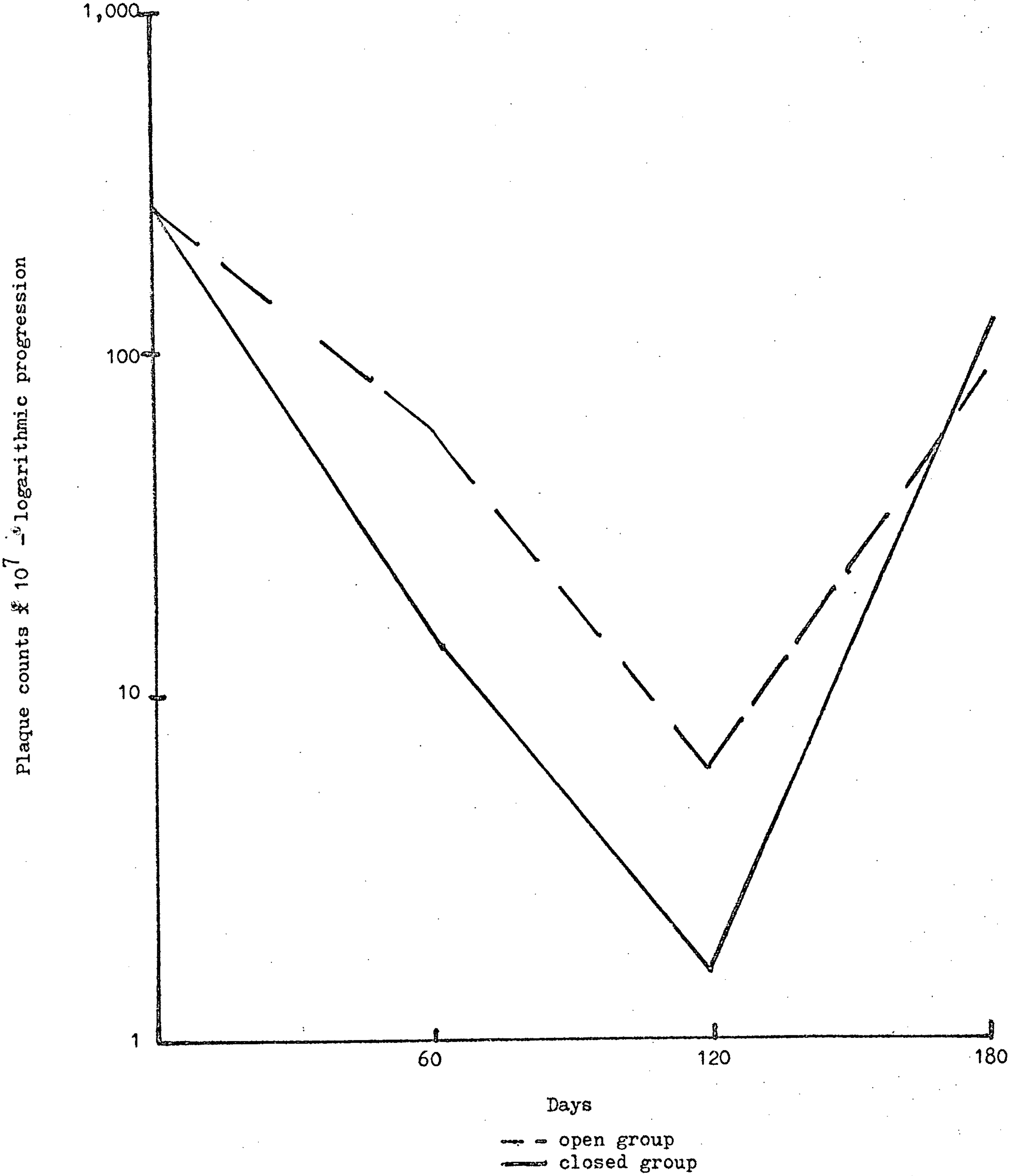
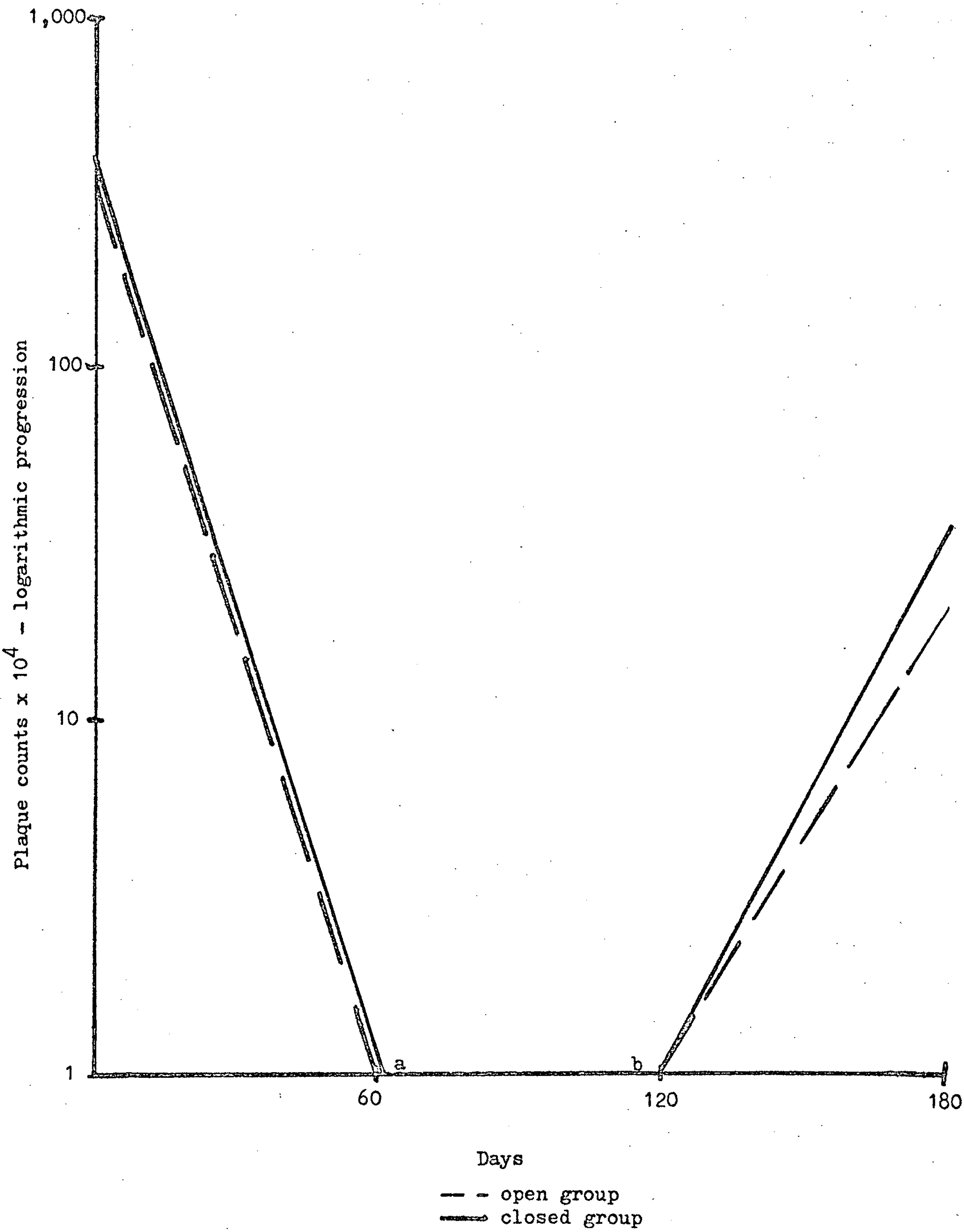


FIGURE 5. Aerobic Lactobacillus



a - no detectable colonies at 10^{-6} dilution.
b - no detectable colonies at 10^{-5} dilution.

FIGURE 6. Anaerobic Lactobacillus

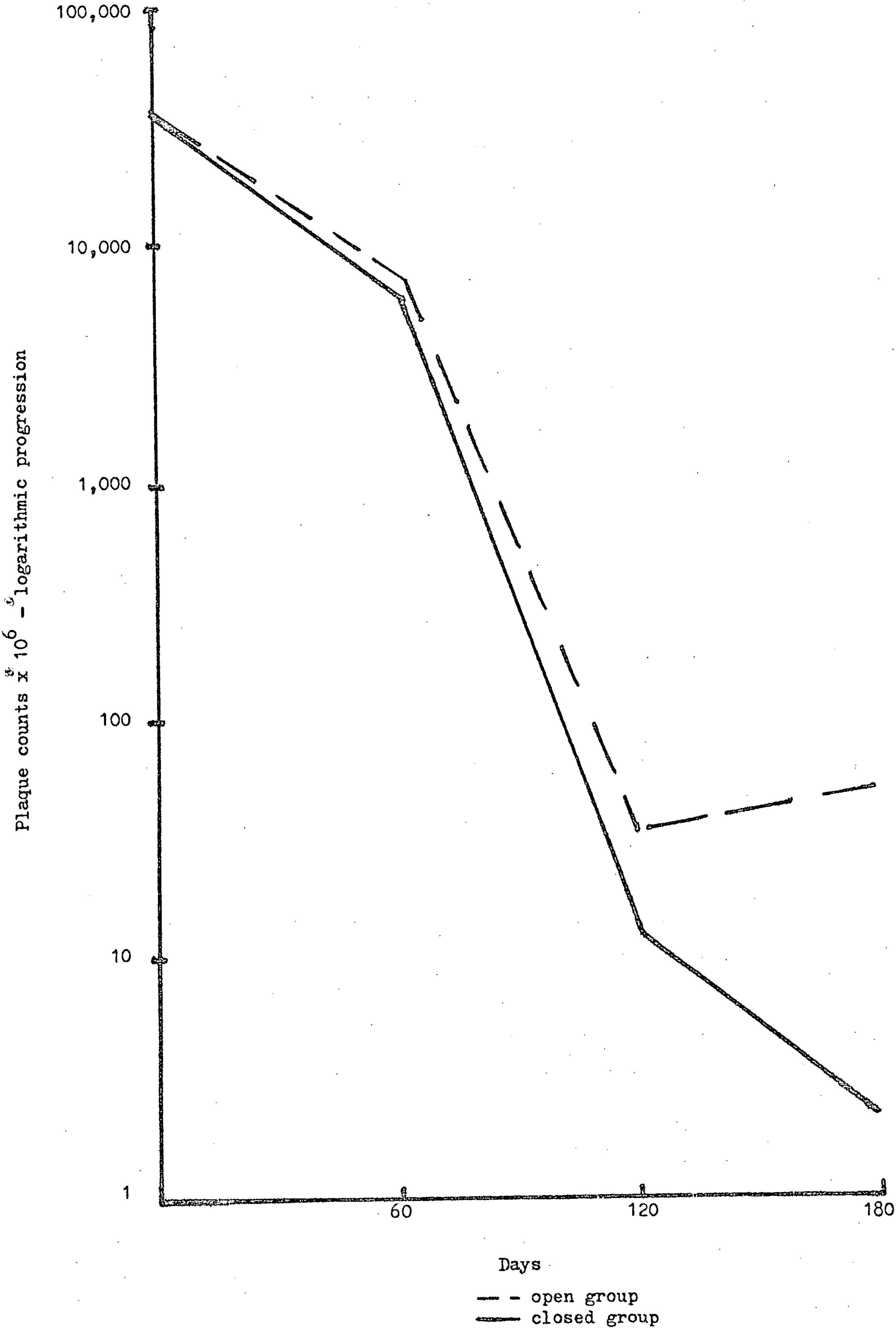
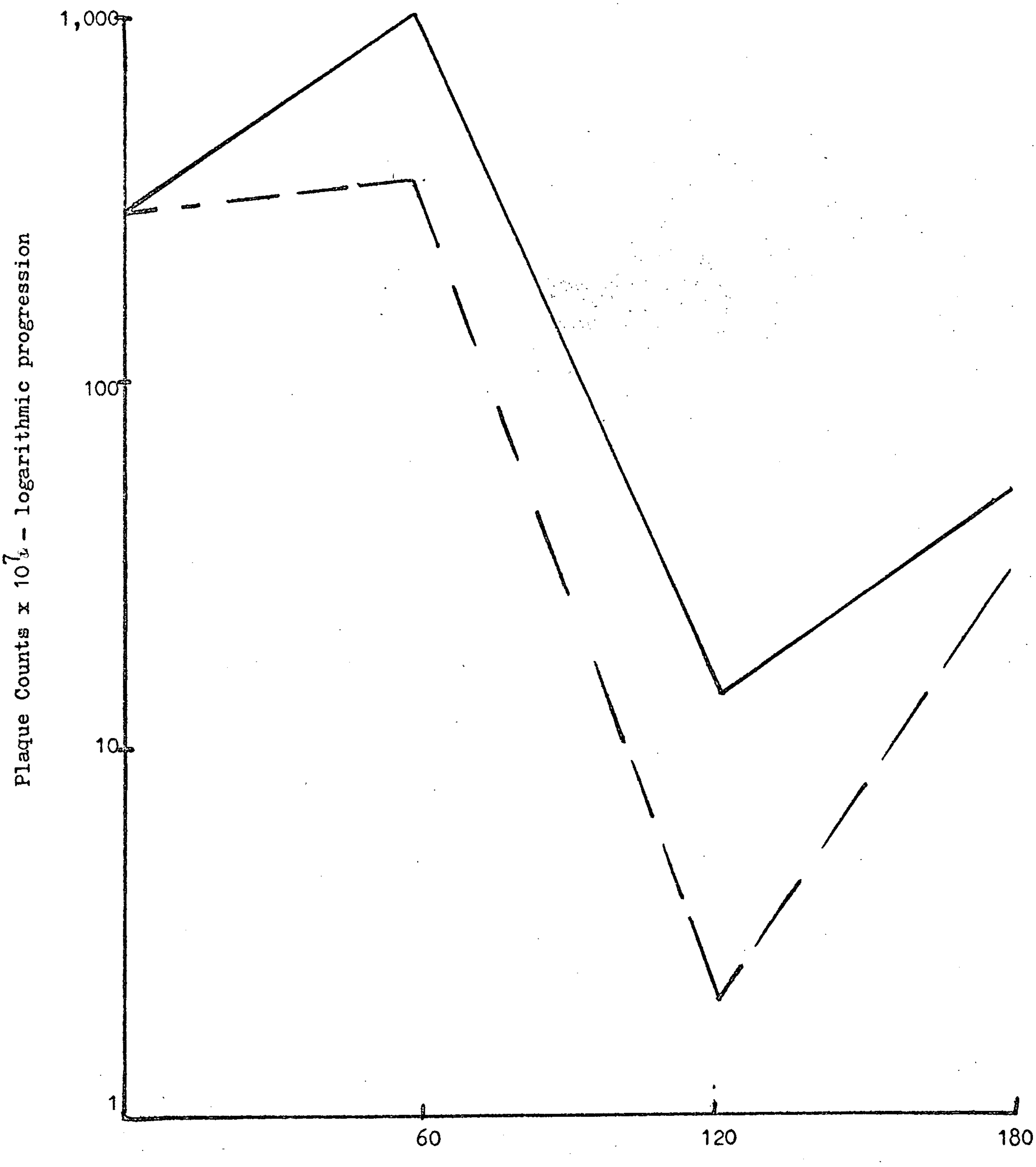


FIGURE 7. Aerobic Streptococcus



- - open group
—— closed group

FIGURE 8. Anaerobic Streptococcus

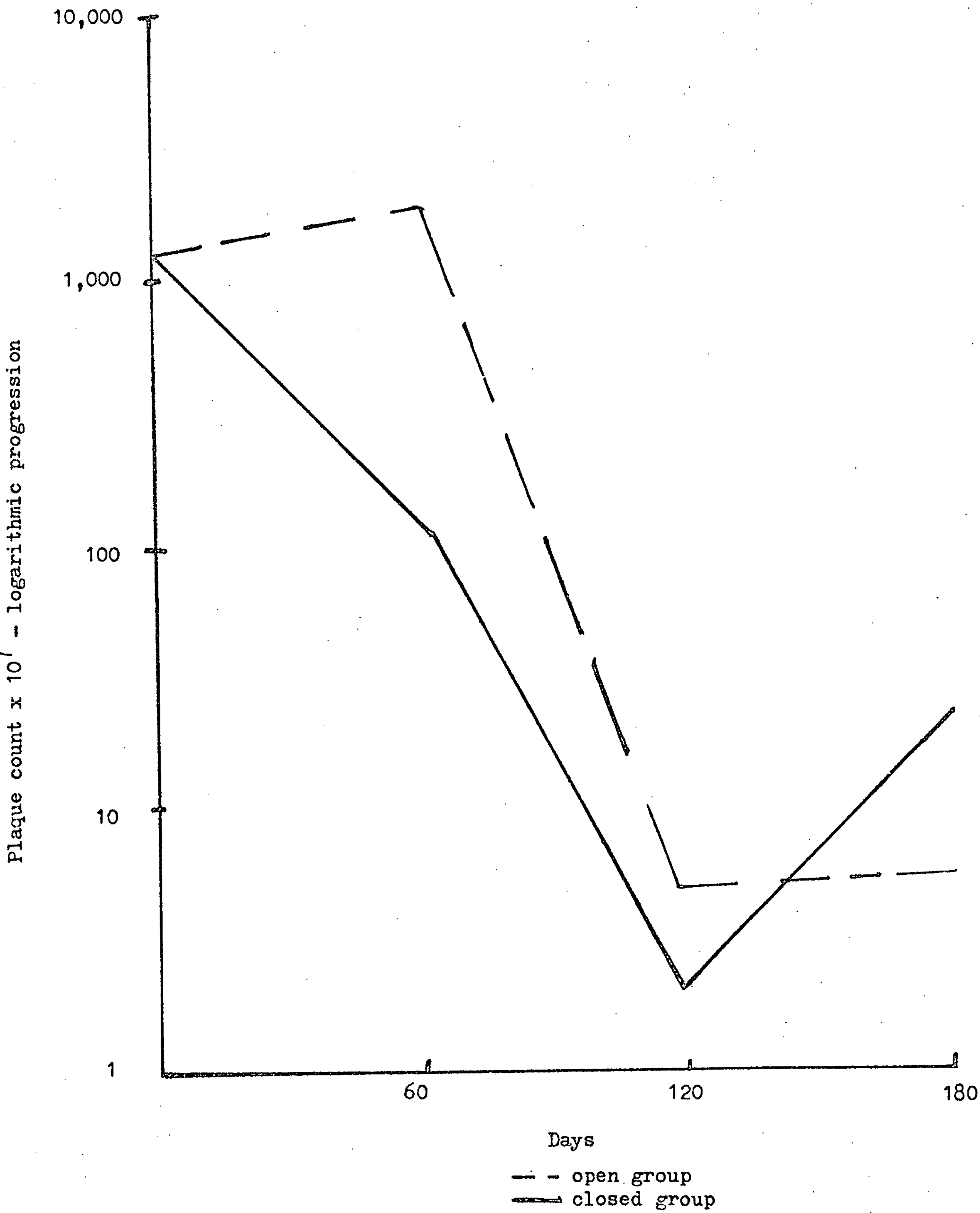


FIGURE 9. Shigella

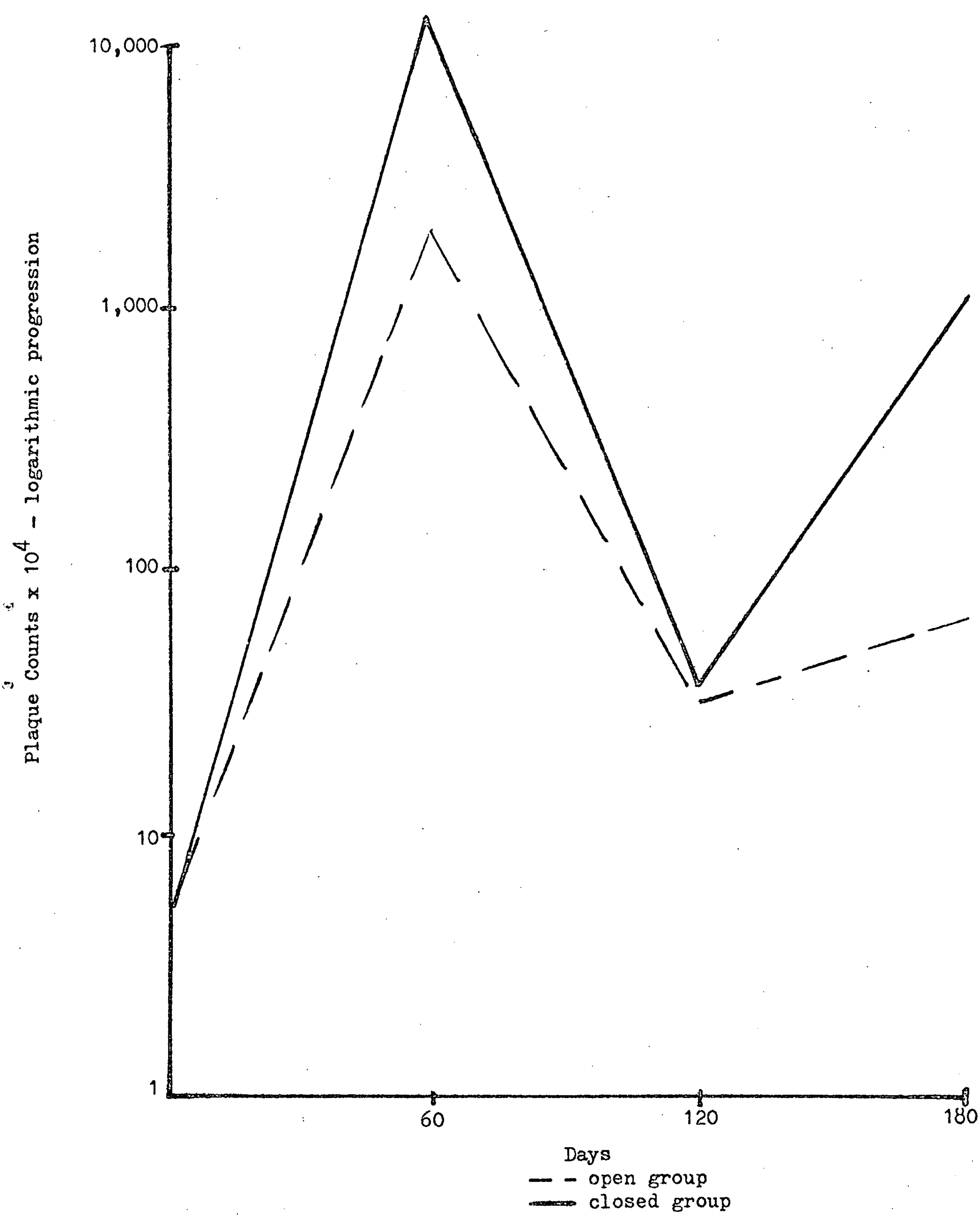
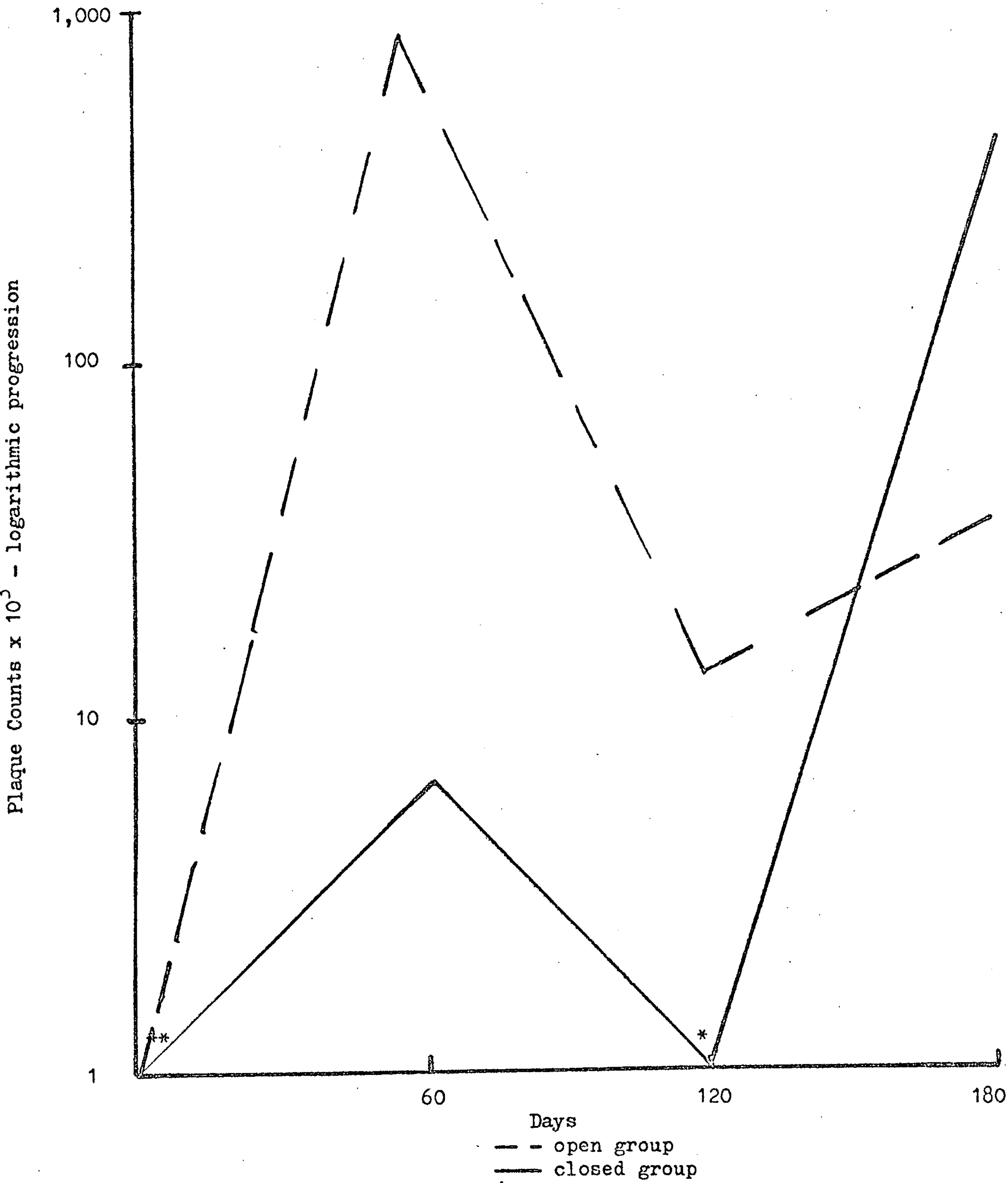


FIGURE 10. Salmonella



*no detectable colonies at 10^{-4} dilution.

FIGURE 11. Body Temperature

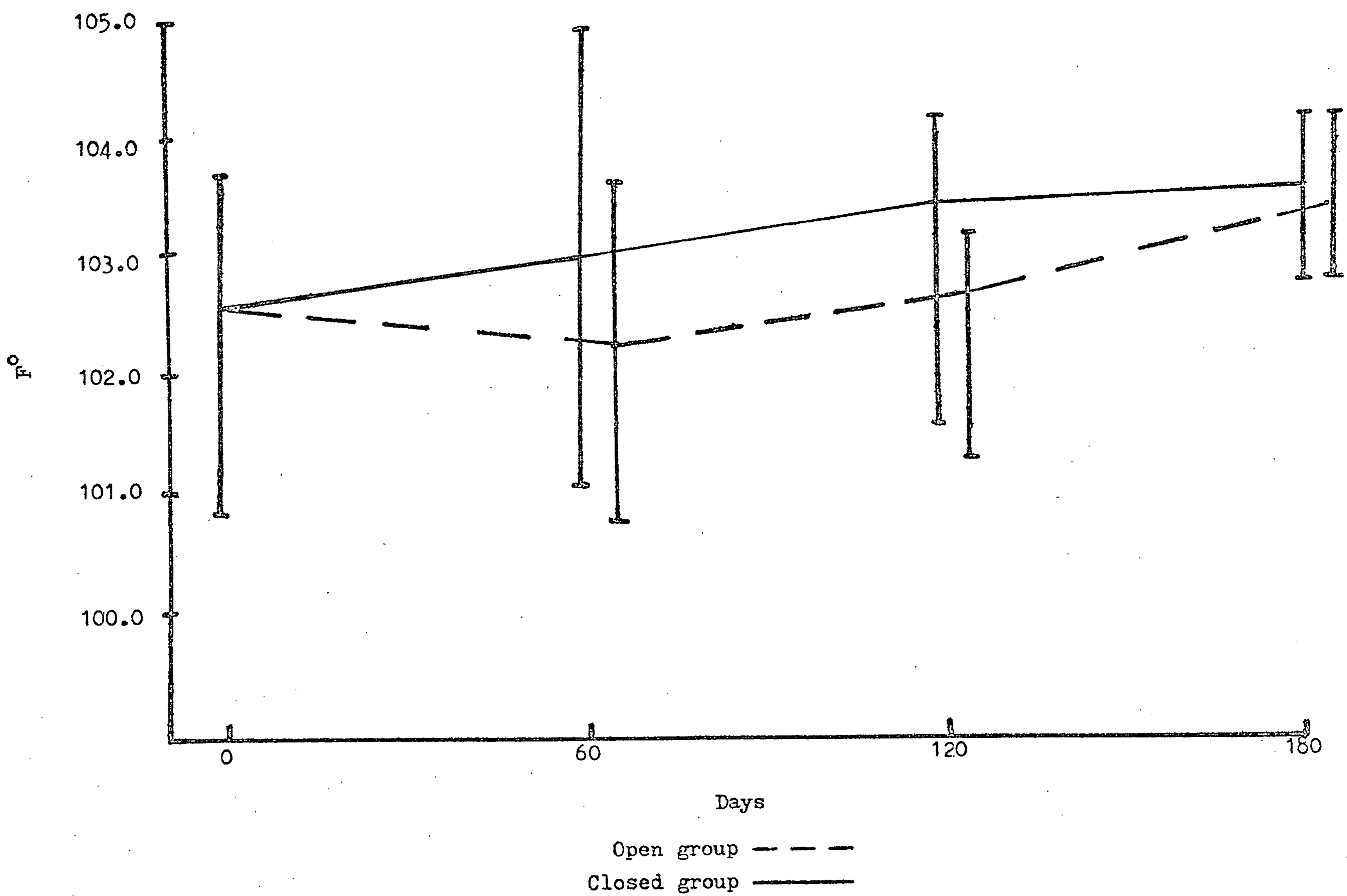


FIGURE 12. Motility

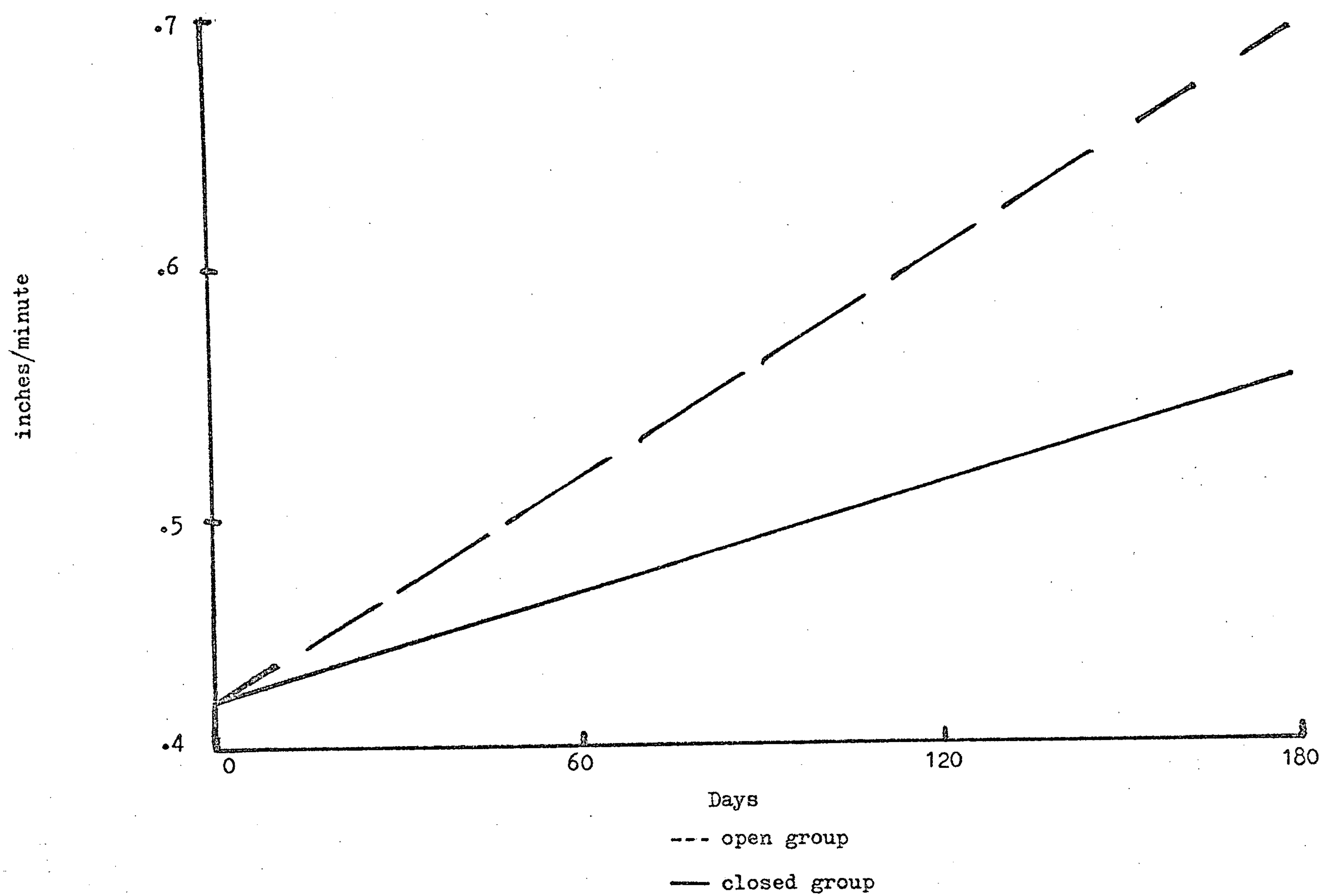


FIGURE 13. Wet Weight: Gut Contents - Duodenum

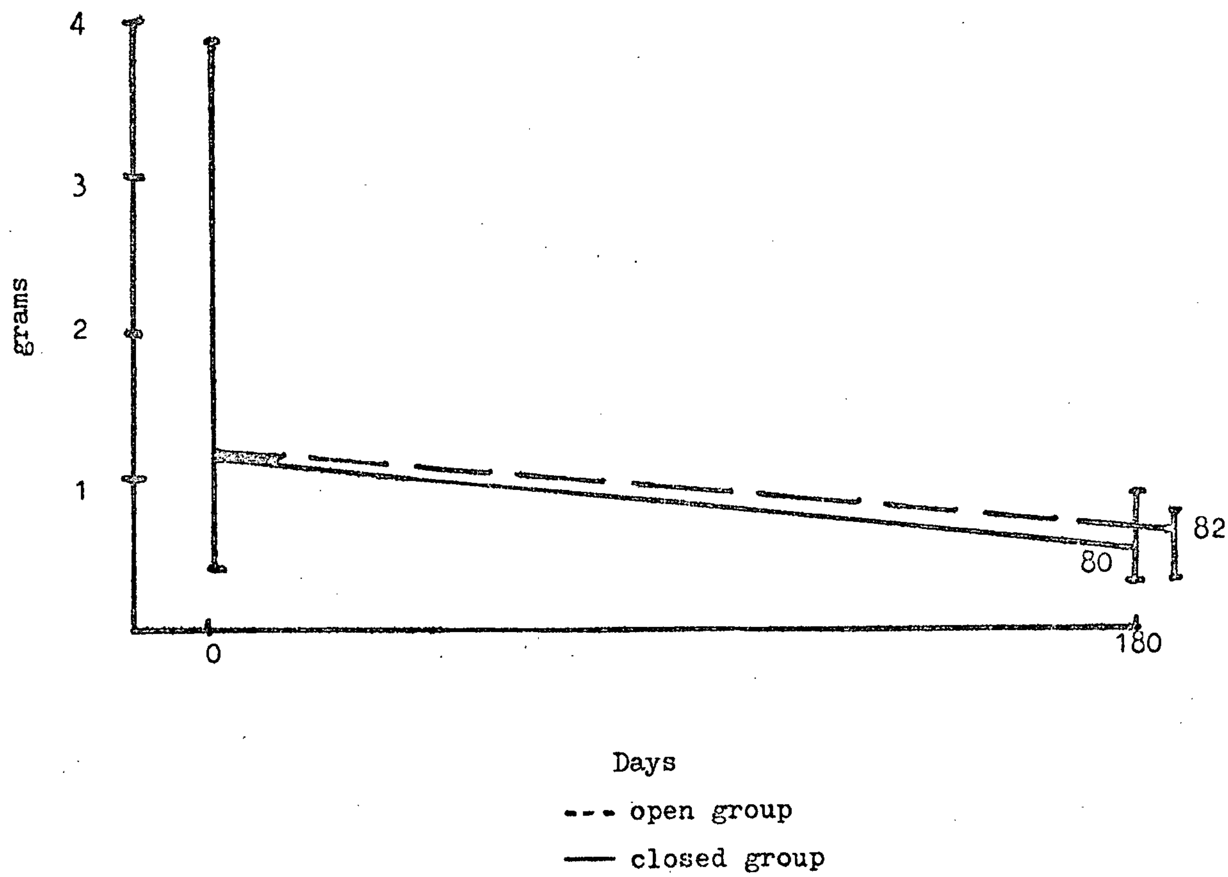


FIGURE 14. Dry Weight: Gut Contents - Duodenum

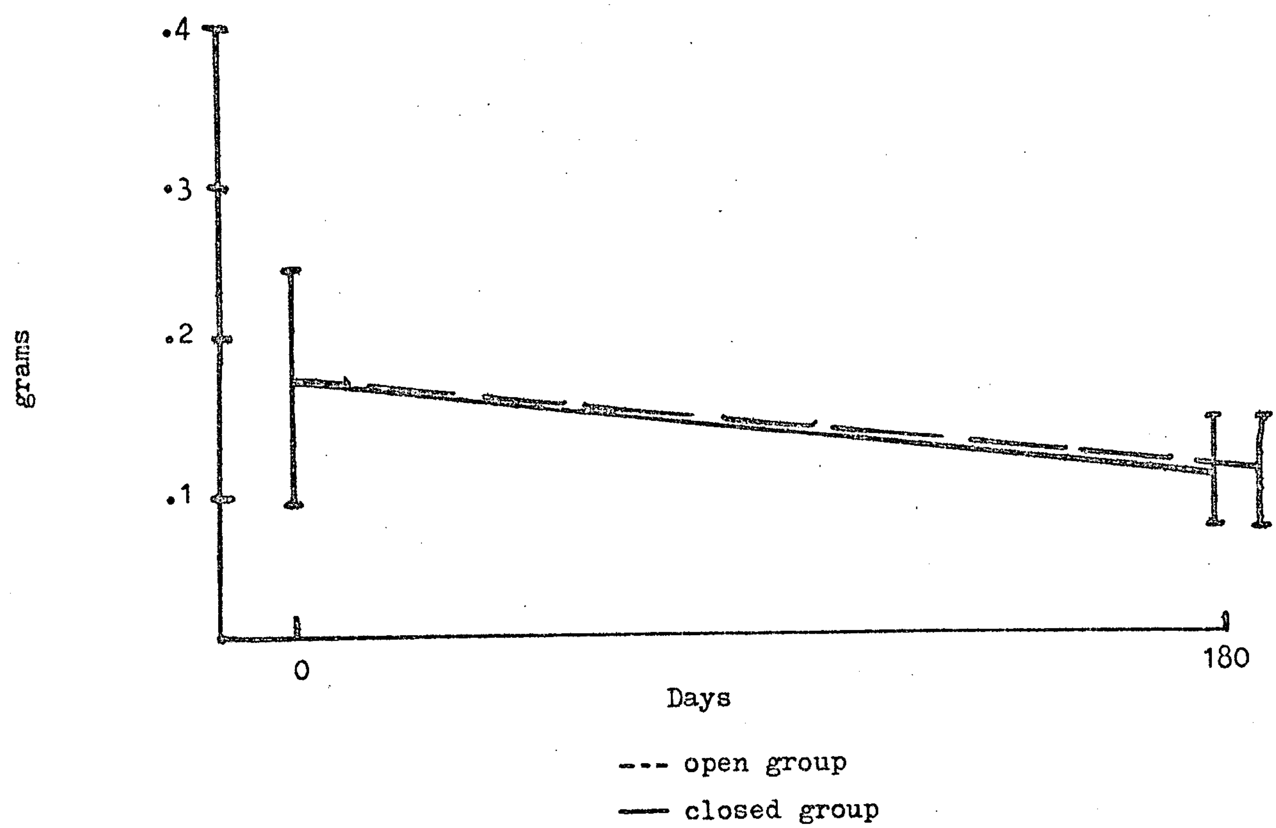


Figure 15. Wet Weight: Gut Contents - Ileum

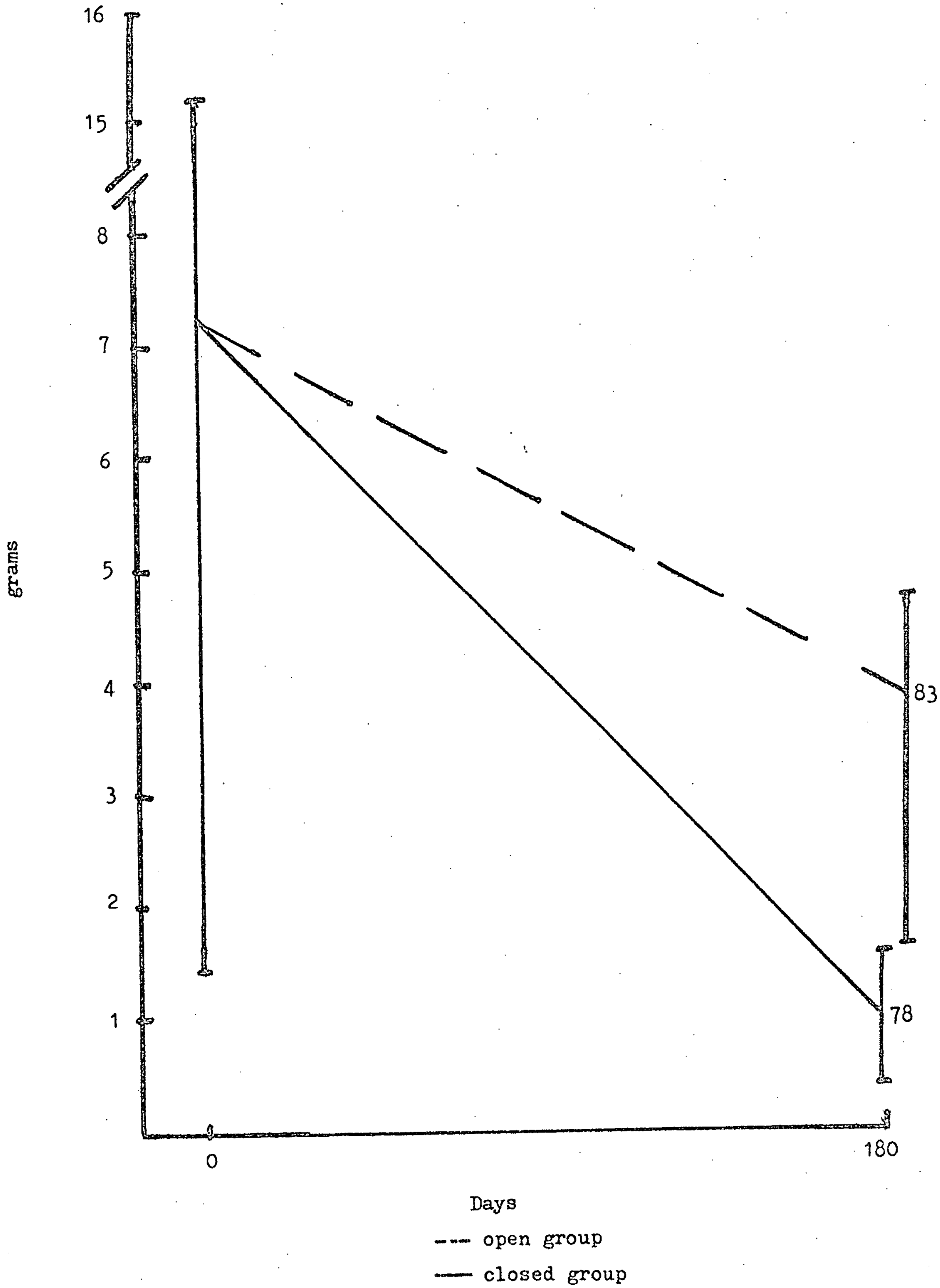


FIGURE 16. Dry Weight: Gut Contents - Ileum

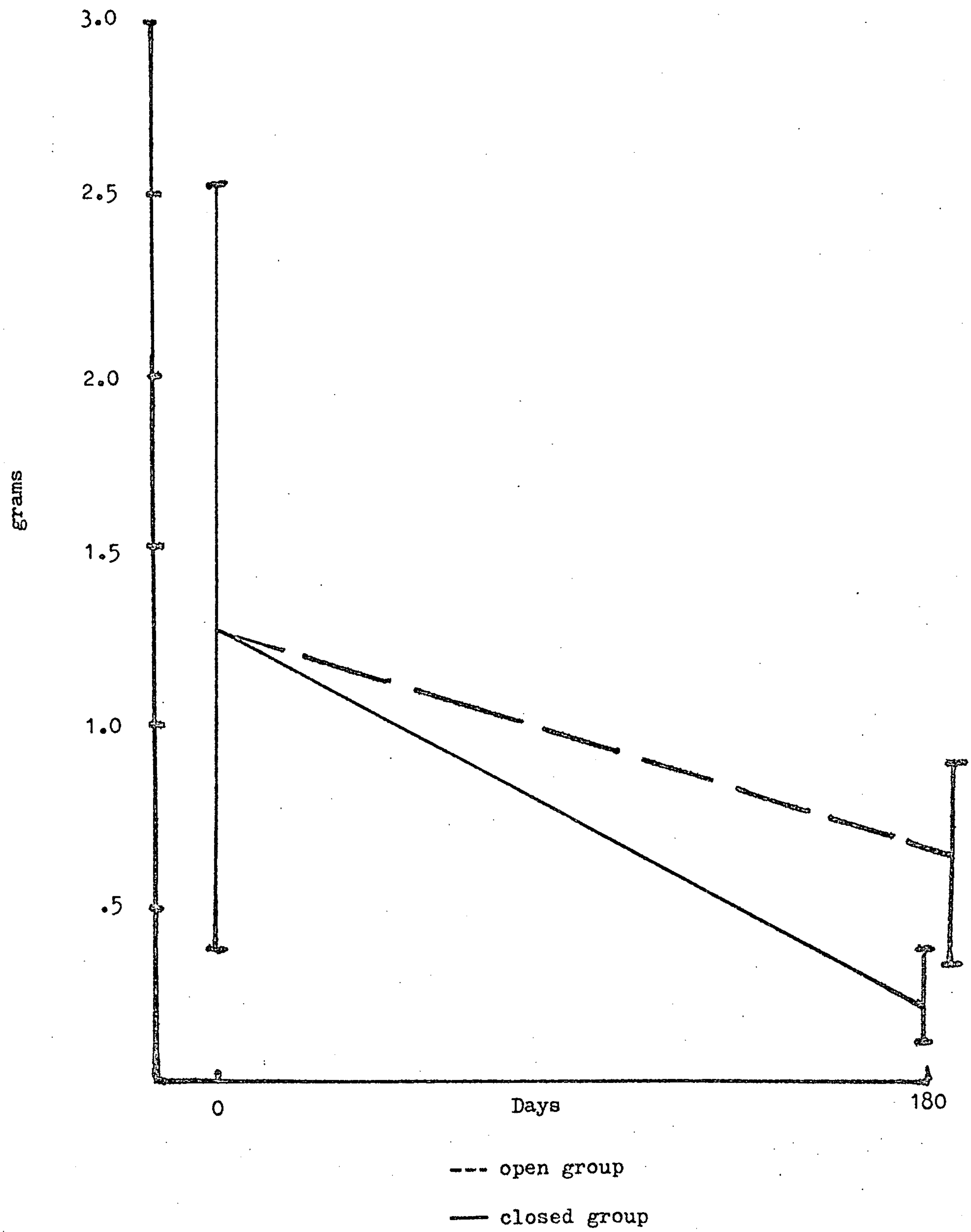


FIGURE 17. Wet Weight: Gut Contents - Colon

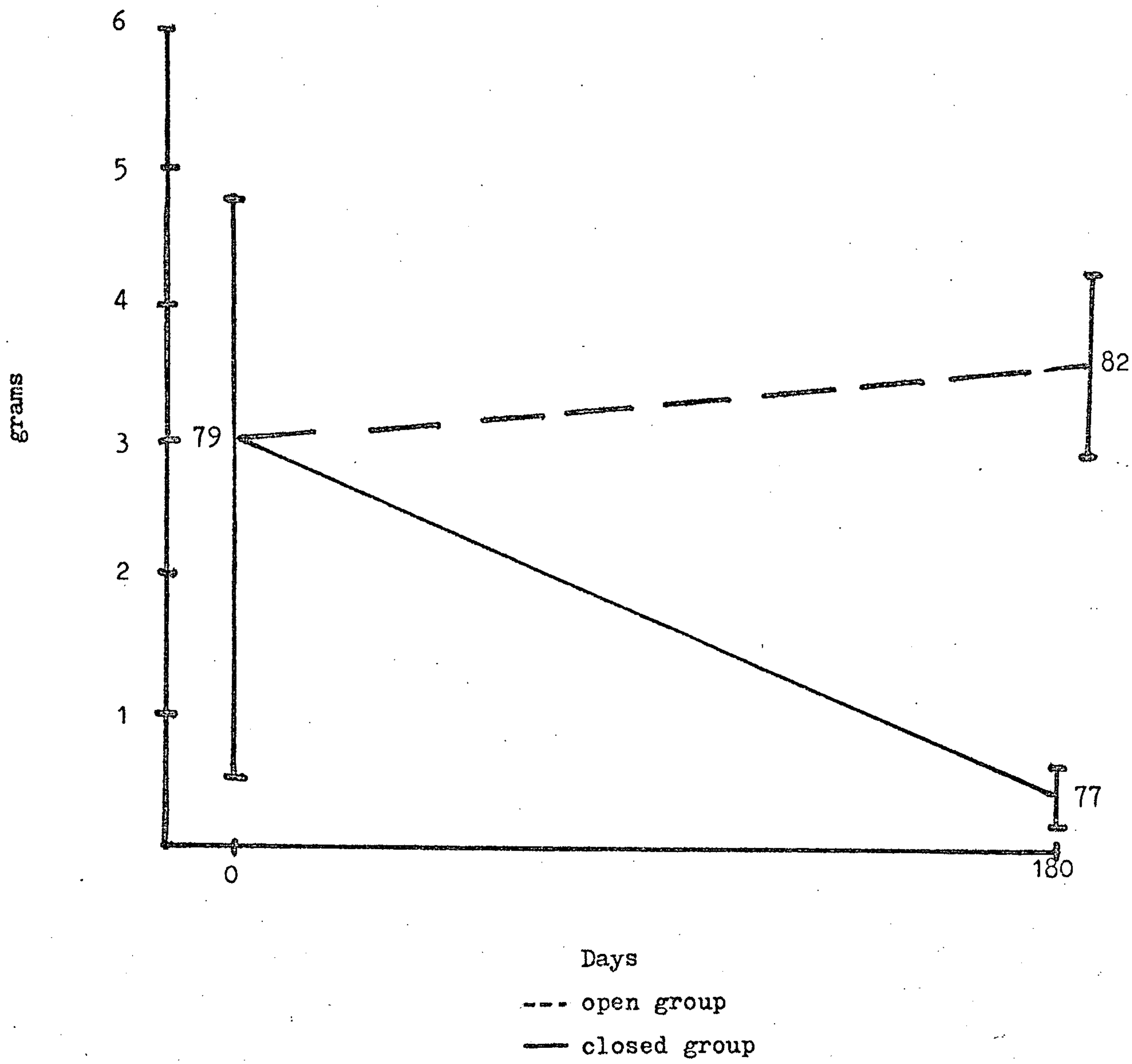


FIGURE 18.

Dry Weight: Gut Contents - Colon

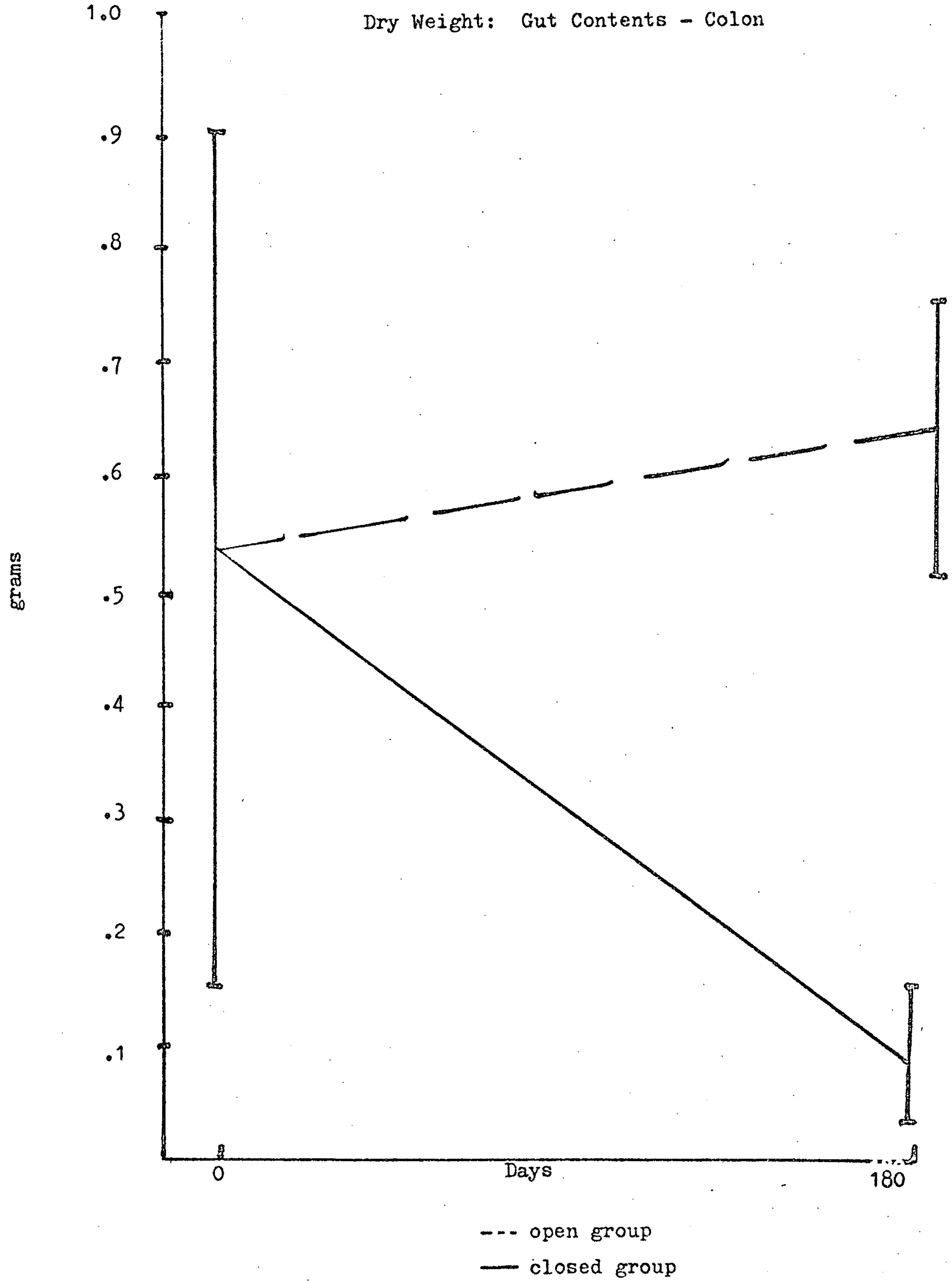


FIGURE 19. Wall Weight: Duodenum

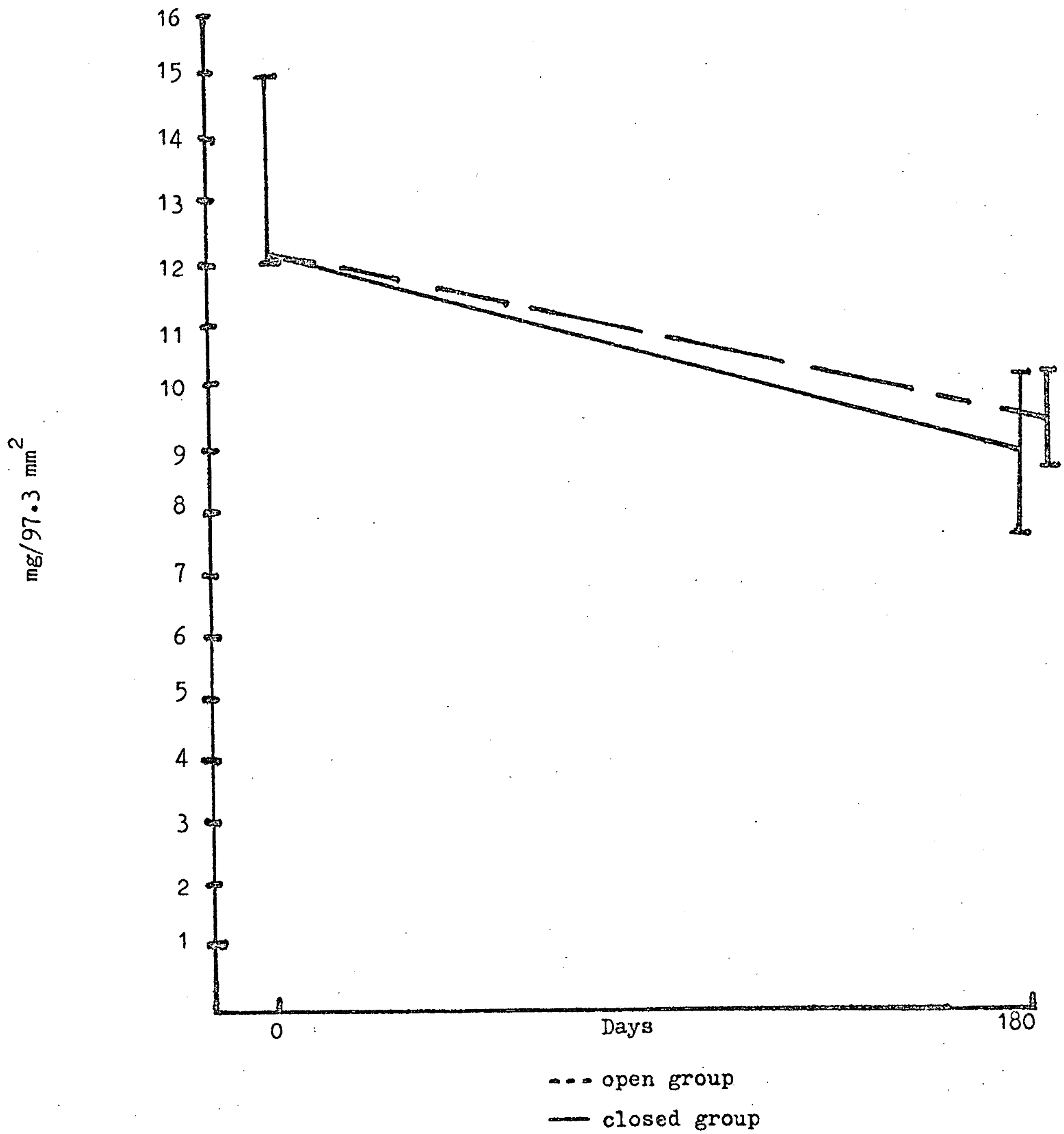


FIGURE 20.

Wall Weight: Ileum

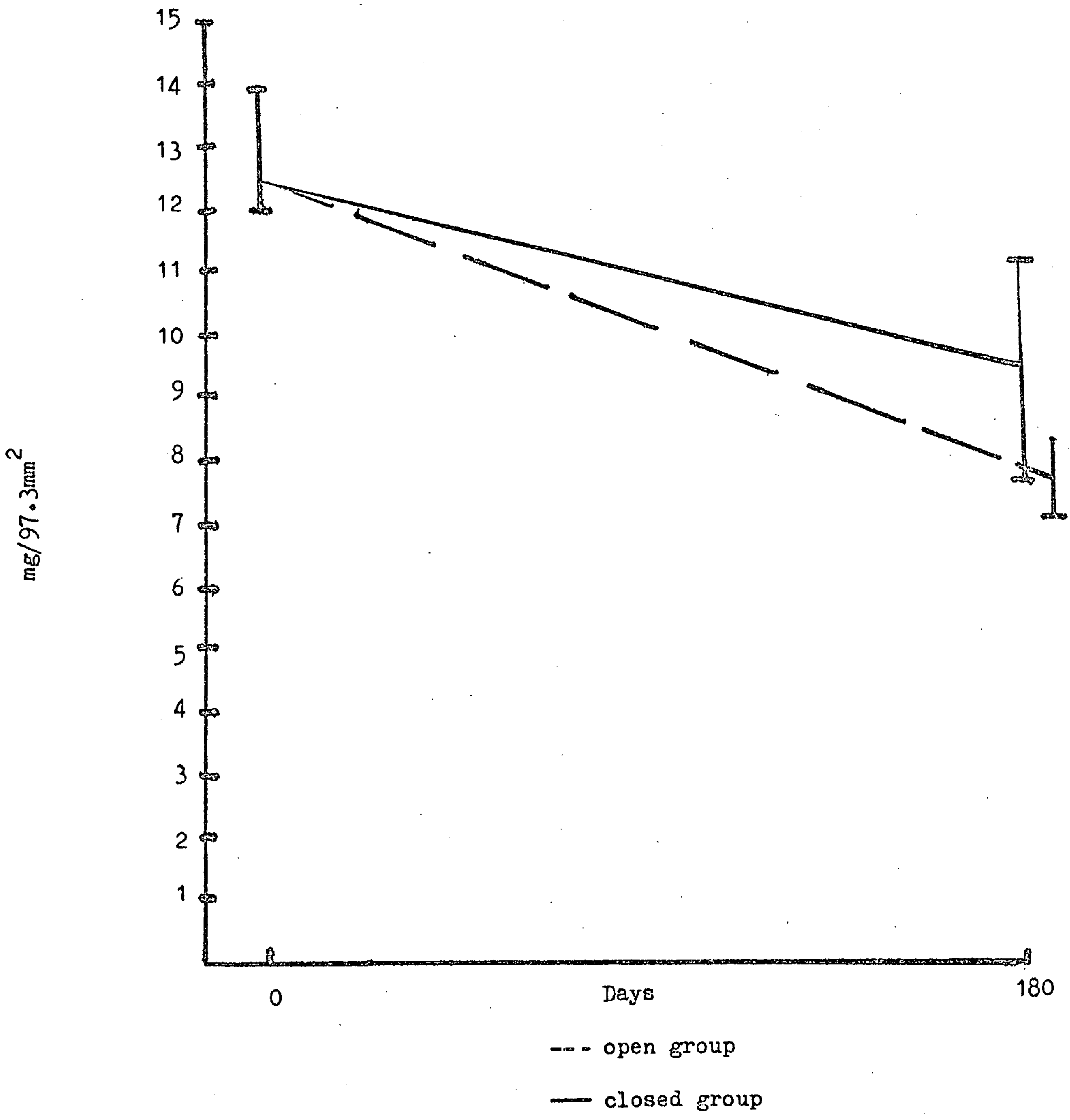


FIGURE 21.

Wall Weight: Colon

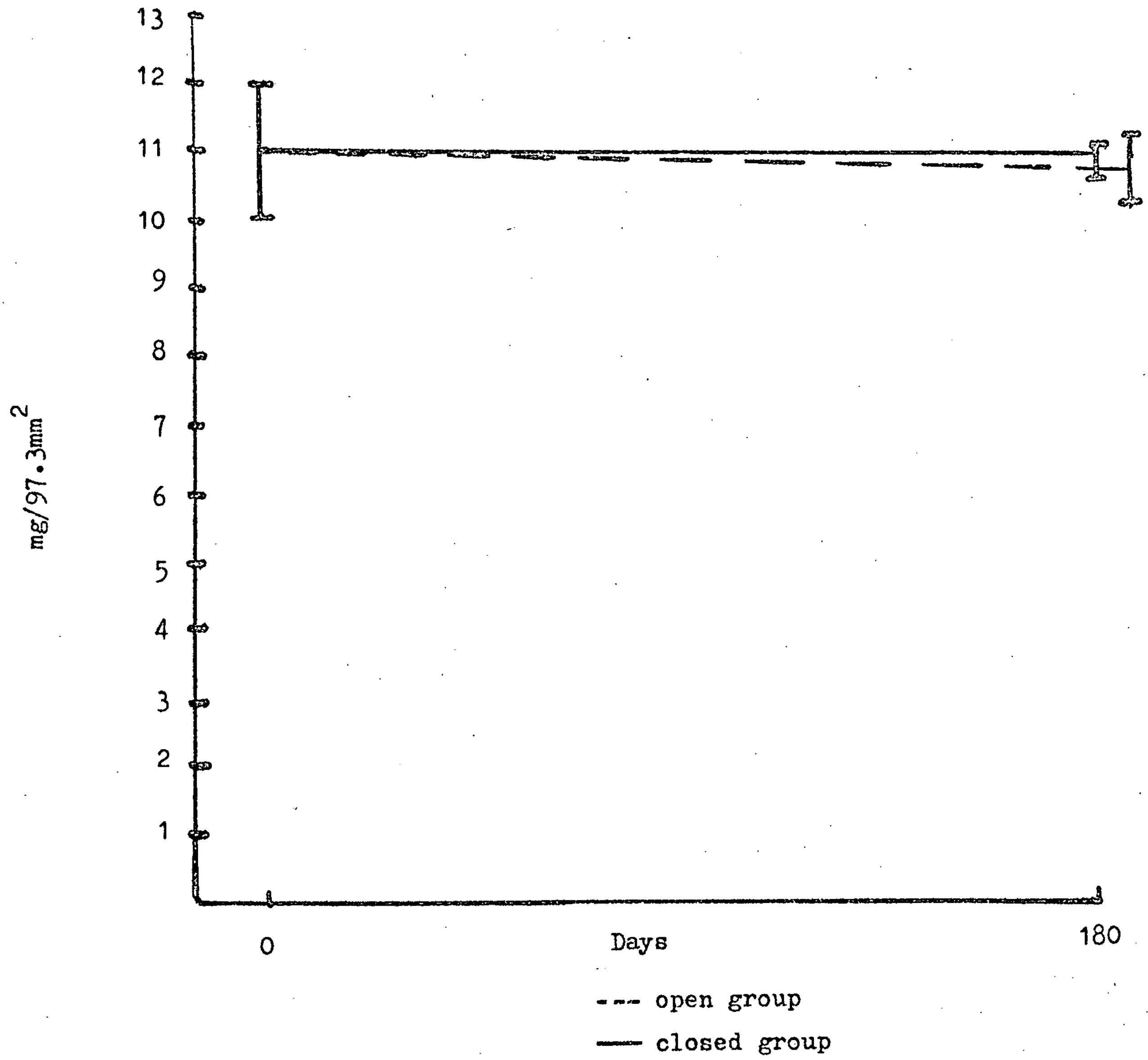


FIGURE 22. Assimilation: Day 0

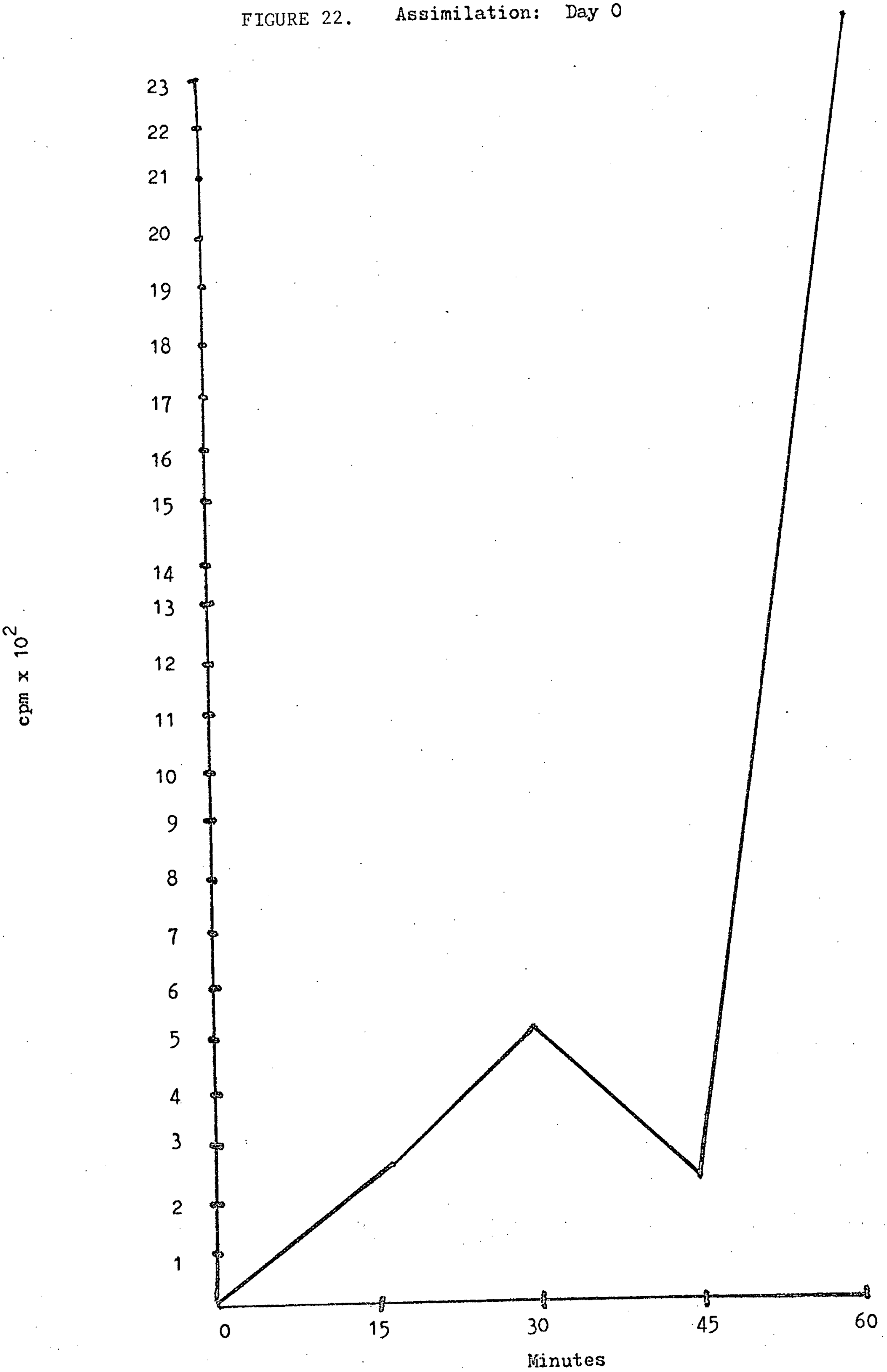


FIGURE 23. Assimilation: Day 180

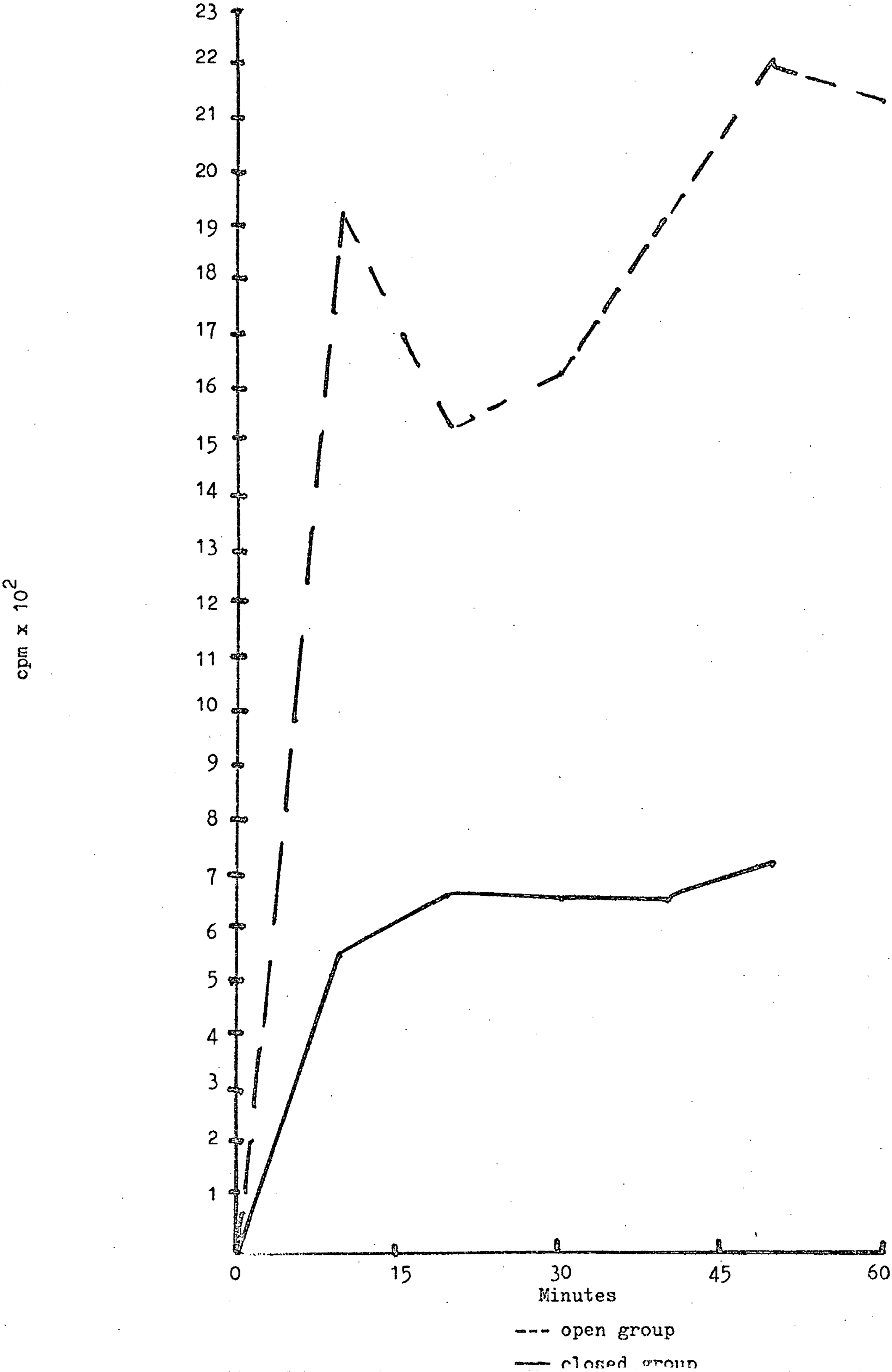


FIGURE 24. Hematocrit

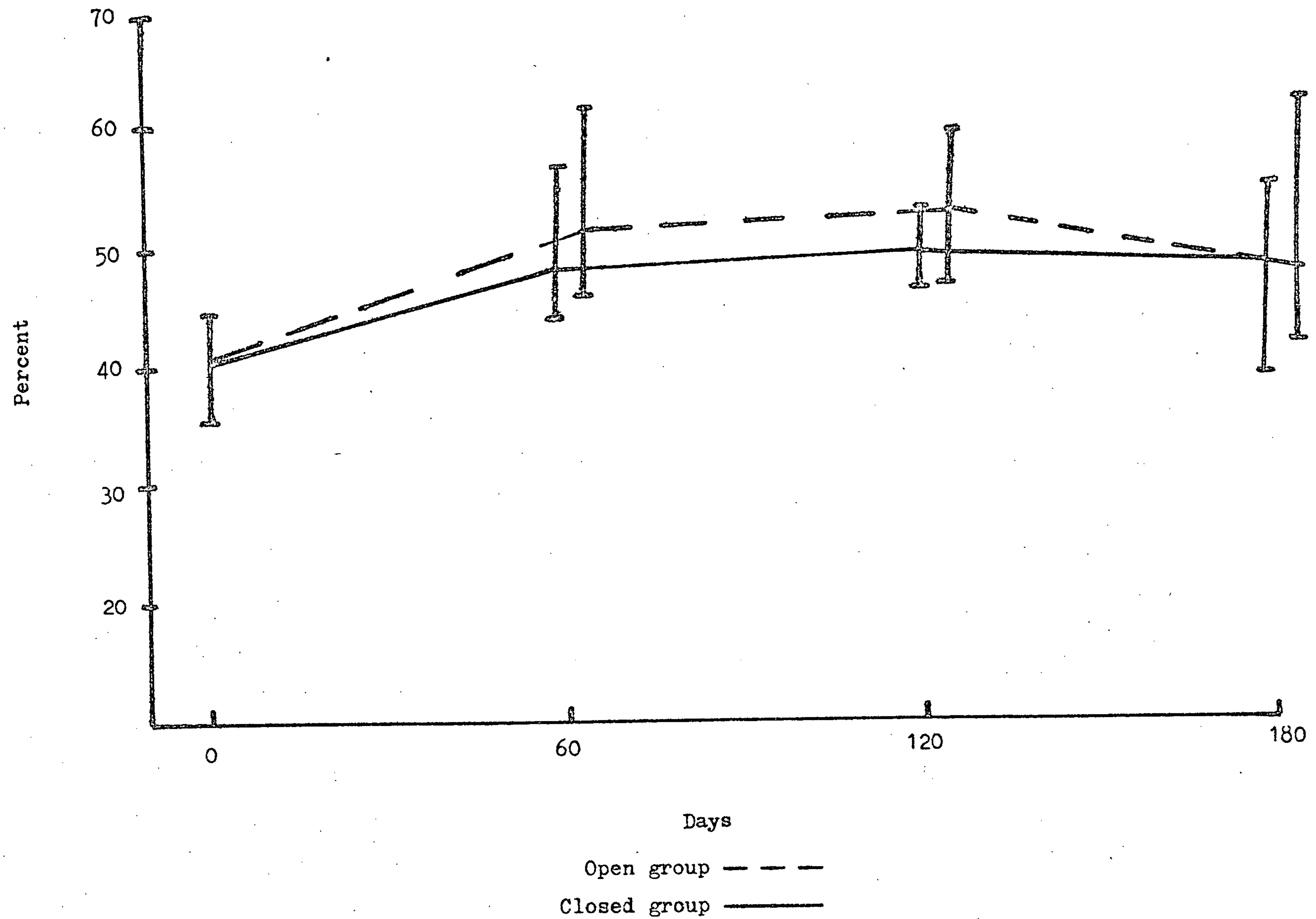


FIGURE 25. Gamma Globulin

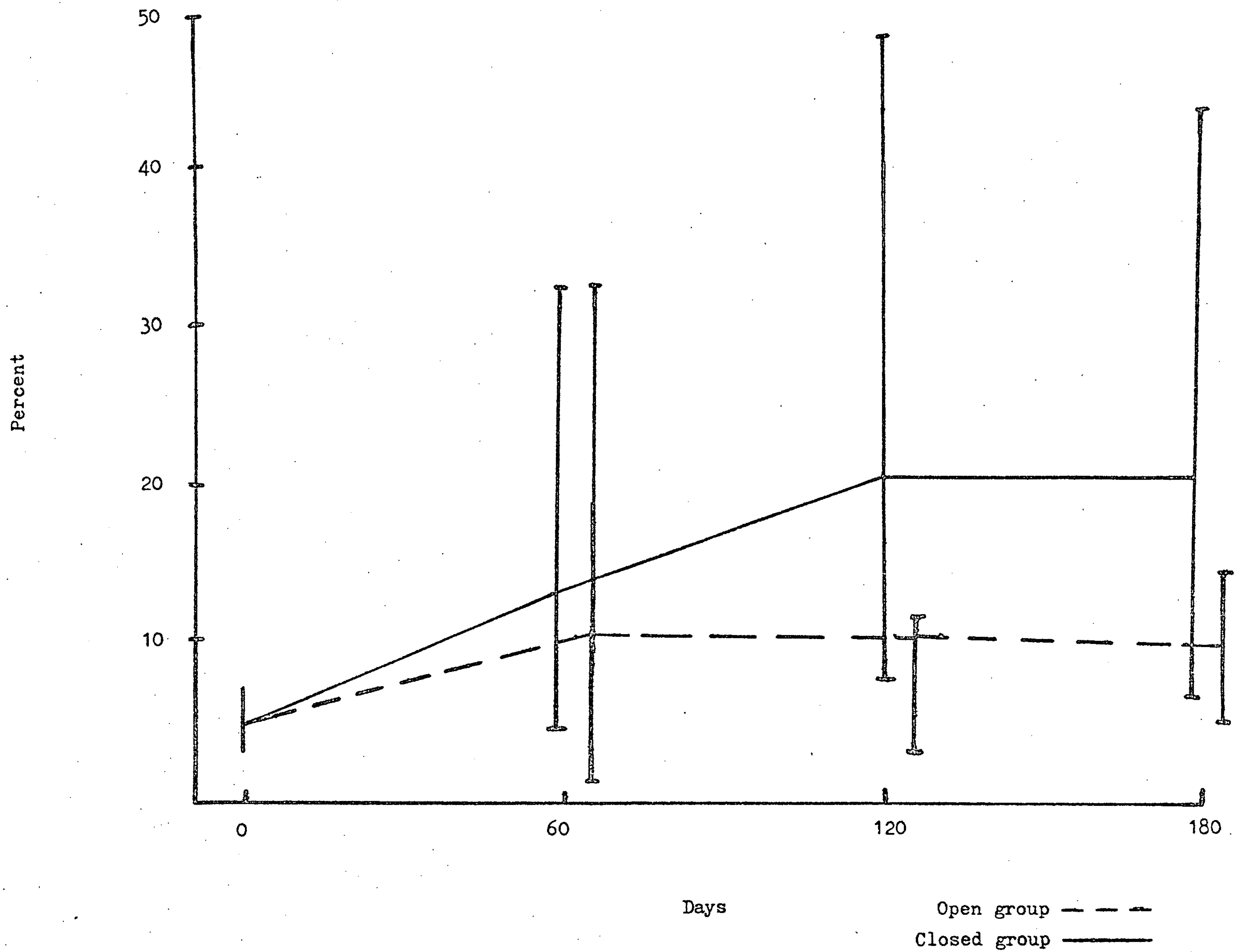


FIGURE 26. Protein

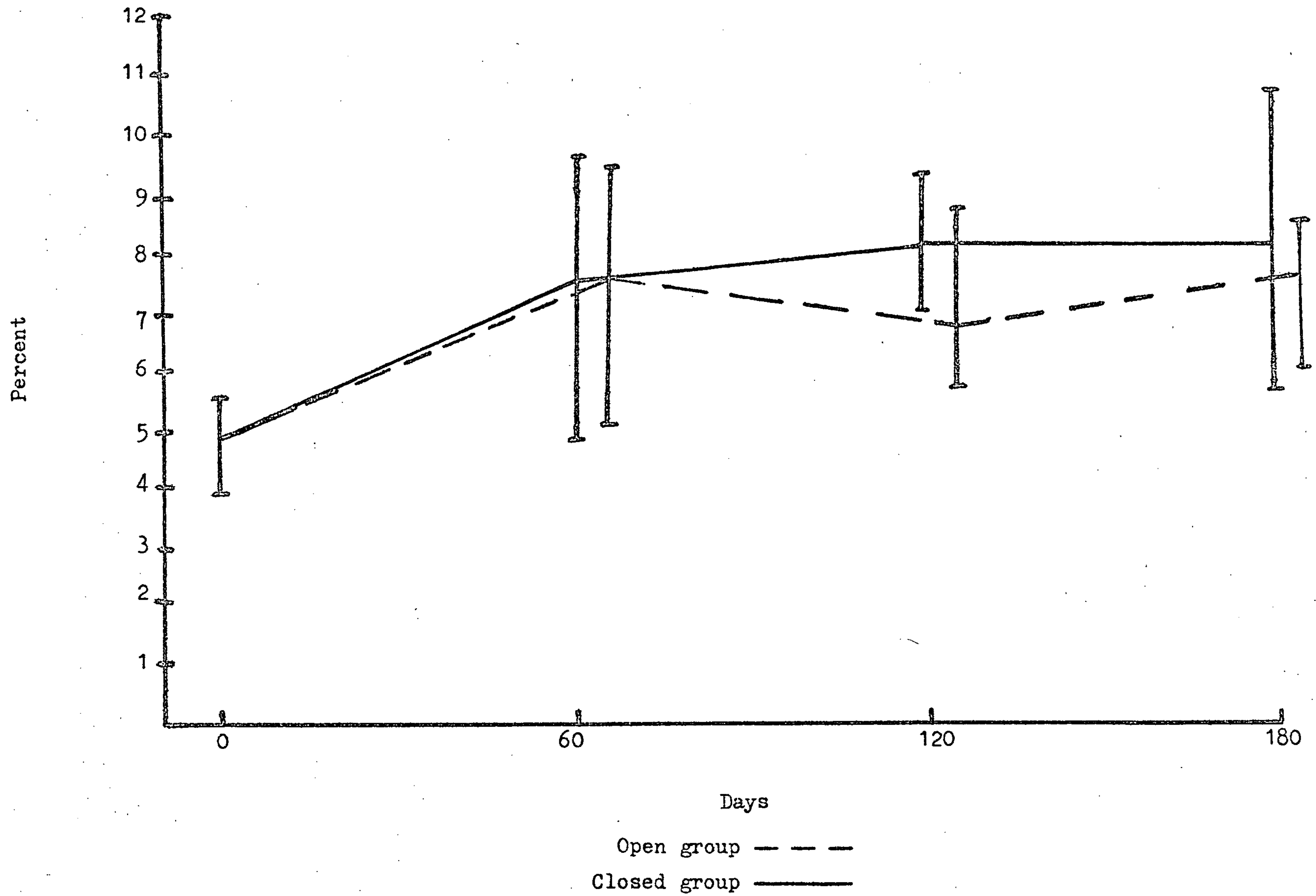


FIGURE 27. LDH

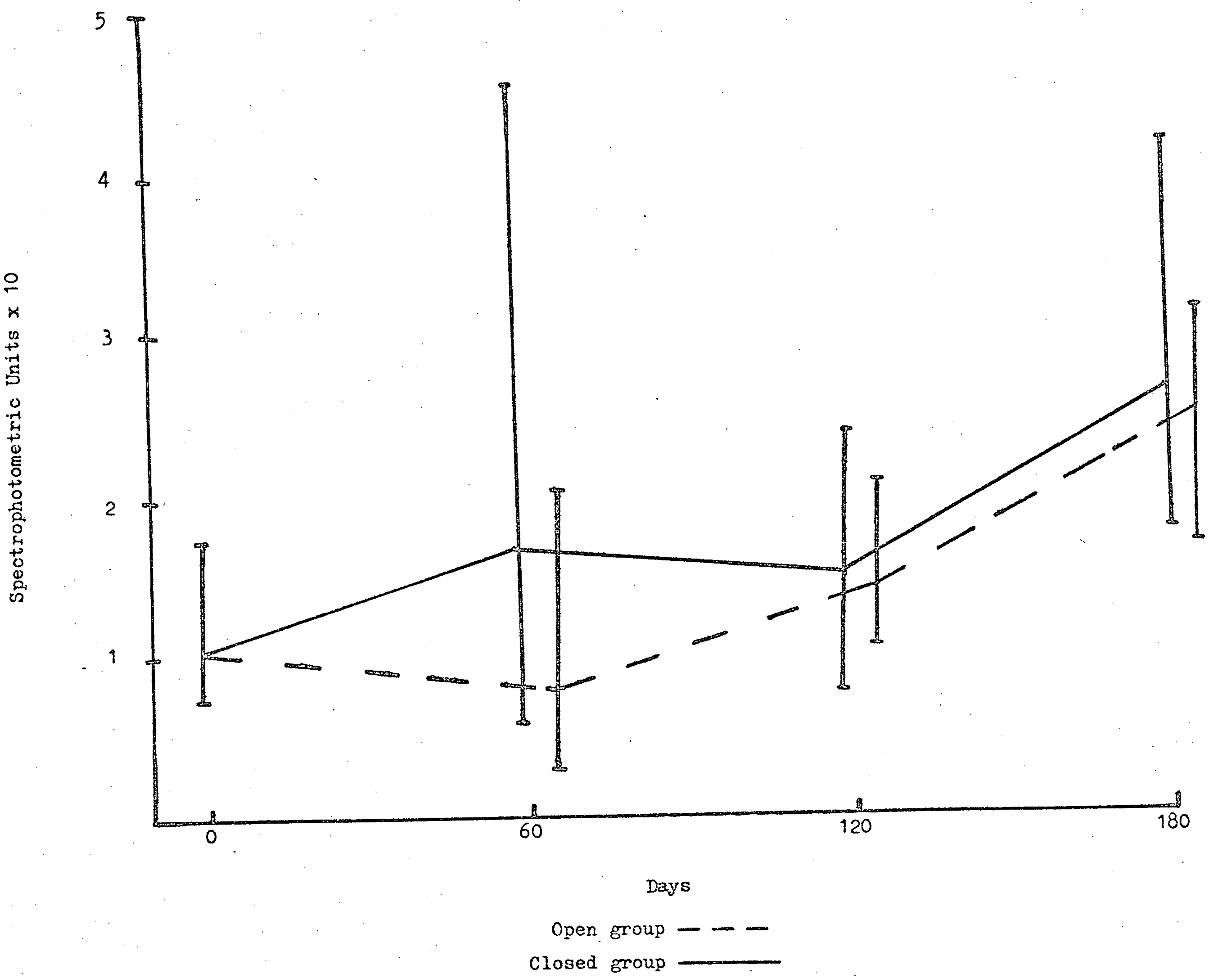


FIGURE 28. Alkaline Phosphatase

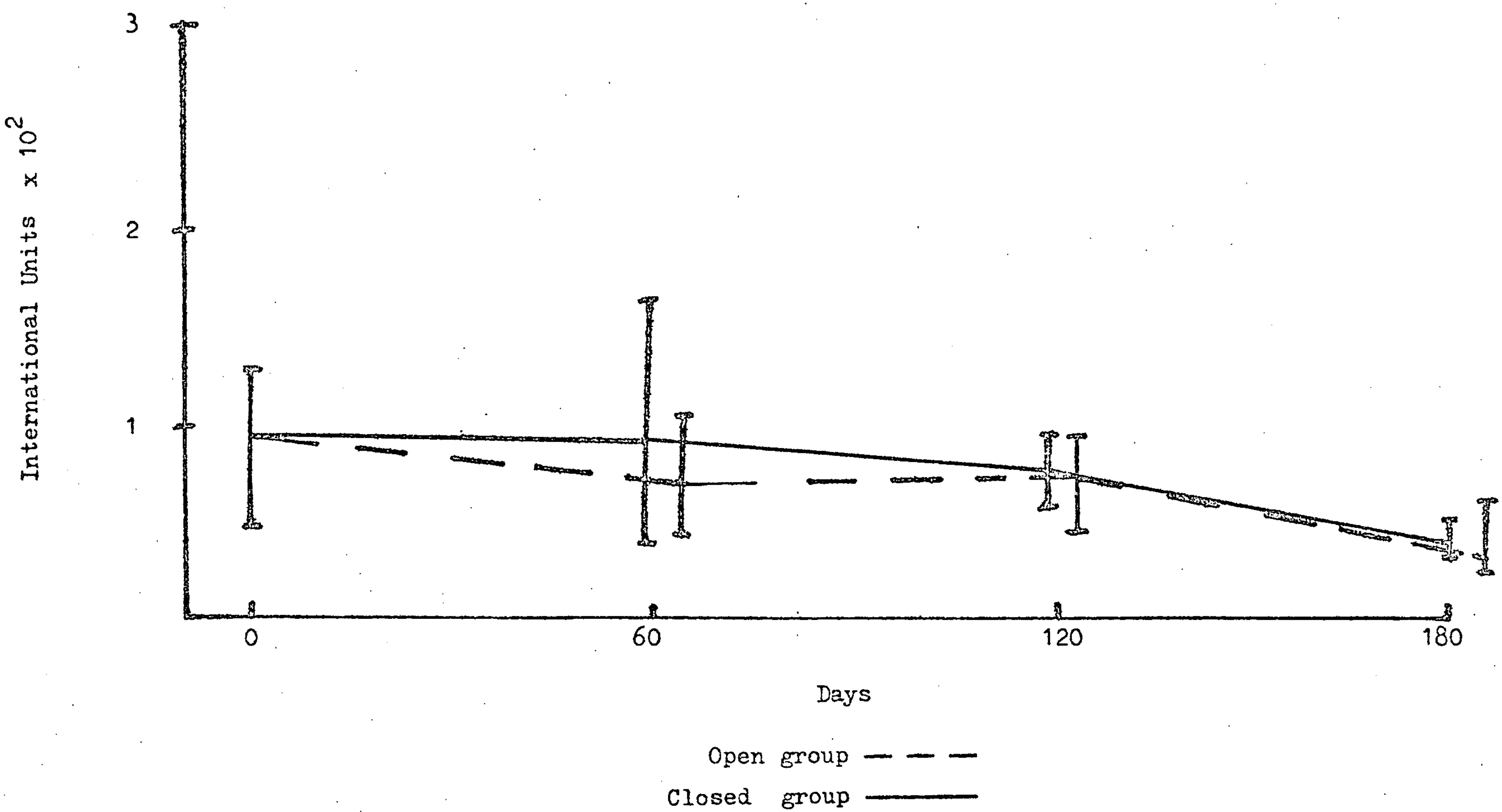


FIGURE 29. SGOT

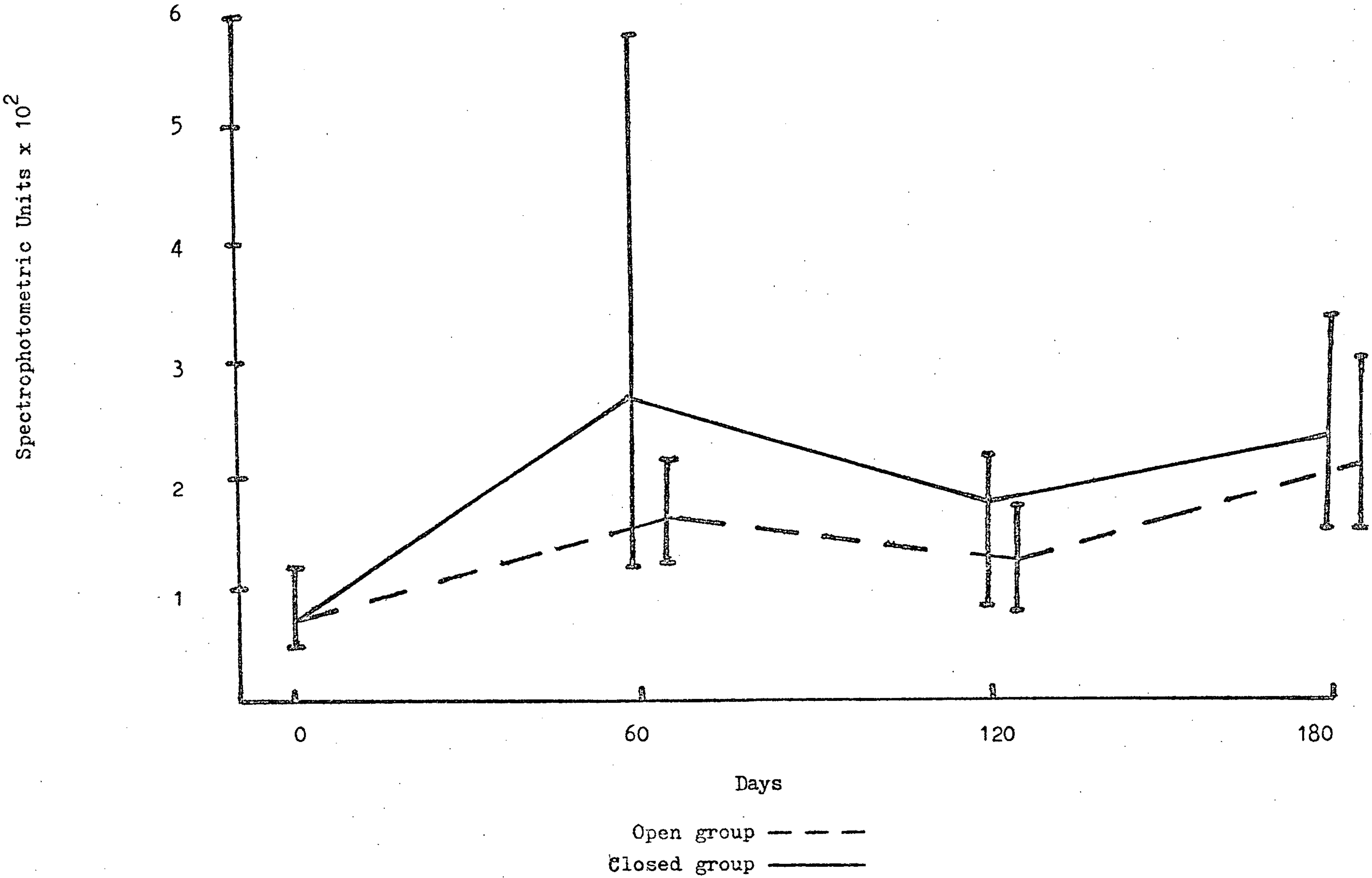
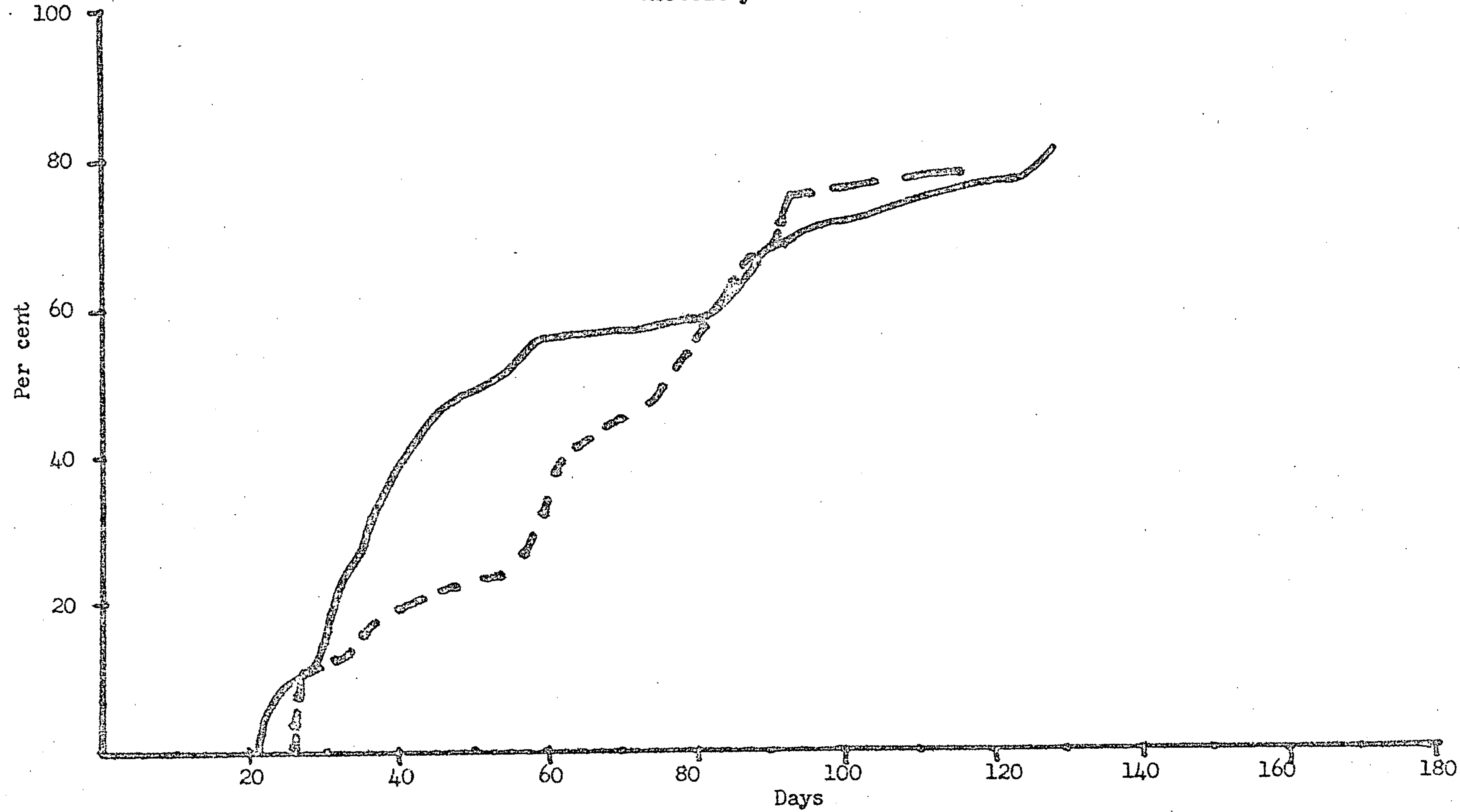


Figure 30

Mortality



Open group = - - - - -

Closed group = ———

Figure 31.

Body Weight

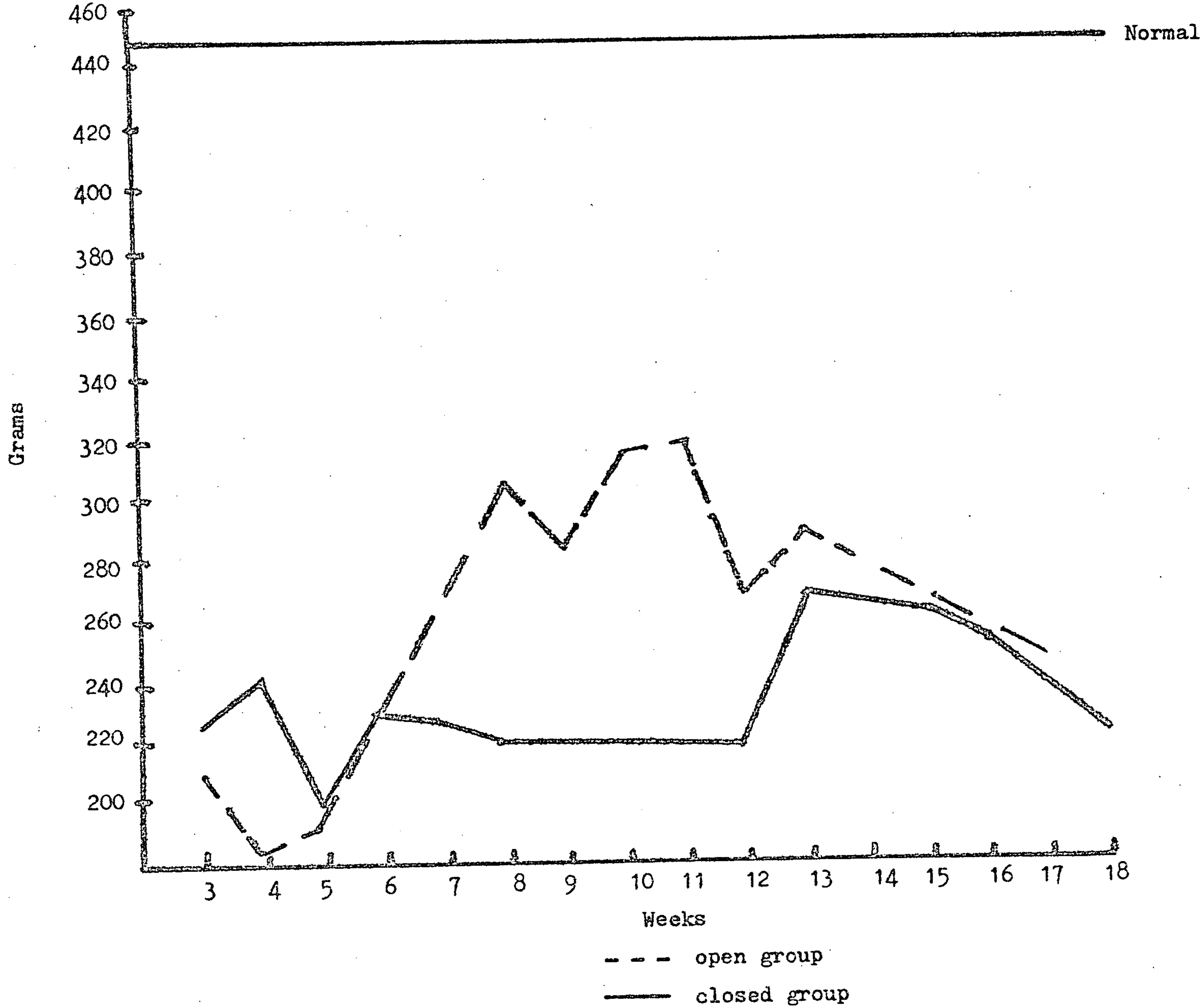


FIGURE 32. Liver

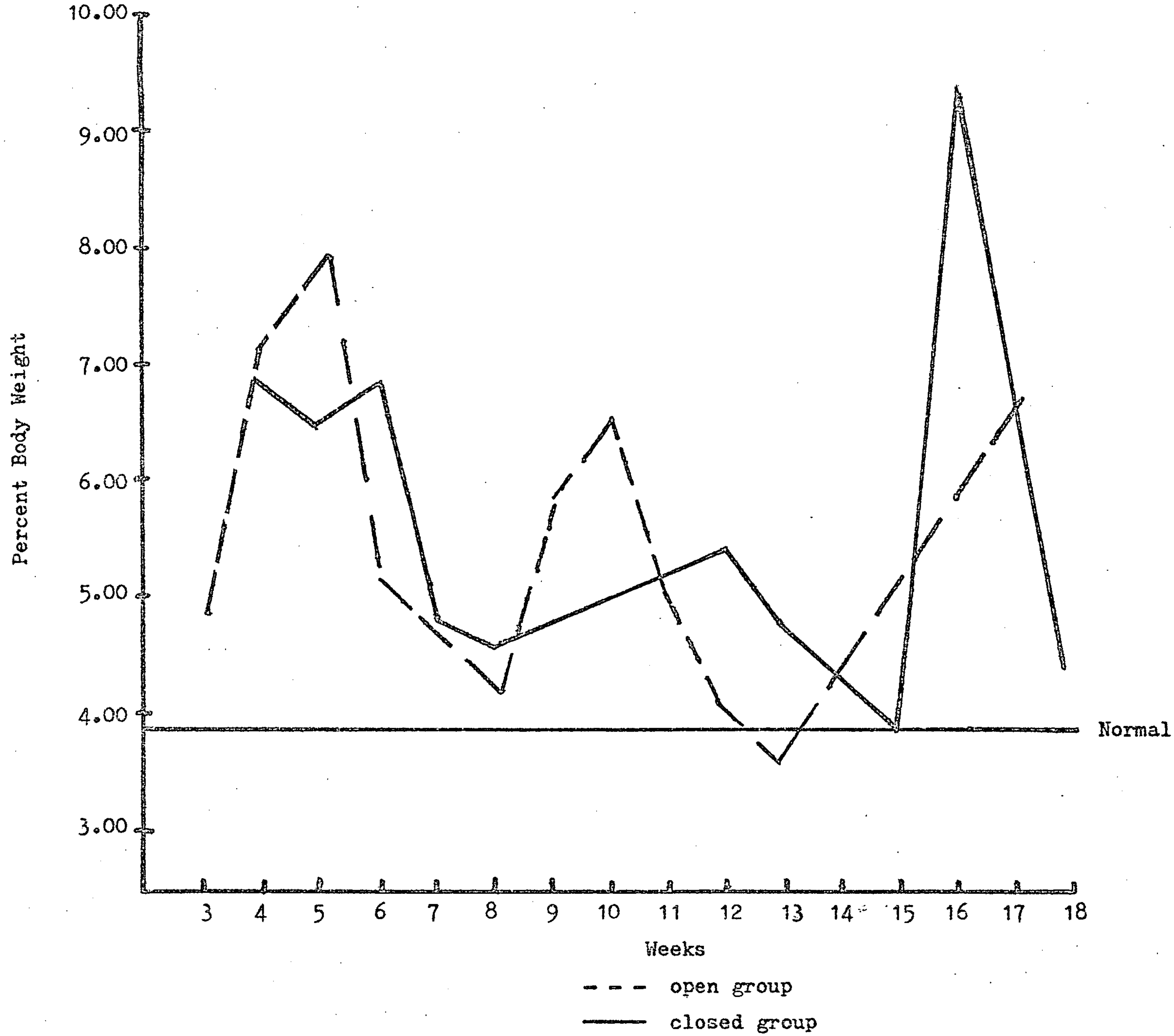


FIGURE 33. Spleen

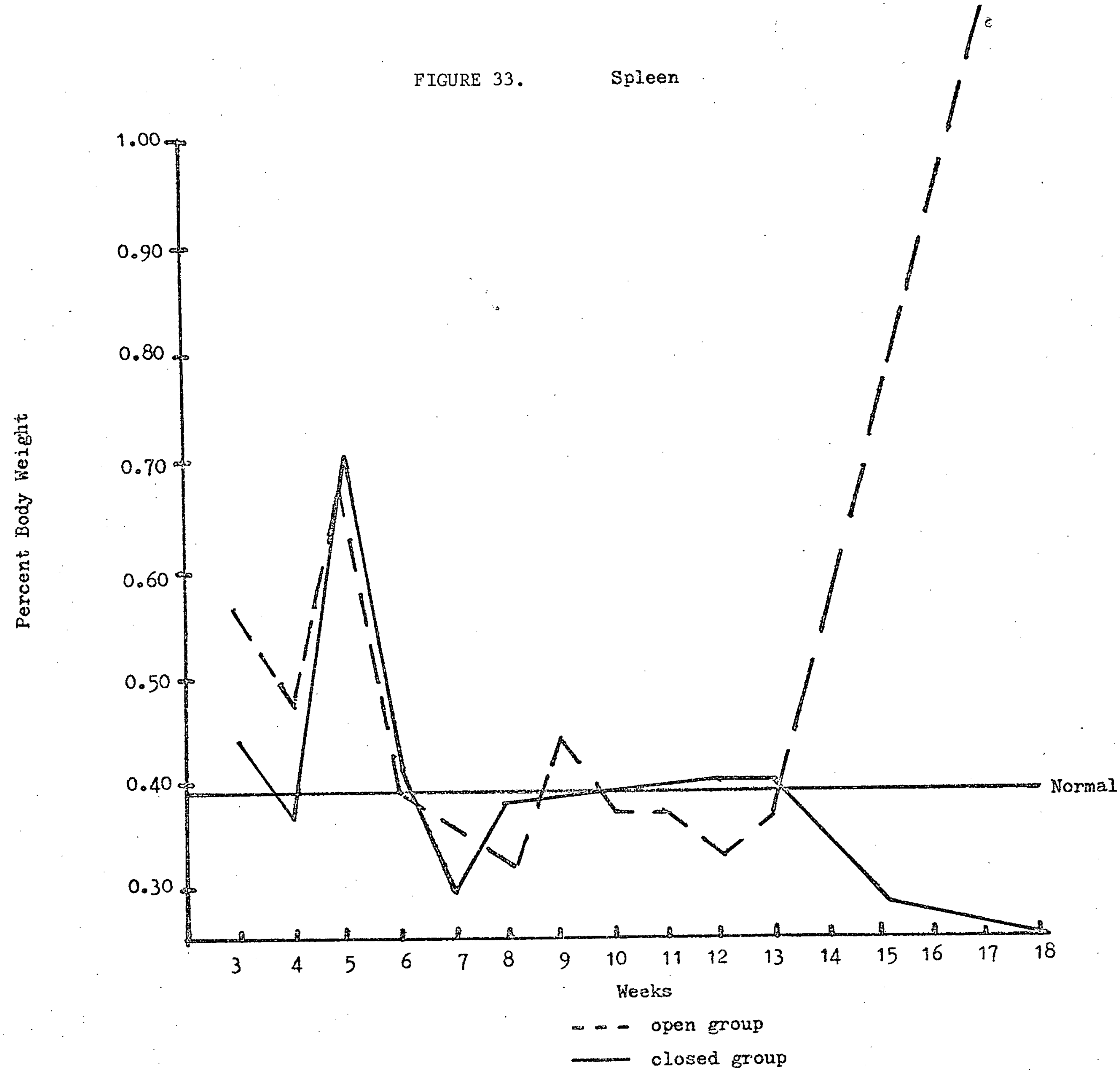


FIGURE 34. Kidney