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ORBITAL FLIGHT EFFECTS ON CALCIUM KINETICS AND FRACTURE HEALING

FINAL REPORT - 15 FEBRUARY 1970

CONTRACT NUMBER NAS2-5245

The Bioastronautics Laboratory
University of California, Davis
John R. Beljan, M.D., Principal Investigator
Associate Professor of Surgery, School of Medicine
Associate Professor of Engineering, College of Engineering

FINAL REPORT

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National Aeronautics and Space Administration
Contract NAS2-5245
with the University of California (Davis Campus) entitled:

ORBITAL FLIGHT EFFECTS ON CALCIUM KINETICS AND FRACTURE HEALING

Principal Investigator: JOHN R. BELJAN, M.D., Associate Professor of Surgery, School of Medicine, and Associate Professor of Engineering, College of Engineering.

Co-Investigators: RICHARD L. BELL, Ph.D., Associate Professor of Chemical Engineering, College of Engineering, and Associate Professor of Physiological Sciences, School of Veterinary Medicine.

RUSSELL R. BURTON, D.V.M., Specialist in the Agricultural Experiment Station.

Collaborators: MARVIN GOLDMAN, Ph.D., Research Radiobiologist, Radiobiology Laboratory, Lecturer in Physiological Sciences, School of Veterinary Medicine, and Professor of Radiobiology, School of Medicine.

FRANK H. KRATZER, Ph.D., Professor of Poultry Husbandry, College of Agricultural and Environmental Sciences.

LEO C. NORRIS, Ph.D., Lecturer in Poultry Husbandry, College of Agricultural and Environmental Sciences.

ARTHUR H. SMITH, Ph.D., Professor of Physiology, College of Agricultural and Environmental Sciences.

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MINERAL KINETICS STUDY

Introduction

Any determination of the effects of an environmental or nutritional perturbation on normal calcium homeostasis requires a basic understanding of some of the mineral kinetics of the system under study. In the avian model used, in studying the effects of weightlessness on skeletal quality, repair of calibrated osteotomies in weightlessness as well as at 1-"G" will include a quantitative evaluation of the dynamics and source of mineral utilized in bone repair. In describing the mineral model a series of studies are planned, the first of which is described below.

Many kinetic models have been published which attempt to summarize the salient features of calcium absorption, deposition and incorporation into bone mineral, and subsequent reutilization and fate. Common to all such models are compartments interrelated by varying influent and effluent rates and estimated by numerous experimental approaches. In many, a calcium balance is attempted by determination of the rates of calcium ingestion and excretion and, by difference, a general diagnosis of positive or negative balance is derived. If a radioactive tracer of calcium is introduced into the system, further information regarding

dilution (pool size) and concentration rate of change (clearance) can be obtained. Perturbation of the system usually alters the values and often assists in determining the most likely causal explanation of the change.

It should be emphasized that the focus of this pilot experiment was to characterize the calcium dynamics of the avian model in our standard laboratory environment, at normal gravity, on a normal diet and in an unstressed condition. The results of this study will be compared, at a later date, with similar data from birds (1) on a decalcifying diet, (2) in restraint, (3) with controlled osteotomies, (4) in hypergravitational fields, and/or (5) combinations of these conditions. The experiments will initially be quantitized using a linear kinetics model, the coefficients of which should reflect changes in the avian's mineral homeostasis.

It is anticipated that, as data accumulates, a clear picture of the bird's calcium kinetics will develop. This will, hopefully, enable a more definitive model to be formulated containing specific physiologic parameters.

The agreement between the mathematical constants derived from similarly treated animals is an indication of population variation essential to separation of treatment effects from biologic variability. The spread in results of treated animals relative to comparable controls thus assists in the evaluation of the quantitative validity of

the data as well as qualitative changes in kinetic parameters.

The object of this first study was to determine the rate and degree of blood calcium exchange, using simple isotope dilution techniques, and to obtain a preliminary estimate of the fractorial skeletal uptake of a labeled pulse of blood calcium.

Material and Methods

Twenty male Single Comb White Leghorns of fifteen months mean age were used in this experiment. For fourteen days before and during the experiment, the birds were housed in 16" x 10" wire cages and maintained on a commercial ration containing approximately 2% calcium. Deionized water was available for drinking for fourteen days in advance of the isotope administration. During this time, evaluations of the health and physical condition of the birds included daily weighing and weekly hematological examination consisting of a hematocrit determination and a differential leukocyte cell count.

The birds were divided into two groups of ten each. One group was used to study blood ^{47}Ca concentrations over the period from five minutes to eight hours after isotope injection. The other group of ten provided data on blood and bone ^{47}Ca and whole body retention of the isotope for the period from four hours to seventy-two hours. The latter

group (the long-term study birds) were not given any special preparatory treatment before isotope administration. Their average body weight before isotope administration was 1.91 Kg., and at time coincident with their last scheduled blood sampling, 1.95 Kg. The short-term birds, however, were subjected to preparatory treatment, as described below.

Pre-injection Preparation - Cannulation

The right jugular vein of each of the short-term birds was ligated and an 8" long Silastic(R) catheter[1] (1.6 mm. I.D., 3.2 mm. O.D.) was inserted into the jugular vein, proximal to the occlusion. The indwelling end of the catheter reached nearly to the left atrium, while the other end protruded about 2" through the skin of the neck. This was occluded by an obturator when the catheter was not used for fluid withdrawal or addition. The cannulation was completed not less than five days before the administration of isotope. In all cases, the birds showed post-operative weight loss, but by the fifth day, the body weight had stabilized or showed definite signs of stabilization. The average weight loss during the recovery period was 0.16 Kg., and the average body weight of the birds was 1.84 Kg., at the time of isotope administration.

The short-term birds received doses of procaine penicillin G during the post-operative recovery period (but not

[1] Dow Corning Corporation, Midland, Michigan.

after isotope injection). Their catheters were flushed daily with ∿10 ml. of heparinized saline (100 U.S.P. units/100 ml.) to maintain catheter patency.

Radionuclide Administration

The radionuclide, ^{47}Ca chloride in HCl (specific activity 12.3 mCi./g., radiochemical purity >99%)[2] was diluted with saline (0.9 gm. NaCl/100 ml.) to provide an injection stock solution with a concentration of approximately 16 μCi/ml. Each of the twenty birds received a single injection of the stock solution, equal to 1.98±0.01 ml. per dose (i.e. 32 μCi). The isotope was injected into the left brachial vein with a 2½ ml. Monoject(R) disposable syringe. Particular care was taken to insure intravenous administration.

Blood Sampling and Sacrifice

Blood sampling was done through the cannula for the ten short-term birds, and where possible, from the right brachial vein of the long-term birds. To prevent mixing of the blood sample with the heparinized saline in the cannula, one milliliter of fluid from the cannula was withdrawn through a 16 gauge needle into a disposable syringe and discarded. A disposable syringe containing 0.05 ml. of heparin (1,000 U.S.P. u./ml.), was then used to withdraw a 3 ml.

[2] General Electric Corporation, San Jose, California.

blood sample for analysis. After blood withdrawal, 3.6 ml. of dextran [(6 gm. dextran-75 + 0.9 gm. NaCl)/100 ml.] was injected to replace the blood volume removed, and 0.5 ml. of heparinized saline (100 U.S.P. u./100 ml.) was then injected into the cannula to prevent clotting.

The "sample time" for calculation purposes was measured from the mid-time of the tracer injection to the mid-time of the blood withdrawal step (a 45-second procedure). The cannula was closed by an aluminum obturator between sampling periods, with precaution taken to insure no heparinized saline was allowed to seep out of the cannula. The birds in the short-terr group each had samples taken at 5, 15, 30 minutes and 1, 2, 4 and 8 hours following ^{47}Ca administration.

Three-ml. blood samples were taken from the ten long-term birds' right brachial vein with a syringe previously heparinized with 0.05 ml. of heparin. In a few cases the left brachial vein or cardiac puncture was used when the vein(s) became obscured by hematoma formation from earlier blood sampling. Birds were sacrificed with an injection of Beuthanazia$^{(R)}$, immediately after their last scheduled blood sampling as shown in Table I.

Each carcass was placed inside a thin-walled polyethylene bag for whole body counting of ^{47}Ca. When the counting was completed, the two tibiae and the two humeri of each bird were removed, scraped and trimmed of soft tissue and

cartilage. The bones were subsequently measured for their ^{47}Ca content and then analyzed for their total calcium content.

Blood Samples

Duplicate hematocrits for each blood sample were determined on an International micro-capillary centrifuge[3] by the method used by Burton, Sahara and Smith (1966).

A drop of whole blood from the syringe was used to make a smear for differential cell counting. The remaining blood was transferred into a 10 mm. diameter x 70 mm. plastic tube which was then placed inside a thick-walled centrifuge tube and spun for ten minutes in a clinical centrifuge[4], at 4,000 R.P.M. About 1 ml. of the plasma was withdrawn with a Pasteur pipette and placed in a previously-weighed 10 mm. x 70 mm. plastic tube. After being weighed, the tube of plasma was placed inside a 16 mm. diameter x 125 mm. well counting tube for subsequent gamma-ray assay.

Bone Samples

The right tibia (with fibula removed) and humerus were stripped of soft tissue and weighed. The bones were then wrapped in a small piece of aluminum foil, and fragmented

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[3] International Equipment Company, Needham Heights, Mass. Model MB.

[4] International Equipment Company, Needham Heights, Mass. Model CL.

with a hammer. The fragmented bone was divided into three portions to fit into 25 mm. diameter x 80 mm. glass bottles designed for the well counter.

Measured sections of the midshafts of the left tibia and humerus were also excised, weighed, and assayed for their ^{47}Ca content. After cleaning, the length of the intact bone was measured, and the midpoint marked. A fine saw cut was made one-half inch on either side of the midpoint. A specially designed jig was employed to insure uniformity of the length excised. The midshaft section was then weighed and placed in a glass counting bottle.

Radioactive Assays

PLASMA AND BONES. The samples described above were assayed for their ^{47}Ca content in an automatic well counter[5] which utilized a 3" diameter x 3" NaI(Tl) scintillation crystal.[6] The machine was programmed to count a maximum of ten minutes, or 20,000 counts per sample, whichever was attained first. The values that were recorded and used for calculations were net counts per minute, in the gamma energy from 1.2 to 1.4 mev. The maximum number of samples that could be counted in any one cycle was 99. Where possible, samples were cycled more than once.

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[5] Nuclear Chicago Corporation, Des Plaines, Ill. Model 4227. Automatic Gamma Counting System.

[6] Nuclear Chicago Corporation, Des Plaines, Ill. Model 972.

The effect of radioactive decay of ^{47}Ca (half-life 4.7 days), was eliminated by use of a ^{47}Ca standard with each batch of samples assayed by the well counter. A standard solution was made by diluting a single dose of the isotope solution, i.e., 1.98±0.01 ml. of the stock solution used for bird injection, into 100 ml. An aliquot of the dilution containing 1% dose/ml. was transferred into a 100 mm. x 70 mm. plastic tube and its weight determined. By comparing the activity of samples against the activity of the standard counted at the same time the amount of tracer in the biologic samples was determined, i.e.,

$$\%\text{Dose} = \frac{\text{Net c.p.m. in Sample}}{\text{Net c.p.m./1\% of Injected}}$$

WHOLE BODY ^{47}Ca ASSAY. The ^{47}Ca retained in the bird carcasses was determined in a large whole body counter located in the Radiobiology Laboratory, University of California, Davis. Each carcass was accurately placed one meter from the face of the detector, an 8" diameter x 4" NaI(Tl) crystal. The multi-channel analyzer was calibrated to a value of 10 kev per channel. The net count rate from 150 kev to 2,000 kev was recorded. The reference standard utilized consisted of a single dose of isotope, used for bird injection, diluted with 2.3 Kg. of water and contained in a one-gallon square polyethylene jug and counted in the same geometry as the birds.

Determination of Total Calcium in Plasma and Bone

Plasma calcium was determined on 0.2 ml. aliquots after radioactivity counting. To the aliquots were added 9.80±0.05 ml. of a 1% Lanthanum Oxide + 5% v/v HCl solution and the calcium in solution was measured at 2120 Å on an atomic absorption spectrophotometer[7], which used an acetylene-air flame.

The bone samples were individually wrapped in cheese cloth for defatting in a solvent extractor. Following an 18-hour ethanol reflux and a 16-hour ether extraction, the bones were weighed (fat-free weight) and dried in an oven at 100°-140°C. for six hours. The weight of the bones on cooling in a desiccator was determined, and was designated fat-free dry matter (FFDM) weight.

The bones were ashed in a muffle furnace at 500°-600°C. for 24 hours, and weighed after cooling. The bone ash was dissolved in about 2 ml. of 5% HCl and transferred quantitatively to a 100 ml. volumetric flask and brought to volume with distilled water and about 25 ml. of 2.5% HCl used for rinsing of the crucible. Two ml. of this solution was further diluted into 100 ml. using distilled water, and finally a 2 ml. aliquot in 25 ml. using a 1.1% Lanthanum Oxide +5.5% v/v HCl solution was assayed for calcium content on the atomic absorption spectrophotometer as described above.

[7] Perkin-Elmer, Norwalk, Conn. Model 303.

Results and Discussion

Blood Disappearance Data

The averaged ^{47}Ca blood disappearance data are presented in Figure 1 in terms of per cent of original dose. The raw data exhibited a significant spread in absolute magnitudes between birds which was not related to bird size (body water content). However, the tracer disappearance data for a given bird were internally consistent and data which were initially high or low relative to the mean remained so throughout the experiment. This observation suggested that despite the care taken to insure uniform dosage to each bird, there existed actual differences in initial whole body specific activity (i.e., μCi ^{47}Ca/ml. plasma) which were not accounted for by simple hemodilution. A factor was therefore calculated for each bird which would normalize the blood disappearance data for that bird to the mean value for all birds. The correction factor as described below was subsequently used to correct both the whole body and the bone data in accordance with the assumptions of a linear kinetics model.

The first step in computing the correction factors was to average the data obtained at a specific time over all birds. The means thus obtained are plotted in Figure 1. The mean of the data from the short-term birds taken at 4 hours was 0.061% of dose and the mean of the data from the long-term

birds at the same time was 0.062% of dose. The two sets of data, therefore, matched precisely, indicating a high degree of response reproducibility. The second step was to compute the ratio of the mean to the actual value of the data point for each bird. This gave a correction factor, relative to the mean, for each bird at the specific time being considered. Correction factors for all other times were computed in a similar manner. Finally, all correction factors for each bird were averaged over time to obtain the best estimate of deviation from the mean. The correction factors for one bird at different times were generally in very close agreement and indicated a very similar dynamic response independent of individual amplitude (i.e., dose of ^{47}Ca).

Whole Body ^{47}Ca Retention

The birds sacrificed according to the schedule shown above were counted in a whole body counter. The net activity for each carcass was first normalized to unit weight by dividing by the bird's body weight at the time of sacrifice. The normalized data were then corrected for initial blood activity by multiplying by the appropriate blood correction factor. In Figure 2 are shown the average relative burden at each time period.

The data follow, very closely, the exponential form which would be predicted by a first order dynamic model. This mode is consistent with the notion that the excretion rate is constant, and the loss per unit of excreta is

linearly related to whole body specific activity.

Bone Uptake Data

The uptake of ^{47}Ca based on the entire bone was about 1%/gm. in the humerus and about 0.3%/gm. in the tibia. These data were corrected for initial blood specific activity by the same correction factors used above for the blood disappearance curves. The specific activity of the humeri seemed to pass through a slight maximum and then decline slightly with time. The specific activity of the tibiae remained virtually constant after four hours.

Mathematical Model

Analysis of calcium kinetics can provide a mathematical estimate of the relative rate with which calcium enters and leaves physiological compartments as well as estimating the size of the compartments. It is essential that any such compartmental analysis be regarded as an adequate mathematical representation of the system without the additional restraint of associating morphologic specificity to the definition of each compartment (Heaney, 1963). Caution in interpretation of such data does not, however, limit its usefulness in skeletal mineral experimentation. In the current study, an injection of "carrier free" ^{47}Ca provides an accurate assessment of plasma disappearance. The disappearance curve does not follow first

order kinetics and thus is compatible with a multiple compartmental analysis. The general shape of the curve for the rooster is similar to that observed for mammals and man (Cohn, et al., 1965; Green, et al., 1968). Most studies utilize modifications of the Bauer multiple exponential model to estimate compartment sizes and flux rates (Bauer, et al., 1955).

The preliminary study, reported herein, provides information on the relative size of the plasma pool, the rate of loss from plasma and the body, and the fractional uptake into the skeleton, as well as the total body fractional retention of the tracer dose. From these measurements a model was devised which estimates the extravascular calcium pool, the exchangeable bone calcium pool and their associated rate constants. The use of a single radiocalcium pulse labeling of the plasma pool eliminates the need to determine calcium intestinal absorption coefficients. The model can easily be expanded to include these values by feeding a labeled meal and measuring the subsequent plasma concentration curve.

The general calcium kinetics model used assumes that the radionuclide rapidly mixes with a plasma calcium pool of constant size. The tracer then is lost from this pool at a rate proportional to the calcium efflux rate into an extravascular pool, and hence into the skeleton, first in an exchangeable compartment which is reversible, and then

unidirectionally into a relatively slow, but large, "deep" or "non-exchangeable bone" compartment which contains most of the skeletal mineral. A fraction of the label is also lost to the entire system by excretion in the intestinal tract and kidneys. The magnitude of the body burden loss was determined by total body counting of birds at intervals following ^{47}Ca injection, thereby eliminating the need for tedious excreta analyses. The return of radiocalcium from non-exchangeable bone was assumed to be negligible over the short-term of the study since this compartment is about 10^3 that of the sum of the others, and the effect of such a large dilution of the radionuclide would not introduce any sensible error into the model. This factor, however, can be determined in longer term studies and is thought to be a measure of the rate of bone resorption and exchange (Cohn, et al., 1965).

In Figure 3 a model for the birds is shown which is similar to the Bauer model (Bauer, et al., 1955).

The data are normalized to a 1 Kg. bird (the birds were actually all about 2 Kg.). A value of 10 mg. per cent of plasma calcium was determined [0.1 mg. Ca/ml. (∿gm.) plasma]. The primary value, based on extrapolation to zero time of the plasma disappearance curve, indicated an initial concentration of 14% of the dose per mg. of calcium. Thus, the kinetics study actually traces the time course distribution of the 14 mg. of plasma calcium circulating at the time of labeling (i.e., 7 mg. plasma calcium/Kg.)

The ^{47}Ca plasma disappearance curve over the 72 hours of the study is graphically determined such that

$$P = 0.84e^{-14t} + 0.075e^{-0.492t} + 0.069e^{-0.128t} + 0.016e^{-0.0216t}$$

where P = per cent dose per 100 nanograms plasma calcium, and
t is in hours post-injection.

Total body ^{47}Ca retention between 4 and 72 hours indicated a half period of 7.7 days; about 0.4%/hour of the dose was being excreted (i.e., 1 mg. Ca/day lost of the originally labeled 14 mg.).

There was no significant difference in the radionuclide concentration of humeral and tibial ^{47}Ca content at 4, 24, 48 or 72 hours which was interpreted as 1) the non-exchangeable label was essentially complete by 4 hours, and 2) resorption and long-term exchange (loss) was negligible.

The time course difference between the plasma disappearance and the bone uptake provide data on the total extravascular and extraskeletal "compartments". The values, as summarized in Table II, show that the transfer is essentially complete in 24 hours and is compatible with the kinetic model parameters. The four component curve for plasma disappearance would suggest that the first three terms were related primarily to the plasma-body fluid pool, while the slower component with an effective half period of 32 hours might be a reflection of the exchangeable bone pool.

The continuous nature of the blood calcium data suggest that, with increasing time, additional compartments may be required to get a fit to long-term data. It may well be that a power function of the form,

$$P = -\left(\frac{R_0}{E_x}\right) \frac{dR}{dt}$$

where R_0 is the injected dose, R is retained dose at time t and E_x is the rate of mineral lost by excretion

will best summarize the long-term kinetics of the system.

The theoretical model of Marshall provides such a model (Marshall, 1967). When longer term data are available, the two approaches will be further compared in the bird. At this time, the analog computer derived model shown in Table III appears to fit the data. The bone analyses further indicate that the humeri (0.7%/gm.) contained twice the concentration of ^{47}Ca as did the tibiae (0.3%/gm.), and that this relationship showed no change with time following administration of ^{47}Ca. By 72 hours, over 95% of the ^{47}Ca was in bone. Longer term studies incorporating absorption measurement as well as osteotomy mineralization rates are in progress.

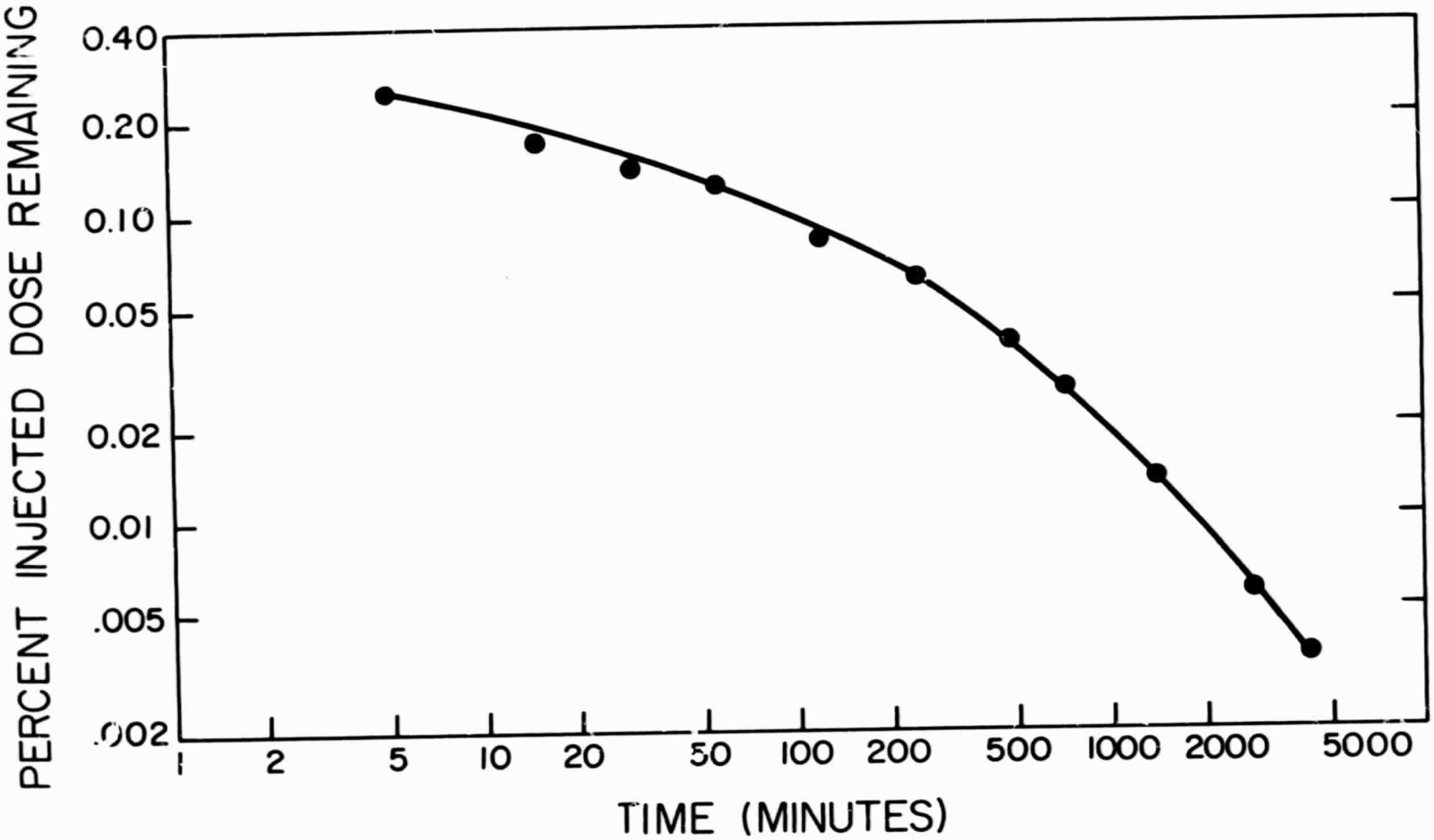

Figure 1: ^{47}Ca blood disappearance curve following intravenous administration of the radionuclide. The plot depicts percent of total injected dose remaining per gram of plasma versus time in minutes.

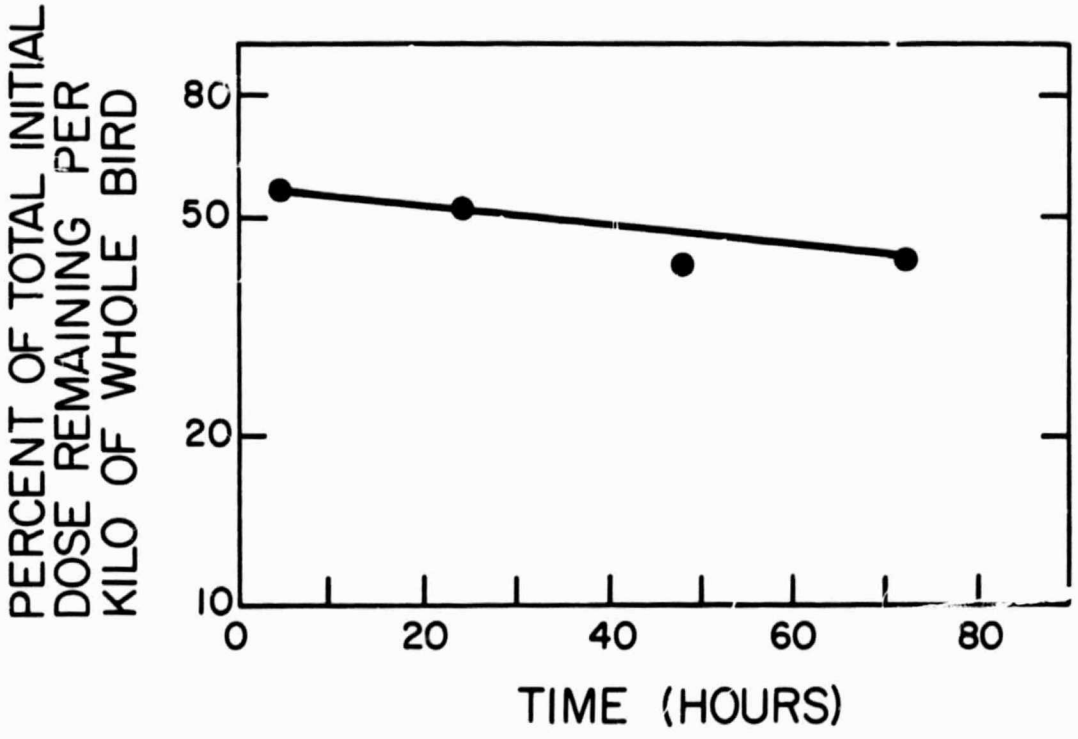

Figure 2: ^{47}Ca whole body retention following intravenous administration of the radionuclide.

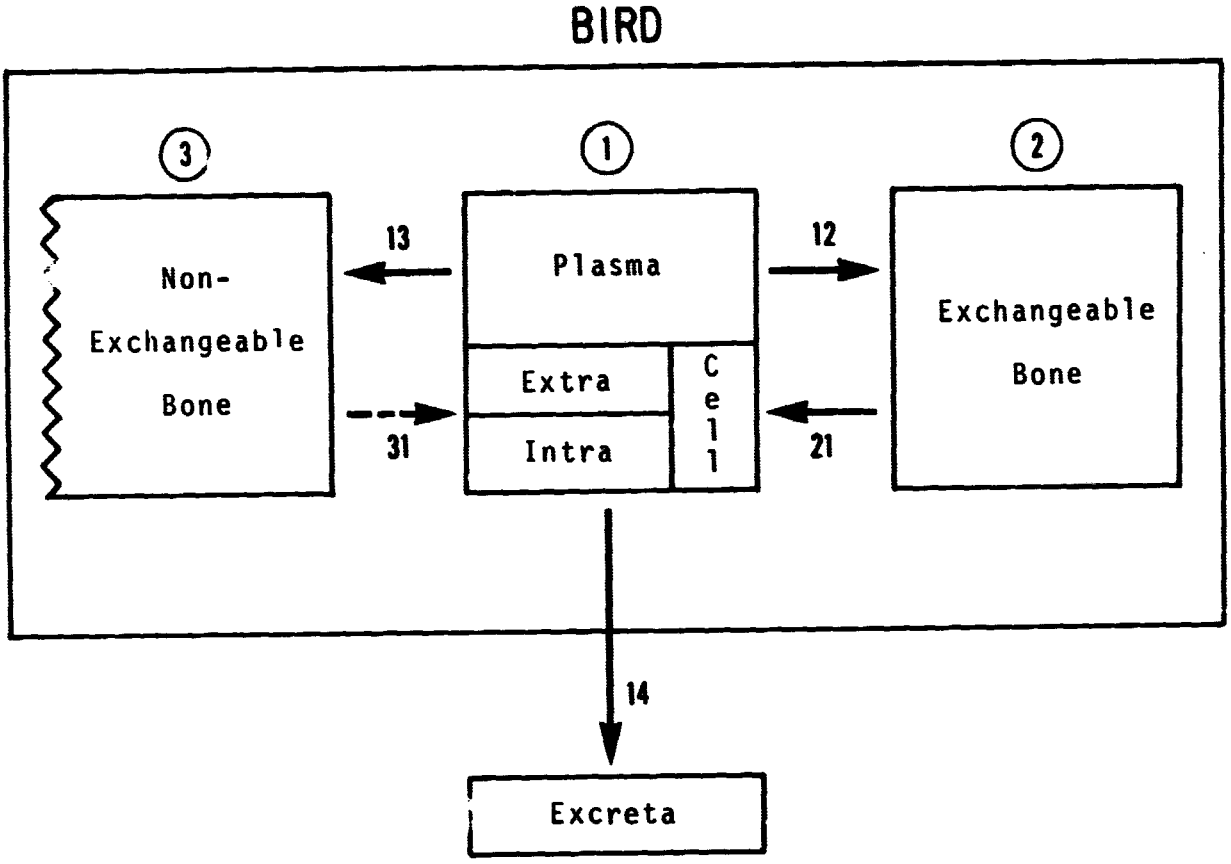

Figure 3: Provisional Calcium Kinetics Model. The rate constants and compartment sizes are shown in Table III. The present model assumes that the rate from compartment 3 to 1 is small and is negligible in this 72-hour study. Compartment 2 is assumed to be ten times that of the plasma compartment. (The numbers associated with the arrows indicate flux direction; e.g., "13" is "from compartment 1 to compartment 3."

TABLE I

Post-Injection Time	Number Sacrificed	Number Birds Sampled, Blood
4 hours	3 birds	10 birds
12 hours	0	7 birds
24 hours	2 birds	7 birds
28 hours	2 birds	5 birds
72 hours	3 birds	3 birds

TABLE II

Post Injection Time (Hours)	Per Cent Dose in "Body Fluid Compartments"
4	36.0
24	7.8
48	3.4
72	2.1

TABLE III

PARAMETERS IN CALCIUM KINETICS MODEL*

Parameters	Size	Flux
Plasma Calcium	4 mg. Ca	
Body Fluids	40 mg. Ca	
Sum of "Fluid Components"	44 mg. Ca	
Accretion (13)		∿4 mg. Ca/day
Fluid Exchangeable Bone Interchange (12, 21)		∿500 mg. Ca/day
Excreta (14)		∿1 mg. Ca/day

*Numbers in parenthesis indicate flux direction between compartments; see Figure 3.

BONE DENSITY MEASUREMENT AND QUANTITATION OF RATE OF FRACTURE REPAIR

Introduction

Of the various methods available for measurement of bone mineral content, the photon attenuation system described by Sorenson and Cameron (1967) has been selected for use in this study. This technique provides bone density quantitation in vivo in a rapid, precise, and non-destructive manner. Calibration of attenuation data with bone analysis will provide a linear quantitative conversion to cross-sectional bone mineral mass.

Method

A prototype bone density scanner similar to that of Sorenson has been fabricated. The system consists of a collimated beam (<1 mm.) of 27 kev photons from a 200 millicurie point source of ^{125}I, a small NaI (Tl) crystal, suitably shielded and collimated, and a single channel analyzer. Data are recorded in digital form using a teletype printout and paper punch unit, and in analog form using a linear ratemeter and a direct writing Sanborn recorder.

The system is being designed to perform two separate functions. One function is to statically monitor the pulsed

photon beams, passing through the osteotomy site and a second site approximately 1.5 cm. distal to the osteotomy. A present attachment to the system between the upper and lower collimators allows accurate placement of the osteotomy in the beam for monitoring *in vitro*; a second attachment now being fabricated will precisely locate and monitor the osteotomy *in vivo*.

The second function is to transversely scan the bone at a right angle to obtain cross-sectional bone mineral mass of both normal and wounded bone. A preliminary study was performed on twenty-one birds sacrificed in groups of three at one week intervals post-osteotomy in which the relative ^{125}I photon attenuation of tibial shaft over, and adjacent to, the osteotomy site was determined.

Results and Discussion

Preliminary results have been used only as a guide in the modification and refinement of the mechanical part of the system; no effort has been made to quantitate the results at this time. Reproducibility was within a 5% range of measurements. The three and seven week samples indicated an increase of about 10% in relative bone mass over that of the unwounded tibial segment. After one week, however, the osteotomy site contained only about 60-70% of the relative density of the normal tibial segment. The qualitative appearance of the radiographs were in substantial agreement with these findings.

Thus, it would appear that the osteotomy repair requires about one to three weeks before sufficient osteoid is laid down to form nucleation sites for marked mineral accretion. The appearance of a peripheral endosteal hyperostosis precedes centripital osteotomy repair. These preliminary observations indicate that quantitation of fracture repair (osteotomy) is quite feasible.

CANNULATION STUDIES
ON THE SINGLE COMB WHITE LEGHORN CHICKEN

I: CHRONIC CANNULATION

Introduction

Previous studies have shown (Beljan, Bell, and Burton, 1969) that the chicken can tolerate parenteral administration of fluids (0.5% saline at a dosage rate of 8-10% of body weight daily) as its sole source of hydration for at least thirty days without exhibiting evidence of physiological stress.

Chronic cannulation of the animal is considered necessary to administer this fluid in a weightless environment and to sample the circulating blood volume. Various techniques have been under study for the past year in order to best accomplish these goals.

Method

Originally an arterio-venous shunt-cannulation between the carotid artery and jugular vein was employed, similar to that developed successfully in the dog (Frasher, 1967). The stainless steel cannulas used in these experiments were machined from Frasher's design, with several modifications.

The second cannulation technique investigated was a by-pass venous "T" cannula. The original cannulas were machined out of stainless steel tubing, and the workmanship was relatively crude. Certain birds were administered standard 50 mg./day doses of Dicumarol(R) (Charles, et al., 1966; Harms and Tarver, 1957) until the cannulas were closed by thrombus formation. The patency of these cannulas was tested three times daily by withdrawing blood with a syringe. When the withdrawal of blood was not easily accomplished, the cannula was considered occluded, the animal sacrificed, and the site of cannula implantation examined grossly.

The original stainless steel cannula was replaced by an improved version, incorporating a decreased wall thickness and a more highly polished interior surface. However, because of the continuing unsatisfactory long-term results obtained through use of the stainless steel "T" cannulas, an alternate cannulation method was developed using Silastic(R) intravenous tubing.

An initial group of eighteen birds was cannulated using 8" lengths of Silastic(R) tubing (0.062 in. I.D. x 0.125 in. O.D.). The chicken was restrained on the surgical table and the surgical area infiltrated with 2% procaine. The feathers were then plucked from around the surgical site, the skin scrubbed with Zephiran(R) and a surgical drape applied. A skin incision of approximately 1½ inches was made on the

right side of the neck in the area of the right jugular vein (Figure 1). The vein was located (Figure 2), isolated from its surrounding tissues (Figure 3), and ligated cranially (Figure 4). An incision was then made in the vein and an 8" piece of Silastic(R) tubing was threaded down to the area of the anterior vena cava (Figure 5). When the cannula was in place, the loose suture placed previously was then tightened and tied (Figure 6). A second ligature, parallel to the first, maintained the cannula in position (Figure 7). A stab incision was next made approximately 3/4" laterally from the skin incision site, and the end of the cannula was delivered through it (Figure 8). The surgical area was then flushed with 100,000 units of potassium penicillin and the skin incision closed by means of a continuous cotton suture line (Figure 9). Two stay sutures were then placed through the skin and around the cannula in the area of the stab incision (Figure 10).

The cannulas were checked for patency and flushed with 1% heparinized saline twice daily. The cannulas were considered to be patent only as long as it was possible to both flush saline into the birds and to withdraw blood from the animals through the cannulas.

A second group of thirty-four birds was cannulated in the same manner as the initial group of eighteen, with particular emphasis given to asepsis during both the operative

procedure and the post-operative flushing of the cannulas. The birds were given procaine penicillin daily for ten days post-operatively. Body weights were recorded daily and hemograms were performed weekly.

Results

1) Arterio-Venous Shunt:

Patency was maintained for several days; however, the birds lost body weight and developed physiological difficulties, such as cardiac decompensation.

2) Original By-Pass Venous "T" Cannulas:

The longest patency period was sixteen days and the shortest was one day. The mean patency period was 6.4±0.6 days for thirty-two birds. Thrombus formation occurred within the main portion of the cannulas, usually at the distal end. The administration of fluids without the necessity of blood withdrawal was always possible, and this cannular function did not appear to have any limitation as to life expectancy. All of the experimental animals showed marked weight loss and "stress" hemograms (Table I).

3) Modified By-Pass Venous "T" Cannulas:

The modified cannulas prolonged the patency period, but the birds continued to lose weight and show physical and hematological signs of stress (Table II).

4) Silastic(R) Cannulas:

The average life span for the cannulas in the initial group of eighteen birds was ten days with a minimum of zero and a maximum of twenty-eight days. All of the birds exhibited "stress" hemograms (Table III) and lost body weight (Table IV). Blood samples taken five to eleven days after cannulation showed marked neutrophilia and lymphopenia; buffy coats, a crude index of leukocyte count, were two to five times normal values. Both these results are indicative of an infectious process. Body weight losses ranged from 1.3 to 3.6 per cent per day (Table IV).

The most prominent finding at necropsy of those birds which died, and of those which were sacrificed when their cannulas were no longer functional, was splenomegaly, possibly as a result of septicemia. One bird had developed endocarditis with vegetative lesions on the right A-V valve. The ends of the cannulas were found in the precava, the atrium, and the postcava. There were no clots in any of the cannulas but those birds sacrificed with non-functional cannulas had thrombi surrounding the ends of the cannulas.

The average life span of the cannulas in the second group where Silastic(R) cannulas were used was seventeen days, with a minimum of three and a maximum of forty-five days. Eight of the thirty-four birds (24%) in this study had cannulas which remained patent for thirty or more days (Table VI). All of the birds at one time or another showed

"stress" hemograms, i.e., neutrophilia, lymphopenia, increased buffy coats, and variable anemia (Table V), and weight loss similar to, but of less severity, than the original eighteen birds reported previously (Table VI). Of the thirty-four birds, twenty eventually died (all with cannulas still patent), nine managed to dislodge the cannula, and five had cannulas which were occluded.

Various lesions seen during autopsy of the birds were:

1. Organized clots around the cannulas.
2. Antemortem clots in the anterior vena cava, right atrium, and right ventricle.
3. Petechiae and plaques (urates?) on the pericardium and endocardium.
4. Vegetative lesions on the right A-V valves and endocardium.
5. Right heart enlargement.
6. Pericardial effusion.
7. Enlarged, mottled spleen and/or liver with or without adherent fibrin.
8. Ascites.
9. Small testes.
10. Suppurative pneumonia.

Discussion

The difficulties which developed with the use of the arterio-venous shunt appeared to be related to increased cardiac load, and the arterio-venous shunt was therefore

abandoned. The by-pass venous "T" cannulas were unsatisfactory because of the short life span of the cannulas themselves, and the unacceptable stress which occurred in the birds as a result of their use.

Initial results indicated that the Silastic(R) cannula might have a longer functional life expectancy than the more complicated stainless steel jugular cannula. Through steps taken to eliminate infections caused by the operative procedure, the post-operative cannula flushing procedure, or both, it was possible to increase the life span of the cannula and to reduce somewhat the weight loss and stress (as shown by the hemograms) exhibited by the birds cannulated by previous methods.

The clinical signs and post-mortem lesions observed were felt to be due to a combination of three major factors:

1. Cardiac and pulmonary embolism secondary to the cannula flushing procedure.
2. Inadvertent introduction of bacteria during the cannula flushing procedure.
3. Traumatization of the endothelium and endocardium by the catheter in situ.

CANNULATION STUDIES
ON THE SINGLE COMB WHITE LEGHORN CHICKEN

II: CONSEQUENCES OF CHRONIC CANNULATION

Introduction

A short study was initiated to determine the causative factors for the clinical signs and necropsy findings observed in the thirty-four chronically-cannulated birds discussed previously.

Method

Seventeen birds were divided into three groups:

Group I: The right jugular vein was ligated in each animal but no cannulas were placed.

Group II: A shorter (5 inch) Silastic(R) cannula was placed in the right jugular vein of each bird. These cannulas were not manipulated or flushed post-operatively.

Group III: These birds were cannulated in the same manner as the birds in Group II and the cannulas were flushed twice daily with ½ to 1 cc. of 0.9% saline (heparinized).

All birds received procaine penicillin daily for seven days post-operatively and body weights were taken daily.

Hemograms were taken on all birds nine to thirteen days post-operatively. The four birds which appeared most stressed in Group III were sacrificed twenty-one days post-operatively and sections of lung, heart, liver, and kidney tissues were preserved in formalin for histological section and future study, if necessary.

Results

The birds in Groups I and II showed no appreciable loss in body weight (Table VII) and eleven of the twelve birds showed normal hemograms ten to thirteen days post-operatively (Table VIII).The birds in Group III showed a steady loss of body weight averaging 12% of their pre-operative body weight by the tenth day (Table VII). Three of the five birds in Group III showed "stress" hemograms (less than 50% lymphocytes) on the ninth post-operative day (Table VIII).

Discussion

This experiment illustrated that the "stress" hemograms and body weight loss observed in cannulated birds are due to complications which arise during the post-operative flushing and maintenance of the cannulas.

These complications are most likely secondary to two main factors: 1) the introduction of bacterial contamination during the flushing procedure, and 2) vascular embolism resulting from clots being flushed into the circulatory system from the tip of the cannula. It is anticipated that these

complications may be avoided or minimized through the use of a peristaltic pump in a closed system which will deliver a continual water input over a 24-hour period. This technique will greatly reduce the introduction of bacterial contamination post-operatively. It is also anticipated that the continual water input will keep the cannulas flushed, and minimize clot formation at the tips of the cannulas. Such a pump has been obtained; a watering system has been developed and preliminary studies on birds have begun.

CANNULATION STUDIES
ON THE SINGLE COMB WHITE LEGHORN CHICKEN

III: PARENTERAL SALINE AS THE SOLE SOURCE OF HYDRATION BY HYPODERMOCLYSIS

Introduction

Because of certain problems initially encountered in administering saline via the jugular catheter, an alternate back-up method of parenteral hydration was developed.

Method

Subcutaneous catheters (Jelco(R) I.V. catheters) were implanted in the posterior cervical region of five male chickens. The birds were given 10% of their pre-operative body weights daily of 0.5% saline (divided into two equal doses and given in the morning and afternoon) as their sole source of hydration for thirty days. Feed was offered ad libidum in the usual manner. The birds were weighed daily and blood work was done periodically.

Results

All birds exhibited normal hemograms throughout the experiment. Four of the five birds showed no appreciable

loss in body weight. All birds appeared normal on a clinical basis.

The experimental results are summarized in Table IX.

Discussion

The chicken was able to tolerate the injection of saline through a subcutaneous catheter as its sole source of hydration for a period of thirty days without exhibiting signs of physiological abnormality.

CANNULATION STUDIES
ON THE SINGLE COMB WHITE LEGHORN CHICKEN

IV: HYPODERMOCLYSIS IN THE RESTRAINED CHICKEN

Introduction

After the chicken had demonstrated tolerance to the prolonged subcutaneous injection of saline as its sole source of hydration, hypodermoclysis was employed in restrained animals, using the method of restraint which had previously been successfully demonstrated.

Method

Subcutaneous cervical catheters were implanted in four laterally restrained chickens in the manner described in the preceding section, and saline was similarly administered. The birds were weighed daily and hemograms were recorded periodically. All birds were sacrificed at the end of the experiment, and organ weights obtained.

Results

All of the birds showed some loss in body weight and "stress" hemograms (below 50% lymphocytes) at one time or other (Table X). However, with the exception of one bird

(#2525), the hematological signs of stress were not severe, and all birds appeared normal on a clinical basis. On day 21, it was discovered that bird #2525 was being pecked by his neighbors when he attempted to eat; after separation from the other animals, his body weight (which had been steadily decreasing from day 1) showed a slight increase until termination of the experiment.

This experimental group of animals was afflicted by a infestation of mites during this experiment. Since these mites can stress birds, the slight loss of body weight and occasional low hemograms could have been a result of the affliction.

The following abstract titled "Parenteral Administration of Fluids as the Sole Source of Hydration: Application in Space (Weightless) Environment", and based on this study, has been submitted and accepted for presentation at the Aerospace Medicine Association's 41st Annual Scientific Meeting (Ray, Burton, and Beljan, 1970).

> Animal care (husbandry) in a weightless environment (space) involves a number of problems which may not be considered in caring for animals living in a (1-"G") gravity environment. Germane to this is the handling of water and its administration to laboratory animals living in space. Watering problems, however, would be essentially eliminated if hydration could be maintained by the long-term

administration of fluids (water or saline) via parenteral routes. The tolerance of laboratory animals to the chronic administration of water subcutaneously has not been clearly defined. Therefore, the entire fluid intake of a restrained laboratory animal (chicken) was given parenterally for one month and various physiological parameters measured.

Parenteral administration of 0.5% saline by subcutaneous injection was well tolerated as the sole source of hydration for 30 days by restrained and non-restrained adult chickens of both sexes. Administration was performed two to three times daily by hypodermic injection or via a permanently implanted cannula. Body mass, feed intake, egg production, and standard hematological determinations indicated no differences from control (orally hydrated) animals. It was demonstrated that fecal consistency could be controlled by hydration--a consideration for waste management in a weightless environment. The birds were sacrificed immediately after the 30-day restraint period and gross and microscopic tissue determinations were made. Organ weights indicated no difference from controls. No consistent pathological lesions were found relative to the cannula implantation site.

It was concluded that restrained chickens could tolerate chronic parenteral fluid administration as their sole source of hydration. The experimental birds were not physiologically different from orally hydrated, non-restrained controls.

Figure 1: Site of surgical incision.

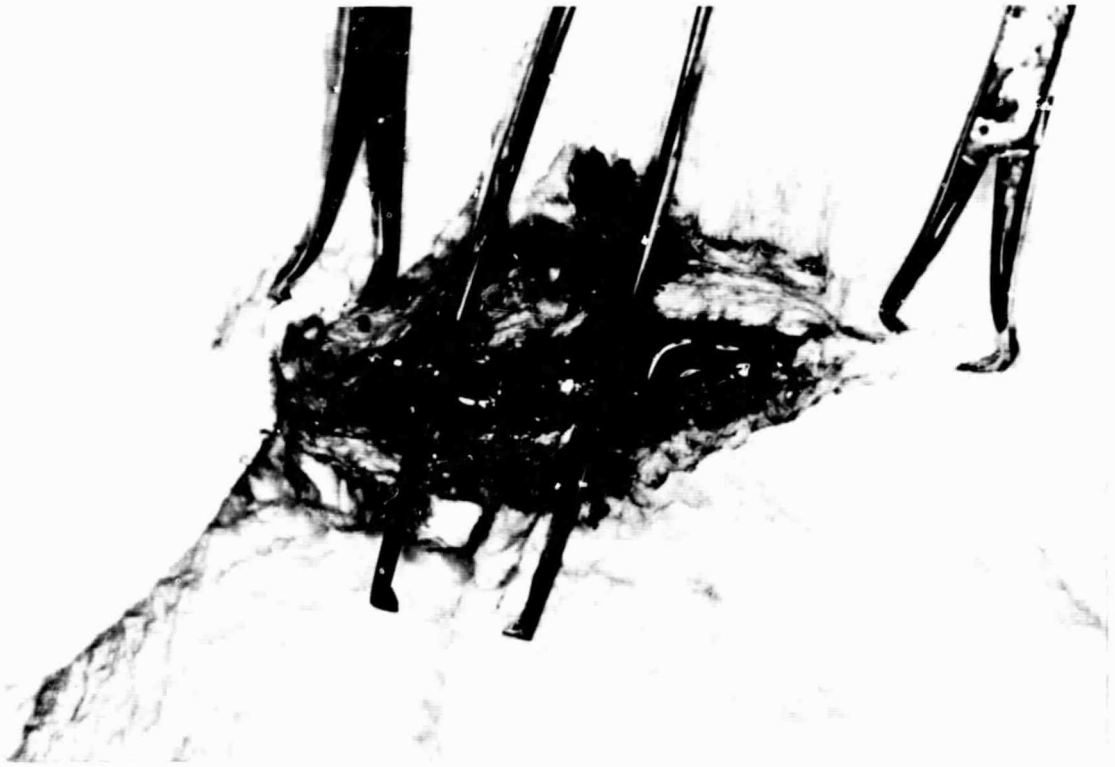

Figure 2: Jugular vein and its surrounding tissues.

Figure 3: Jugular vein after isolation.

Figure 4: Jugular vein after cranial ligation.

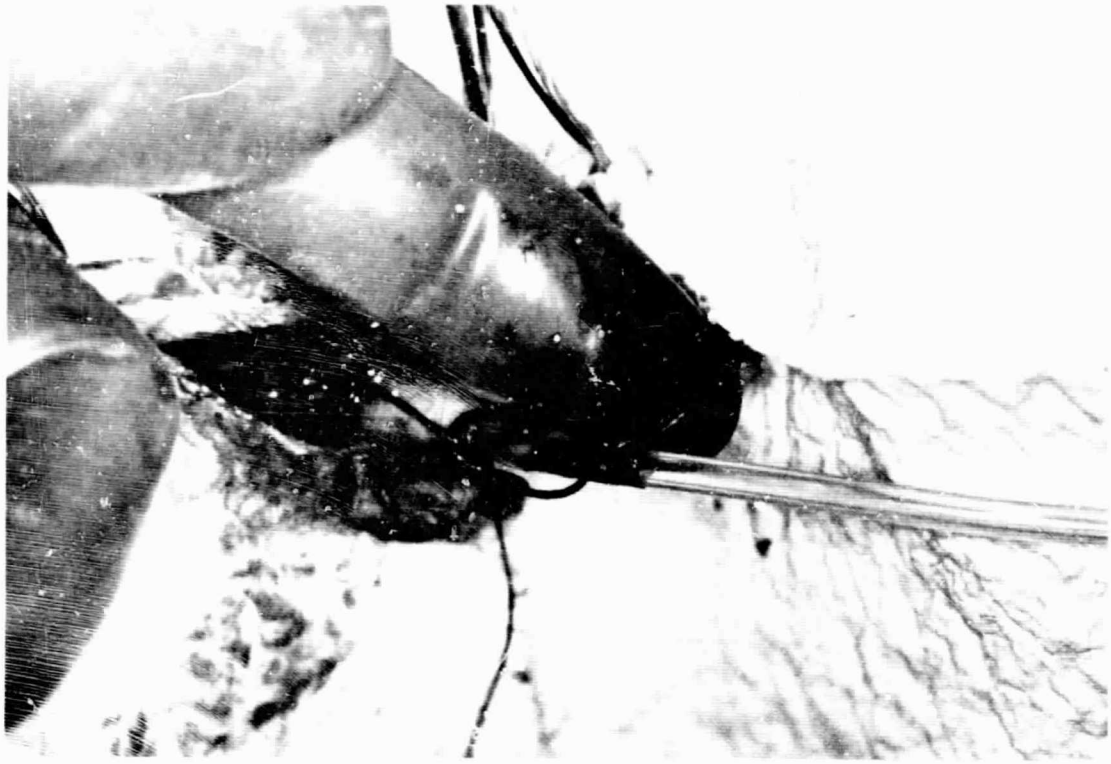

Figure 5: Cannula being placed in jugular vein.

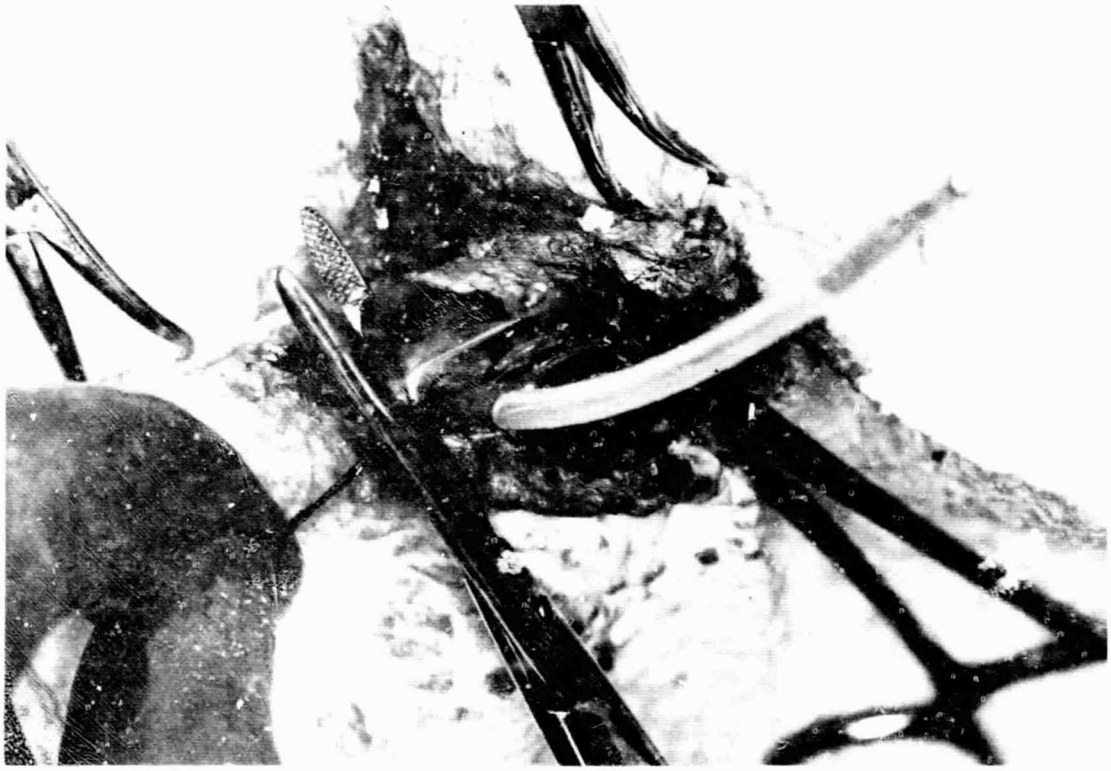

Figure 6: Securing cannula in position.

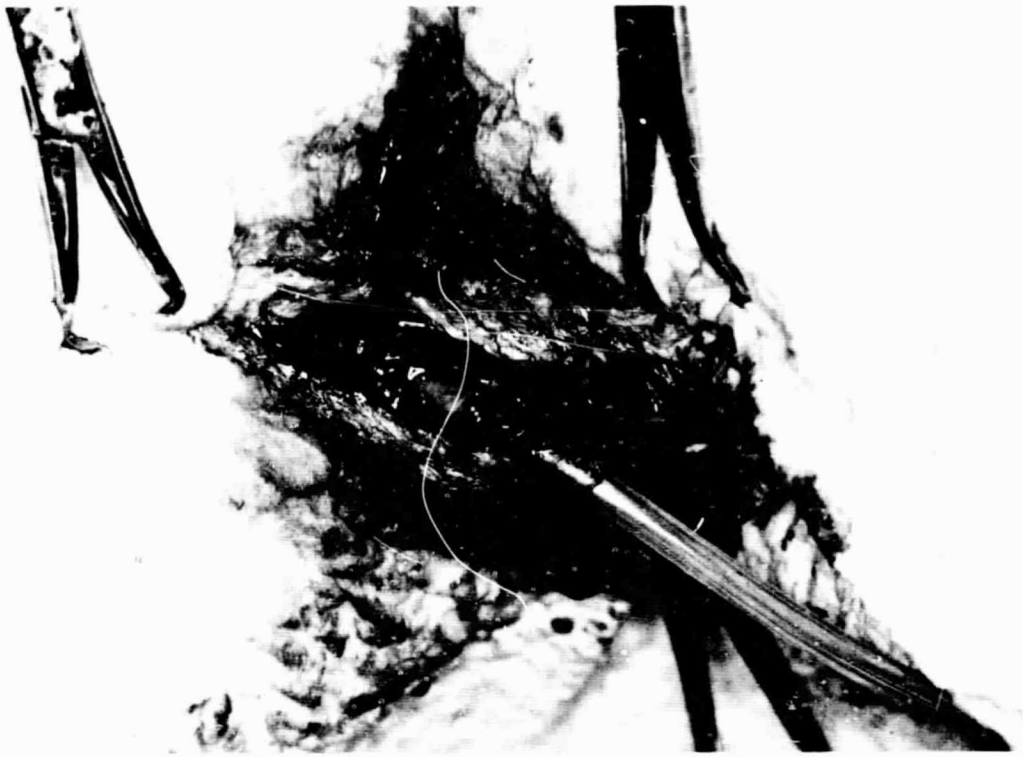

Figure 7: Securing cannula in position.

Figure 8: Cannula after delivery through stab incision.

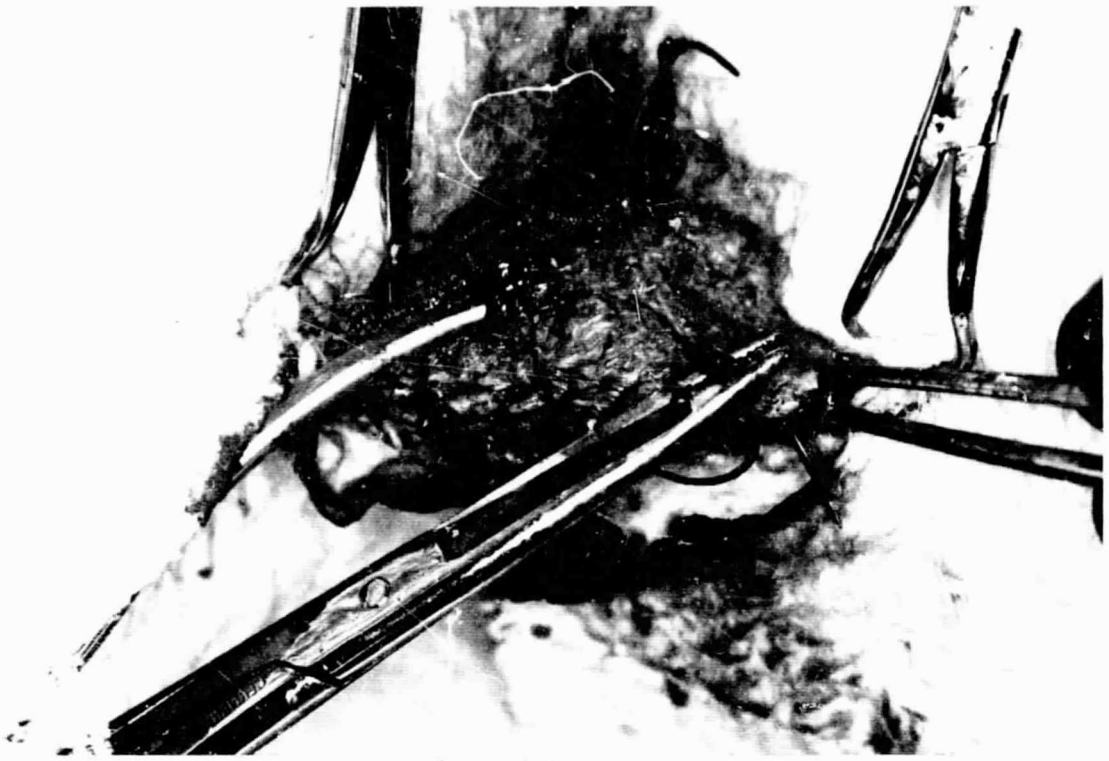

Figure 9: Post-operative skin closure.

Figure 10: Dermal fixation of cannula.

TABLE I

ORIGINAL "T" TYPE CANNULAS

Wing Band Number	Dicumarol(R) Days	Blood Withdrawal W/O Injection (Days)	Blood Withdrawal After Flushing Cannula (Days)	Injection only possible (Days)	Termination	Blood Samples With < 50% Lymphocytes / Total Bleedings	% Body Weight Lost or Gained
8213	0	-	8	42	Plugged	4/7	-25.7
9500	2	-	4	37	Died	5/7	-44.8
8678	1	-	10	36	Plugged	4/6	-44.2
9439	(Varizyme(R)6)	-	10	48	Died	2/5	-43.4
8660	2	-	9	10	Died	1/2	-14.6
9197	0	-	4	9	Died	1/1	-19.5
8637	1	-	3	9	Died	1/2	-17.9
9089	0	-	5	15	Died	2/4	-20.7
8958	1	-	9	16	Died	3/4	-32.4
8926	4	-	8	21	Died	4/6	-30.2
8242	0	2	5	--	Sac.	0/1	- 4.6
8226	0	2	6	--	Sac.	0/1	- 5.2
8125	0	1	7	--	Sac.	1/1	- 2.8
8158	0	1	11	--	Sac.	1/2	- 3.0
8073	0	0	9	--	Sac.	0/1	-10.9
35404	0	0	3	--	Sac.	0/1	-10.1
8555	0	5	13	13	Died	0/1	-27.9
8234	0	5	8	8	Died	-/-	-14.8
8572	0	6	16	22	Sac.	3/4	- 3.7

TABLE I

ORIGINAL "T" TYPE CANNULAS (Cont'd)

Wing Band Number	Dicumarol (R) Days	Blood Withdrawal W/O Injection (Days)	Blood Withdrawal After Flushing Cannula (Days)	Injection only possible (Days)	Termination	Blood Samples With <50% Lymphocytes / Total Bleedings	% Body Weight Lost or Gained
9137	0	-	6	6	Sac.	2/2	-15.3
9031	0	1	1	1	Sac.	---	- 8.0
9123	0	-	2	2	Sac.	1/1	- 6.6
9221	1	-	5	7	Sac.	1/1	- 4.7
8723	0	-	2	3	Sac.	---	- 6.8
9180	2	-	4	14	Sac.	1/2	+ 1.9
8960	3	-	6	6	Sac.	---	- 2.4
8767	2	-	7	7	Sac.	0/1	-10.7
9094	4	-	8	8	Sac.	1/1	-17.1
9201	4	-	9	9	Sac.	0/1	-15.0
9102	4+ 4 Varizyme (R)	-	4	4	Died	1/1	-10.1
9436	3	-	2	2	Died	0/1	- 4.4
9145	3	-	2	2	Sac.	---	- 9.2

TABLE II

MODIFIED "T" TYPE CANNULAS

Wing Band Number	Dicumarol(R) (Days)	Blood Withdrawal W/O Injection (Days)	Blood Withdrawal After Flushing Cannula (Days)	Injection only Possible (Days)	Termination	Blood Samples With <50% Lymphocytes / Total Bleedings	% Body Weight Lost or Gained
8008	0	13	28	28	Died	1/6	-34.1
8203	0	2	17	22	Sac	1/3	-15.9
8521	0	3	9	20	Sac	3/3	-27.6
8358	0	6	11	19	?	3/3	-37.7

TABLE III

LYMPHOCYTE/HETEROPHIL RATIOS IN BIRDS WITH SILASTIC(R) CANNULAS (GROUP 1)

Wing Band Number	Day 5	Day 6	Day 7	Day 10	Day 11	Day 12	Day 14	Day 21
9477	20/67	-----	-----	-----	-----	12/75	------	------
8917	-----	-----	8/85	------	------	------	12/77	------
8914	1/89	-----	-----	------	------	------	------	------
8357	-----	-----	-----	------	4/88	------	------	------
8028	-----	-----	-----	12/72	------	------	------	------
8119	8/82	-----	-----	------	------	------	------	------
8372	7/86	-----	-----	------	------	------	------	------
8487	-----	-----	21/68	------	------	------	------	------
8558	-----	-----	4/83	------	------	------	------	------
8526	-----	-----	9/79	------	------	------	------	------
8474	-----	-----	5/88	------	------	------	------	------
8618	-----	-----	14/65	------	------	------	------	------
8650	-----	-----	-----	------	------	------	------	------
70390	-----	-----	-----	------	------	------	------	------
8853	-----	-----	-----	------	------	------	------	------
8733	-----	-----	-----	------	------	------	------	------
9497	6/90	-----	-----	------	------	------	------	------
9498	-----	3/88	-----	------	------	------	------	------

TABLE IV

CANNULA PATENCY AND % CHANGE IN BODY WEIGHT WITH SILASTIC(R) CANNULAS (GROUP 1)

Wing Band Number	Cannula Patency (Days)	Presurgical Body Weight	Body Weight at Termination	% Change in Body Weight
9477	23	2315	1940	-16.2
8917	28	1995	1130	-48.8
8914	9	1930	1620	-16.1
8357	17	2090	1625	-22.2
8028	15	2070	1515	-26.8
8119	9	1870	1460	-21.9
8372	9	2130	1760	-17.3
8487	7	2860	2500	-12.5
8558	8	2315	1930	-16.6
8526	5	2210	1935	-12.4
8474	4	2450	2195	-10.4
8618	7	2465	2130	-13.6
8650	5	2065	1715	-16.8
70390	7	2190	1805	-17.5
8853	4	1905	1560	-18.0
8733	5	2390	2090	-12.6
9497	9	2605	2410	- 7.4
9498	11	2050	1655	-19.5

TABLE V

LYMPHOCYTE/HETEROPHIL RATIOS IN BIRDS WITH SILASTIC(R) CANNULAS (GROUP 2)

Wing Band Number	Presurgical	2-3 Days	4-5 Days	7-8 Days	10-12 Days	13-15 Days	17-19 Days	20-21 Days	25-26 Days	28-29 Days	32-34 Days	40 Days
8383	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
8402	48/43	-----	-----	-----	26/47	-----	-----	-----	-----	-----	-----	-----
8512	68/24	-----	16/72	10/82	-----	-----	-----	-----	-----	-----	-----	-----
8575	66/29	-----	53/40	-----	-----	-----	------	-----	-----	-----	-----	-----
8445	-----	37/46	22/74	-----	-----	-----	-----	-----	-----	-----	-----	-----
8566	62/27	18/70	15/82	-----	-----	-----	-----	-----	-----	-----	-----	-----
8544	49/49	-----	40/56	-----	31/63	-----	-----	-----	-----	-----	-----	-----
9616	59/35	-----	70/20	-----	65/33	36/62	59/35	-----	-----	-----	-----	-----
8094	29/65	-----	22/66	-----	27/58	38/51	60/35	-----	-----	-----	-----	-----
9112	59/34	-----	55/42	-----	43/42	21/65	71/25	-----	-----	-----	-----	-----
8462	51/42	36/59	-----	26/60	-----	-----	60/36	20/66	38/59	-----	-----	-----
8313	37/51	10/75	-----	31/58	-----	-----	45/55	7/81	23/73	-----	-----	-----
8448	90/ 7	-----	15/66	37/59	-----	51/38	24/69	-----	-----	-----	-----	-----
8342	33/44	-----	10/70	-----	-----	33/63	-----	-----	-----	-----	-----	-----
8360	-----	12/88	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
70394	54/44	-----	54/41	17/77	7/73	-----	48/47	-----	-----	-----	-----	-----
8399	68/26	-----	13/81	20/78	16/75	-----	16/78	-----	-----	-----	-----	-----
8106	50/41	-----	20/66	-----	6/86	-----	-----	-----	50/41	29/63	57/36	29/64

TABLE V

LYMPHOCYTE/HETEROPHIL RATIOS IN BIRDS WITH SILASTIC(R) CANNULAS (GROUP 2)

(Cont'd)

Wing Bird Number	Presurgical	2-3 Days	4-5 Days	7-8 Days	10-12 Days	13-15 Days	17-19 Days	20-21 Days	25-26 Days	28-29 Days	32-34 Days	40 Days
8121	82/15	-----	21/64	-----	11/79	-----	-----	-----	82/15	55/35	57/39	38/61
9440	53/44	-----	30/65	-----	-----	-----	-----	-----	-----	-----	-----	-----
8243	66/28	-----	19/71	-----	7/84	-----	-----	-----	-----	-----	-----	-----
8093	39/46	-----	14/81	-----	23/52	-----	65/31	24/64	33/52	-----	38/59	-----
8422	-----	-----	37/53	-----	61/38	20/69	-----	52/45	24/71	-----	-----	-----
8652	-----	12/83	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
70391	57/38	-----	15/82	-----	-----	-----	-----	-----	-----	-----	-----	-----
8180	-----	20/77	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
8742	61/33	-----	21/78	-----	-----	-----	-----	-----	-----	-----	-----	-----
8782	-----	16/78	------	-----	-----	-----	-----	-----	-----	-----	-----	-----
7566	74/13	-----	32/64	-----	-----	-----	-----	-----	-----	-----	-----	-----
8541	59/34	------	39/56	-----	35/41	-----	-----	-----	-----	-----	-----	-----
8306	85/10	-----	57/30	-----	18/53	-----	-----	-----	-----	-----	-----	-----
8468	80/17	-----	-----	------	-----	-----	-----	-----	-----	-----	-----	-----
9444	54/38	-----	17/76	-----	-----	-----	-----	-----	-----	30/59	-----	-----
8531	-----	-----	19/76	-----	-----	------	-----	-----	-----	63/20	-----	-----

TABLE VI

CANNULAR PATENCY AND % CHANGE IN BODY WEIGHT WITH SILASTIC(R) CANNULAS (GROUP 2)

Wing Band Number	Cannula Patency (Days)	Presurgical Body Weight	Body Weight at Termination	% Change in Body Weight
8383	4	2225	2165	- 2.7
8402	12	1800	1675	- 6.9
8512	10	1845	1650	-10.8
8575	11	2250	1740	-22.6
8445	6	2000	1810	- 9.5
8566	8	1760	1440	-18.1
8544	17	1415	940	-33.6
9616	17	1435	1525	+ 7.4
8094	19	1810	1870	+ 3.3
9112	22	1620	1440	-11.1
8462	31	1855	1420	-23.4
8313	30	1900	1755	- 7.6
8448	19	1840	1400	-23.9
8342	14	1775	1655	- 6.7
8360	6	2100	1680	-20.0
70394	18	1685	1280	-24.1
8399	20	1700	1290	-24.1
8106	45	2065	1600	-22.2
8121	42	1640	1605	- 2.1
9440	8	1595	1410	-11.6
8243	17	1705	1365	-19.9
8093	38	1760	1855	+ 5.4
8422	33	1415	1745	+23.4
8652	7	2245	2070	- 7.8
70391	13	2075	1640	-20.9
8180	12	1850	1630	-11.9
8742	20	1925	1310	-31.9

TABLE VI

CANNULAR PATENCY AND % CHANGE IN BODY WEIGHT WITH SILASTIC(R) CANNULAS (GROUP 2) (Cont'd)

Wing Band Number	Cannula Patency (Days)	Presurgical Body Weight	Body Weight at Termination	% Change in Body Weight
8782	23	2210	1915	-13.3
7566	27	2405	1845	-23.3
8541	17	2000	1930	- 3.5
8306	18	2275	2225	- 2.2
8468	3	1700	1600	- 5.9
9444	31	1675	1440	-14.0
8531	34	1875	1615	-13.8

TABLE VII

PER CENT OF PRESURGICAL BODY WEIGHT
STUDY OF CONSEQUENCES OF CHRONIC CANNULATION

	Wing Band Number	4 Days	7 Days	10 Days
Group I	8335	99%	99%	99%
	8930	99%	100%	101%
	8471	98%	99%	100%
	8822	98%	99%	99%
	8537	99%	100%	100%
	8476	97%	96%	96%
Average		98%	99%	99%
Group II	8288	100%	103%	102%
	8129	97%	997%	99%
	8032	95%	102%	103%
	8230	97%	99%	98%
	8354	97%	96%	96%
	4067	98%	98%	98%
Average		97%	99%	99%
Group III	8170	98%	96%	95%
	8522	97%	94%	90%
	8304	92%	90%	82%
	8177	96%	89%	85%
	8111	95%	92%	90%
Average		96%	92%	88%

TABLE VIII

LYMPHOCYTE/HETEROPHIL RATIOS
STUDY OF CONSEQUENCES OF CHRONIC CANNULATION

	Wing Band Number	Presurgical Control	9 Days	10 Days	13 Days
Group I	8335	52/42	-----	-----	14/75
	8930	68/22	-----	-----	66/18
	8471	56/37	-----	-----	59/36
	8822	65/24	-----	-----	65/24
	8537	76/17	-----	-----	76/15
	8476	68/19	-----	-----	66/27
Group II	8288	46/35	-----	-----	54/33
	8129	67/18	-----	76/12	-----
	8032	60/32	-----	58/27	-----
	8230	69/25	-----	59/30	-----
	8354	70/19	-----	52/42	-----
	4067	67/21	-----	65/25	-----
Group III	8170	-----	65/30	-----	-----
	8522	-----	44/34	-----	-----
	8304	-----	41/49	-----	-----
	8177	-----	46/46	-----	-----
	8111	-----	55/28	-----	-----

TABLE IX

PARENTERAL SALINE AS THE SOLE SOURCE OF HYDRATION VIA A SUBCUTANEOUS CATHETER

Body Mass (gms.)

Bird Number	Control	Day 31	%	Minimum Body Mass
9895	1708	1730	+ 1%	1625 (Day 6)
9728	1840	1860	+ 1%	1770 (Day 4)
9494	2147	2200	+ 2%	2060 (Day 6)
9663	1875	1785	- 5%	1750 (Day 6)
9973	2150	1895	-12%	1825 (Day 26)

Lymphocyte %

Bird Number	Control	Day 14	Day 21	Day 28
9895	27	59	53	53
9728	65	64	57	51
9494	57	74	66	58
9663	85	79	69	60
9973	62	67	63	55

TABLE X

SUBCUTANEOUS CANNULAR HYDRATION
IN THE RESTRAINED CHICKEN

Body Mass (gms.)

Bird Number	Control	Day 35	%Δ	Minimum Body Mass
2525	1900	1760	- 7%	1650 (Day 22)
8925	2110	2010	- 5%	1960 (Day 19)
9884	2065	1865	-10%	1865 (Day 35)
2623	1995	1905	- 5%	1880 (Day 19)

Lymphocyte %

Bird Number	Control	Day 7	Day 21	Day 28	Day 35
2525	34	51	31	17	24
8925	64	39	54	36	51
9884	70	53	44	58	60
2623	43	41	33	40	58

DEVELOPMENT OF AN OSTEOTOMY AS A REPRODUCIBLE FRACTURE; OPTIMIZATION OF SIZE AND SITE

Introduction

To enable the effect of weightlessness on the rate of fracture repair and calcium kinetics to be quantitatively measured, a reproducible induced fracture, or osteotomy, has been developed. The purpose of the preliminary investigation was to evaluate the osteotomy technique and to find its optimal location within the skeletal system. Since the birds will be required to withstand accelerative forces accompanying liftoff and reentry, classical types of osteotomies might be subject to additional fracture or possible failure at the point of weakness, particularly if a weightbearing bone were selected.

Method

The type of osteotomy used throughout the study was a hole drilled through one cortical wall of a long bone of the Single Comb White Leghorn rooster, (Figure 1) using a Jordan-Day Engine, a variable speed motor with a flexible drive shaft. A low drilling speed was used to avoid heat

buildup and possible bone necrosis. The tibia and humerus were selected as sites for the preliminary osteotomies.

In the initial group of twenty-one animals the epiphyseal and diaphyseal areas of both the tibia and humerus received 3/32" osteotomies; the medial cortex of the tibia and the lateral cortex of the humerus were used. Osteotomies performed on a second group of five birds were 3/16" in diameter and placed only in the diaphysis of the tibia and humerus; site selection and fracture diameter were contingent upon the results of the first group. Finally, a third group of twenty-one birds was given 1/8" osteotomies in only the midshaft medial cortex of the tibia.

Except for the second group, three birds were sacrificed each week for seven weeks; both tibiae and humeri were removed from Group I, and only the tibiae from Group III. After the immediate area of the wound was examined for evidence of infection, the bones were removed, stripped of muscle, and frozen.

Radiographs of anteroposterior and lateral views were taken of all extracted bones for qualitative observation of the osteotomy and subsequent callus formation. Bone density and rate of fracture repair will be evaluated by photon absorption densitometry and additionally, if necessary, by conventional densitometry using the radiographs previously obtained.

Results

Group I: Inflammatory reaction at the operative site was minimal, and totally resolved by the second week; no bone necrosis was found. The osteotomies in the humerus appear to have healed more rapidly than those in the tibia; and, as expected, the epiphyseal osteotomies healed more rapidly than those of the diaphyses. (Refer to Figures 2, 3, and 4.) At this time, no effort has been made to quantitate these differences.

Group II: Only five birds of a scheduled group of twenty-one birds were given osteotomies. Use of the 3/16" osteotomy was discontinued after fractures had occurred at the osteotomy site on seven of the ten bones drilled.

Group III: None of the osteotomies of this group exhibited any significant inflammatory response or signs of bone necrosis. Only one animal (#BNR) exhibited a moderate lymphopenia, and one animal (#9885) showed significant weight loss (Table I). Fracture healing in twenty of the twenty-one osteotomies was similar to the examples of one, three, and seven week osteotomies given in Figure 5; the callus formation of one osteotomy extended several centimeters distal to the osteotomy site. Preliminary investigations to quantitate the rate of fracture repair have begun.

Discussion

The preliminary investigations of this study were conducted to develop a particular type of osteotomy, and to provide sufficient information to allow choice of both the site and size of the osteotomy. Criteria for the choice of the osteotomy site include a readily accessible area, a healing time of sufficient length to permit significant quantitation of fracture repair under weightless conditions, and an osteotomy diameter large enough to allow passage of a photon beam of approximately 0.5 mm. in diameter.

Visual interpretation of the radiographs of Group I strongly indicates that both sites on the humerus as well as the tibial epiphysis heal too rapidly. The osteotomy placed in the midshaft of the tibia apparently heals in seven weeks or less, short of final remodeling. In addition to eliminating the epiphyseal osteotomy in the tibia because of known higher level of metabolic activity compared to the midshaft, precise placement of the osteotomy is not as critical in the midshaft as it is in the epiphysis, where the level of activity varies over short distances.

The occurrence of fractures in Group II at the site of the 3/16" osteotomy decidedly established an upper limit to the diameter of the osteotomy. Although no fractures were observed in the animals receiving the 1/8" osteotomy, it was not subjected to any accelerative forces other than those

occurring in normal cage movement. Experiments involving chronic acceleration of the animals receiving the 3/32" osteotomy are anticipated.

Although some variation in producing identical osteotomies is inherent in the technique, results indicate a reproducible technique has been established. Information from radiographs and experience have resulted in the selection of the diaphysis of the tibia as the most useful site for the osteotomy of the four sites that have been explored. Studies have begun investigating the feasibility of other possible sites. The size of the osteotomy, now established at 3/32" diameter, will be used until shown to weaken the tibia sufficiently to cause fracture under increased acceleration.

Figure 1: Osteotomy in tibial diaphysis immediately following operation.

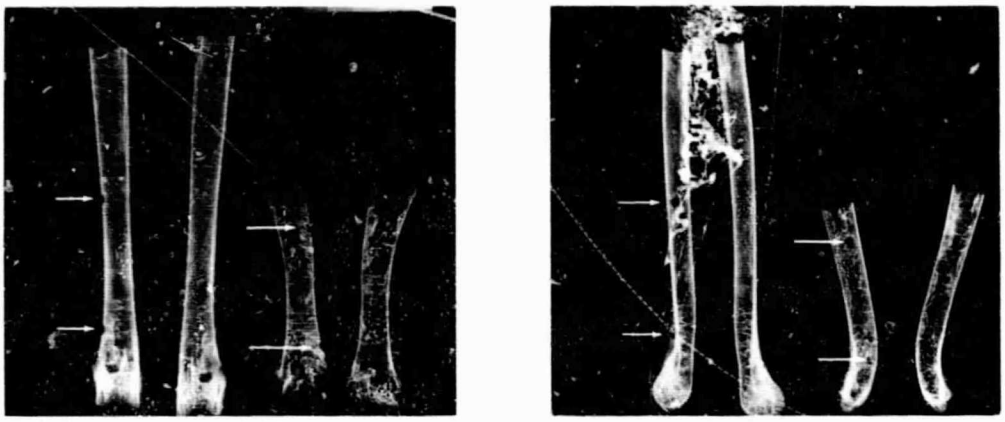

Figure 2: Anteroposterior and lateral X-ray views of tibia and humerus, showing appearance at one week post-osteotomy. The non-wounded left tibia and humerus are shown for comparison.

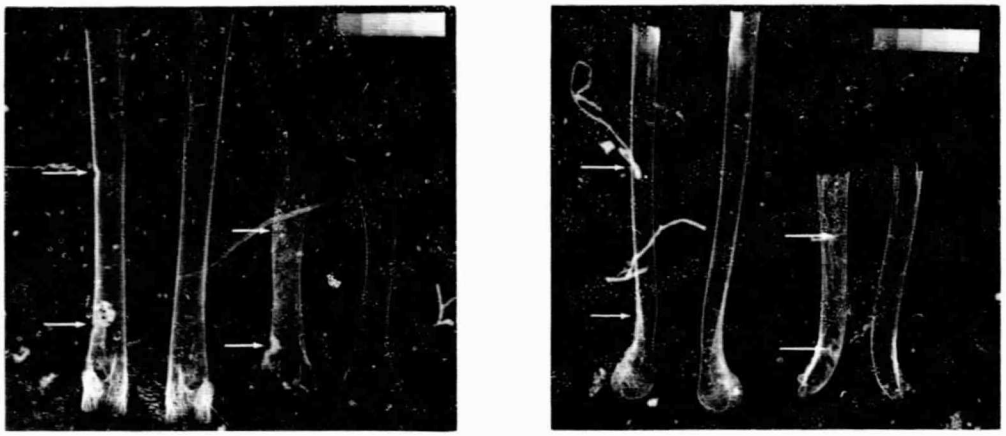

Figure 3: X-ray appearance of the osteotomy at three weeks following injury. Note the periosteal and endosteal thickening, and the sclerosis at the osteotomy site. The pneumatized bone (humerus) is almost completely healed.

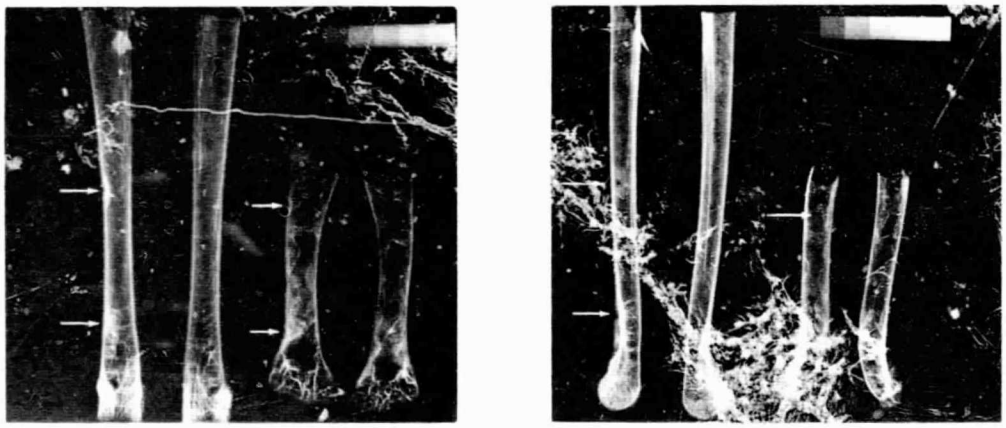

Figure 4: X-ray appearance of the osteotomy at seven weeks following injury. Note that healing is complete except for final remodeling.

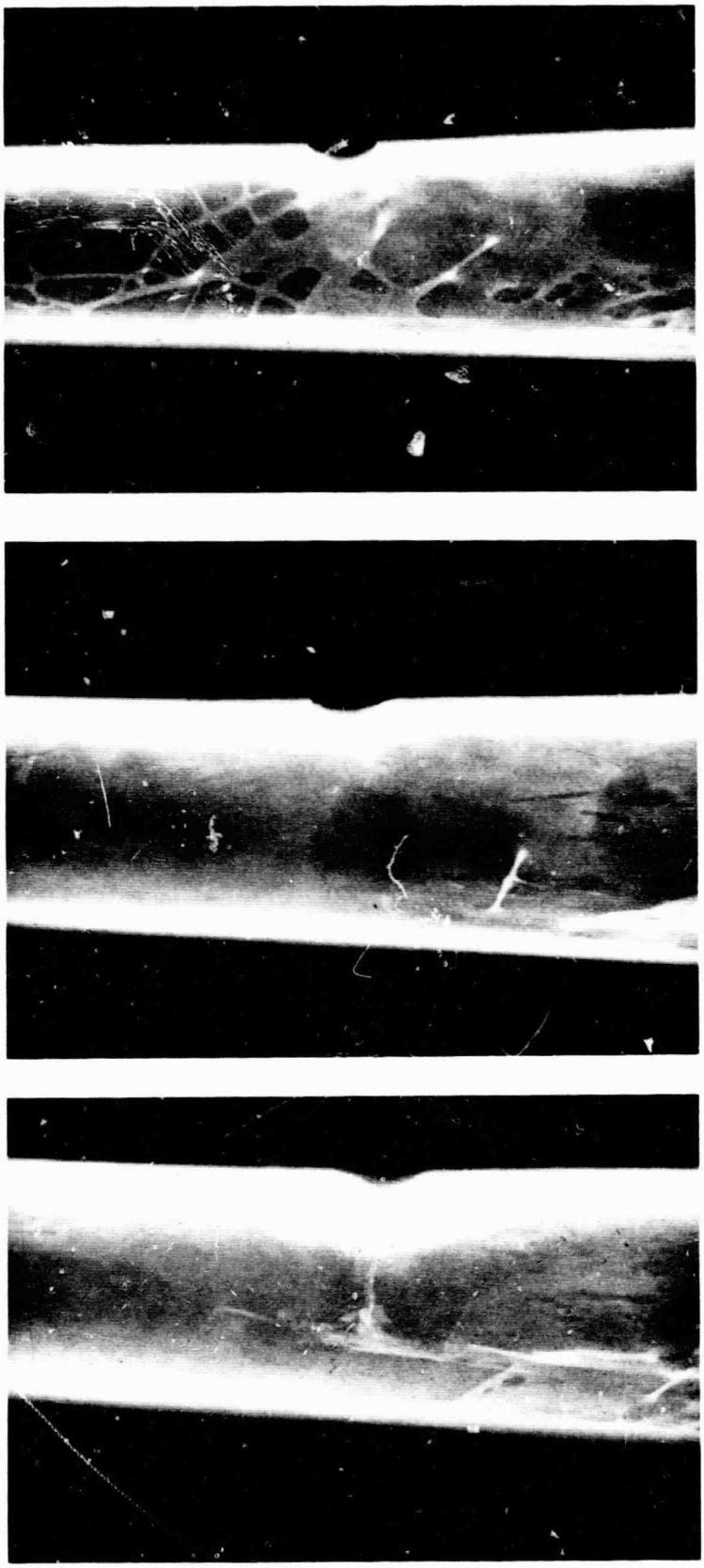

Figure 5: Enlargement of X-rays of the wound sites of three midshaft tibial osteotomies showing typical callus formations at one, three, and seven weeks post-osteotomy.

TABLE I

PER CENT LYMPHOCYTES AND TOTAL BODY WEIGHT CHANGE FOR GROUP 3

Wing Band No.	Wound Time	Per Cent Lymphocytes Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Total Body Wt. Change as % Body Wt.
9726	1 wk	68	-	-	-	-	-	-	- 6.5
9610	1 wk	55	-	-	-	-	-	-	+ 7.2
9847	1 wk	82	-	-	-	-	-	-	- 7.3
BNR	1 wk	26	-	-	-	-	-	-	- 3.9
9701	2 wk	70	77	-	-	-	-	-	+ 1.4
9964	2 wk	98	74	-	-	-	-	-	+ 6.2
8012	2 wk	49	66	-	-	-	-	-	- 1.1
9991	3 wk	69	75	-	-	-	-	-	+ 2.8
9990	3 wk	72	75	-	-	-	-	-	+17.3
9824	3 wk	67	74	-	-	-	-	-	- .3
NBX	4 wk	83	79	70	-	-	-	-	- 7.5
9769	4 wk	67	79	68	-	-	-	-	+ 2.7
9885	4 wk	86	73	87	-	-	-	-	-15.9
9983	5 wk	60	67	75	65	83	-	-	+ 3.7
8289	5 wk	55	56	64	45	69	-	-	+17.2
9988	5 wk	40	61	62	70	69	-	-	+12.2
NB	6 wk	69	58	78	75	81	-	-	+42.4
9882	6 wk	70	83	52	64	52	64	-	+11.8
8515	6 wk	77	80	70	66	67	62	-	+ 2.2
8426	7 wk	41	59	64	68	72	49	-	+ 4.5
8090	7 wk	-	-	46	57	65	50	55	+16.5
8330	7 wk	-	-	52	68	73	63	67	- 8.7

CHRONIC RESTRAINT OF THE SINGLE COMB WHITE LEGHORN CHICKEN

Introduction

The necessity to develop a method of restraint or immobilization of animals living in a weightless environment (space) in order to maintain orientation is well known. It has also been established that restraint or confinement of various species of animals may cause stress and/or alter various physiological functions(Banerjee, 1969; Hoffman et al., 1968; Libke and Mosby, 1968; Bartlett and Altland, 1959; Gass, 1960; Buckner et al., 1932; Latta and Nelson, 1948; Cragg, 1961; Rosecrans and DeFeo, 1965; Sullivan, 1967, 1968; Besch et al., 1967a; Panferova, 1966; Weltman et al., 1966; Gell et al., 1955; Macho et al., 1968; Weltman et al., 1968; Francis; 1957a, 1957b; Sackler and Weltman, 1967; Bartlett and Quimby, 1958; Whittow et al., 1965; Frankel et al., 1958; Bartlett, 1956; Gabel and Clay, 1961). Consequently, animal restraint becomes an important factor to consider in orbital experiments. Therefore, a consideration of the restraint of the chicken has begun early in this project.

Interest in chicken restraint is not new to this group; research in this area began several years ago (Besch et al. 1967a, 1967b). Essentially, it was discovered that chicken

restraint was not easily accomplished; however, research in this area has continued with some success.

Method

Fundamentally the equipment and the original idea of restraint versus immobilization has not changed. The use of the chicken "vest" and 4-way ties have continued with several modifications.

The vest now in use is manufactured in one piece out of nylon materials and costs approximately $6.00 each (Figure 1). The several holes seen along the edge of the vest are for lacing which allow individual bird fittings. The vest placed on the chicken may be seen in Figure 2.

From the original work of Besch et al. (1967a) it became apparent that restraint was an environmental stressor to the chicken which responded with a typical physiological stress reaction. This stress response in the chicken may be quantitated by lymphocyte frequency counts; the occurrence of a lymphopenia is an index of the degree of stress (Burton et al. 1967; Burton and Guion, 1968; Burton and Smith, 1967, 1968).

The chicken is not stressed by wearing the vest when allowed to remain untied in the cage; however, upon the application of restraining ties, it becomes stressed and will die unless the ties are removed. These stressed birds assume

a typical posture which is easily recognized and is best described as a "posture of disorientation" (Figure 3). They also lose body mass and exhibit a lymphopenia.

Since it appeared that the ties (and particularly the tensions exerted by the ties) were acting as a stressor, a method was devised to quantitate the tension developed by the ties. These were initially applied to birds within individual cages. Eventually, birds were restrained out of the cage on a restraining rack which has been termed the "mock-up" (Figure 4).

Known amounts of weights were attached to the bird via nylon cord and by the use of pulleys. The direction of the known tension was directed either dorsally (counterweighing the bird's body weight) or ventrally (adding to the bird's weight) (Figure 5). A lateral tie system is now being developed.

This system has several advantages which have allowed for continued progress in this problem of animal restraint: 1) quantitation of the tie tension; 2) some mobility of the bird although it remains restrained; and 3) allowing a limited increase or decrease of restraint (load or unload).

In order to quantitate the restraint which may be tolerated by the chicken, three criteria of tolerance failure (stress) were developed: 1) loss of 10% body mass; 2) development of the orientation syndrome; and 3) reduction of

the lymphocyte count to less than 50% of the total leukocyte count. A bird was considered stressed and its restraint was reduced if it exhibited any two of these criteria.

A typical restraint tolerance graph is shown in Figure 6.

Results

Initially birds were restrained with a minimum of dorsal or ventral weights. A bird with dorsal and/or ventral restraints amounting to 5% of its body mass, with solid lateral ties, and with "training", may be restrained in the mock-up for at least sixty days without detectable physiological stress. This quantity of restriction at 1-"G" (earth's gravity) is sufficient to restrain a bird to a "perch". Lateral movement is restricted by ± one inch and dorso-ventral movement by ± three inches. This dorso-ventral movement appears to be necessary for continued tolerance by the chicken to the restraint, which allows for resting by "squatting" and for maintaining the bird's center of gravity, which is dynamic.

The tolerance of the chicken to dorsal weights and to ventral weights separately was determined quantitatively to be approximately 30% of its body mass. Too much dorsal weight would precipitate this "orientation syndrome". At the present time, an excess of ventral weight (>30% body mass) only produces a lymphopenia without the complete syndrome.

Experiments involved with dorsal and ventral weights added at the same time and amount (counterweighing each other) are beginning. In conjunction with these will be lateral-tie weight experiments.

Discussion

These studies to date have demonstrated several important points which make the chicken a prime "space" laboratory animal. He is able to tolerate restricted movement for several months, without exhibiting physiological stress, and appearing "normal" in all respects. The chicken can tolerate restraint tension directed dorsally, ventrally, and laterally, which would appear to be of sufficient magnitude to allow the animal to orient itself in a weightless environment.

The greater value of a restrained animal in a weightless experiment as opposed to an immobilized animal (immobilization in itself simulates weightlessness) is quite obvious. The approach to the development of a restraint system at 1-"G" which will be adequate in a weightless environment is now under consideration.

Since tension (acceleration) is quantified in vector units of direction and magnitude, it would appear that tension is additive to the effect of gravity (accleration) without affecting the physiology of the animal (tolerance). The findings reported herein, therefore, suggest that the chicken can

tolerate either a chronic loss or increase of 30% of its body weight. Considering the body mass at 1-"G" (body weight), it may be necessary to "load" the animal in a weightless environment with tension equivalent to approximately 70% of its body mass. This tension (load) may also be increased to 30% over its body mass, i.e., ± 30% load (tension) in relation to its body weight at 1-"G".

At this time, it should be mentioned that a study of a loaded animal in a weightless environment should not detract from the results of physiological effects produced by weightlessness.

Recently, animal restraint has been reviewed by Smith (1970, see Appendix A). He discusses in detail the problems of restraint of several species of animals including various methods of training and selection. This review is given in this report as Appendix A.

The following abstract titled, "Animal Restraint: Application in Space (Weightless) Environment", based on studies funded by contract NAS2-5245, has been submitted and accepted for the Aerospace Medicine Association's 41st Annual Scientific Meeting (Burton and Beljan, 1970).

> In the near future, earth orbiting space laboratories will be a reality. A principal research objective will be to study weightless effects upon physiological functions. This will require the use of laboratory animals which may be restrained,

suitable for animal orientation and experimentation. Specifically, however, the animal should not be immobilized since immobilization is a classical weightless simulation and itself a physiological stress for many animals. Consequently data from immobilized animals in a weightless environment become difficult to evaluate. The choice of the chicken as a prime potential space laboratory animal has been dependent upon its restrainability and a restraint method involving orientation without immobilization.

Birds were restrained in a "natural" $+G_z$ posture using a "fitted body vest". The animal could be anchored by two lateral and ventral and/or dorsal variable tension restraint ties. Repeated restraint experimentation using the same animals indicated that tolerance to the treatment was an individual bird characteristic. Training techniques of selected birds by gradually increasing the degree of restraint severity was successful in developing a number of restrainable animals which could be restrained for indefinite periods of time.

Restraint failure was a typical physiological stress phenomenon, characterized by

lymphopenia, body mass loss, and a specific type of disorientation syndrome. The precipitation of this syndrome was a function of restraint tension, especially of the dorsally directed tie which tends to lift the bird from its perch. This procedure, which was considered to be a simulation of weightlessness, suggested that during space travel the bird should be "weighted" approximately 75% of its body mass by ventrally directed tensions.

Birds which were "adapted" to the restraint procedure were examined physiologically and no differences from non-restrained controls were found. This included hematology, nutrition, body mass, core temperature, and arterial blood pressure. It was concluded that the chicken could be sufficiently restrained for experimental purposes without immobilization. These restrained birds were not considered physiologically altered by the treatment.

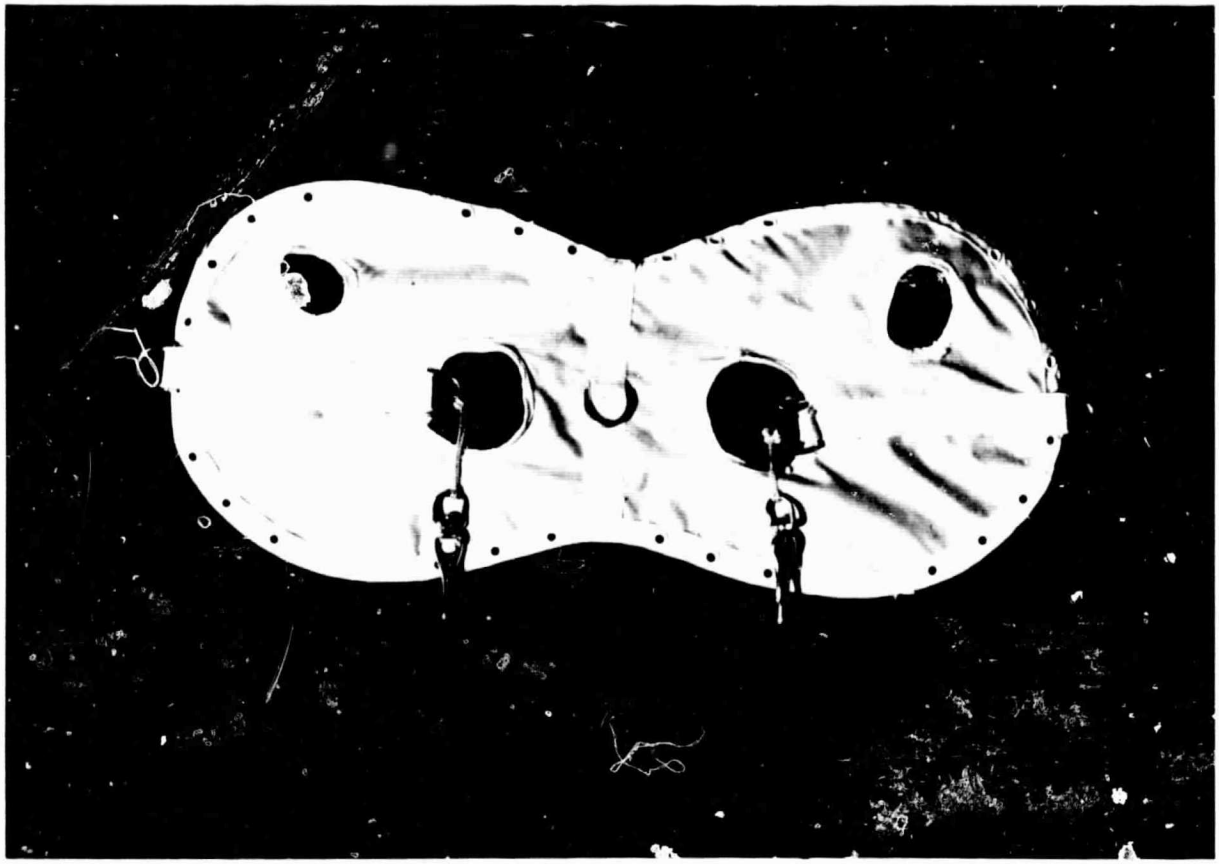

Figure 1: A chicken vest used for chronic animal restraint. The two large center holes are for the legs and the smaller more lateral holes are for the wings. The several small holes along the edge of the vest allow for lacing the vest about the chicken. The metal rings allow for the ties used in the restraint.

Figure 2: The vest in Figure 1, fitted to a male adult chicken.

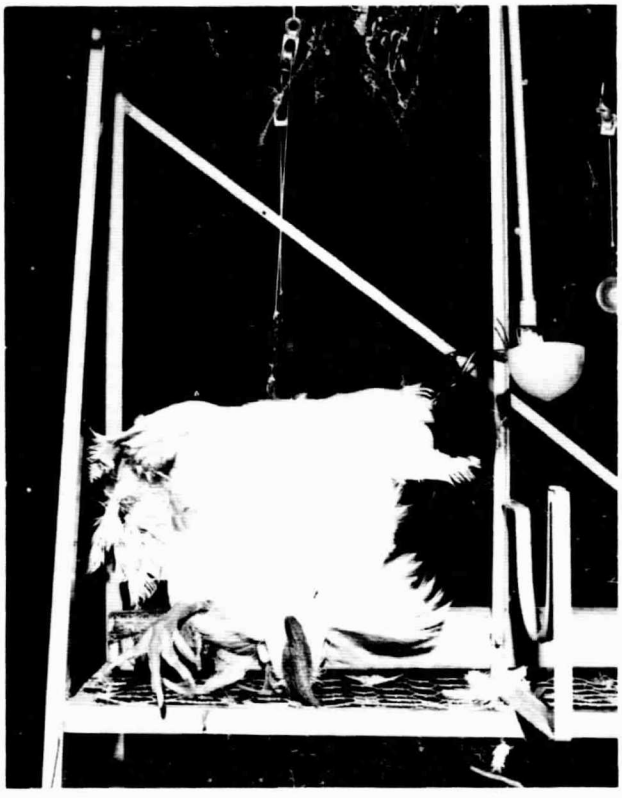

Figure 3: The typical posture of a stressed restrained bird demonstrating the "posture of disorientation" described in the text.

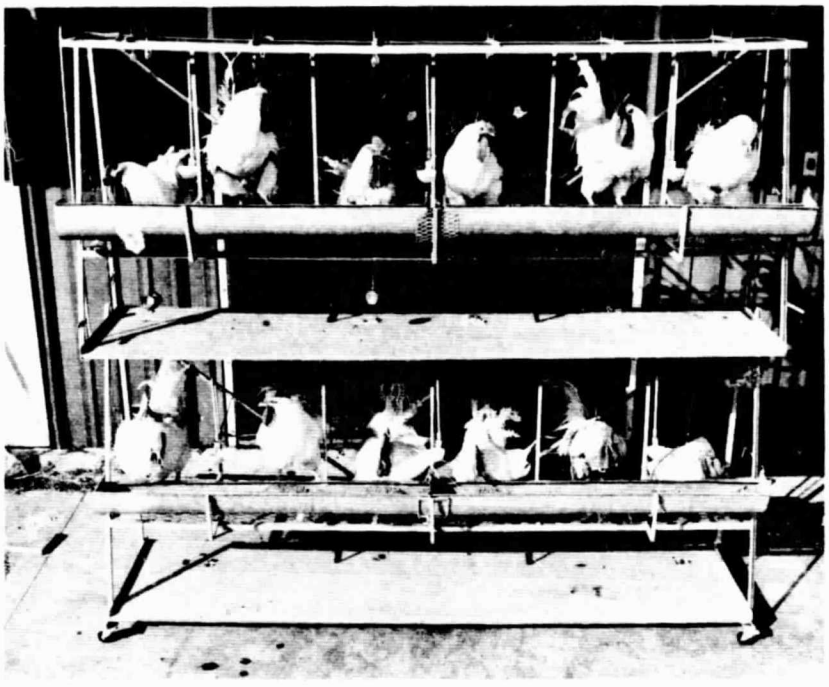

Figure 4: Shown is the "mock-up" used for animal restraint. Constructed of angle iron and having casters for easy movement, it will accommodate twelve adult male chickens. A close up of the automatic waterers, perch, and restraint tie assembly may be seen in Figure 5.

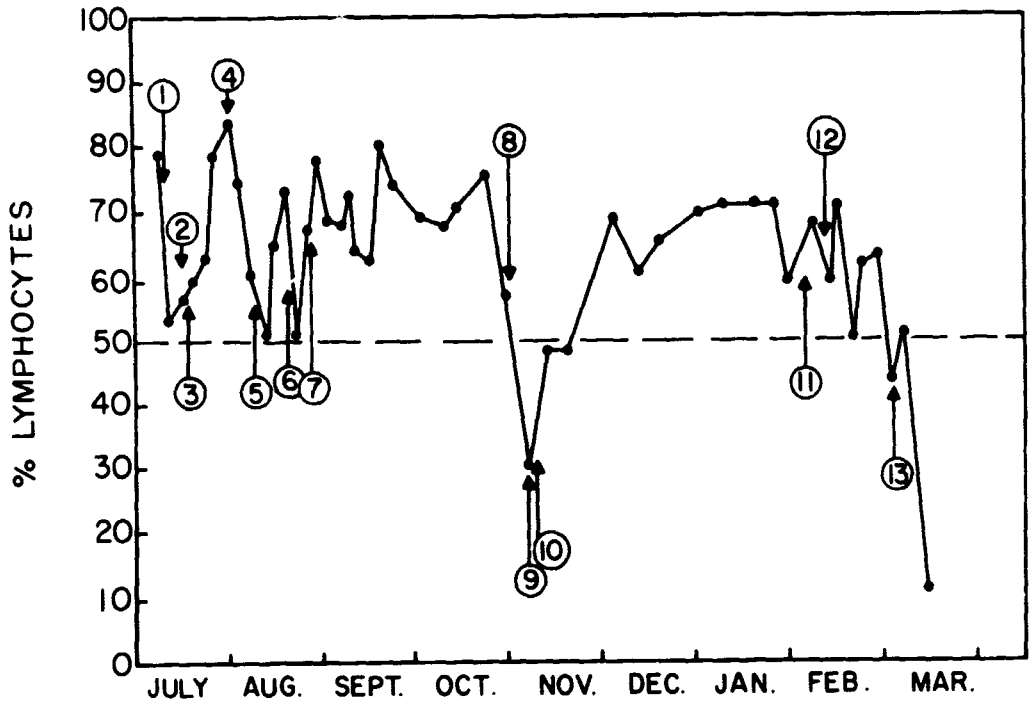

Figure 6: The relationship of the % lymphocytes to the applied chronic restraint as determined in one bird: (1) dorso-ventral static ties; (2) orientation syndrome; (3) bird improved; (4) two static lateral ties; (5) dorsal weight of 4% body mass; (6) weight increased to 17%; (7) increased to 29%; (8) orientation syndrome; (9) weight decreased to 18% body mass; (10) weight released; (11) placed on "mock-up" static lateral ties only; (12) dorsal weights of 5% body mass; (13) orientation syndrome and weights removed; (14) at the present time the bird's lymphocyte count is 52 which is not shown on the Figure. It is about to be placed back in restraint.

DEVELOPMENT OF A PURIFIED DIET WITH CONTROLLABLE CALCIUM AND PHOSPHORUS LEVELS

Introduction

In planning the nutrition phase of this project, the possibility of empirically formulating an experimental diet suitable for maintaining mature male chickens in an adequate nutritional state (except for calcium and phosphorus content) was realized as a consequence of studies made over the years on the nutrient requirements of adult chickens. Studies had not been made on the calcium and phosphorus requirements of adult male chickens, however, because males were generally given the levels of calcium and phosphorus required to maintain egg production in females. Moreover, a diet relatively free of calcium and phosphorus was needed in order to relate levels of these minerals to rate of bone-healing, following the use of osteotomies to simulate bone fractures. Since preliminary experimentation showed that the calcium and phosphorus requirements of adult male chickens were less than the quantities present in diets composed of natural ingredients (commerical diets), it became necessary to develop a diet composed of purified ingredients in which calcium and phosphorus content could be precisely controlled and the quantitative requirements for these minerals be determined by

feeding graded levels. In this way, the relation of calcium and phosphorus intake could be related to rate of fracture repair.

Method

The formula of the purified diet (Diet A) developed for the purpose of studying the rate of bone healing in mature male chickens is given in Table I. Blood fibrin was used as the source of protein because of its extremely low calcium and phosphorus content and adequate amino acid composition. Upon assay this diet was found to contain 0.02 per cent calcium and 0.03 per cent phosphorus. Butylated hydroxytoluene was included in the die. to retard the development of oxidative rancidity in the corn oil and thereby minimize the detrimental effect of this process on the stability of vitamin A, vitamin E and biotin.

The levels of calcium and phosphorus fed in the experiment are given below:

Diet A - 0.02% Ca, 0.03% P

Diet B - 0.2% Ca, 0.133% P

Diet C - 0.4% Ca, 0.267% P

The ratio of calcium to phosphorus in Diet B and C was set at 1.5:1. The added calcium and phosphorus was supplied by pure hydrous dicalcium phosphate ($Ca\ H\ PO_4 \cdot 2H_2O$) and pure calcium carbonate ($Ca\ CO_3$).

Mature Single Comb White Leghorn male chickens were used as the experimental subjects. The males were housed in individual wire cages with a floor space of 12" x 18". Feed and water were also supplied individually, ad libidum. The laboratory in which the males were confined was air conditioned in such a manner as to maintain constant ventilation. Each diet was fed to fourteen males for twelve weeks; their weight and feed intake was recorded weekly.

Blood samples were taken at the start of the experiment and biweekly thereafter. The data collected on the blood were "packed cell volume" (chiefly erythrocytes), relative leukocyte counts, plasma protein, hydroxyproline, and calcium and phosphorus content. Hemoglobin determinations were made approximately ten weeks after the start of the experiment.

At the end of two weeks and every two weeks thereafter, two males from each group were sacrificed and the tibiae dissected. One was used in determining fat-free dry bone, bone ash, and bone calcium, magnesium, and phosphorus; the second was held for reserve purposes.

Results and Discussion

The health of the Single Comb White Leghorn male chickens was well-maintained throughout the experimental period. No gross evidence of disease was observed at any time. The packed cell volume (percentage erythrocytes),

buffy coat (percentage leukocytes, chiefly) and percentage plasma protein decreased slightly during the experiment, but this appeared not to affect the physical state and was unrelated to the experimental plan (Table II and Figure 1). No changes of any significance were observed in blood differential white cell counts (Table III). The counts, moreover, were approximately the same as those of White Leghorn males of the same strain and age, fed a commercial ration composed largely of natural ingredients.

The hemoglobin values obtained were 12.7±0.8 gm. per cent, 13.0±0.3 gm. per cent and 13.3±1.0 gm. per cent for the low to high calcium and phosphorus levels, respectively. The differences in the values are not significant and appear normal according to results on the hemoglobin content of adult male chickens reported by Dukes and Schwarte (1931).

A slight drop in plasma calcium was observed at the end of the sixth week of the experiment but the males had recovered by the eighth week. This, therefore, was apparently of no significance. The levels of calcium had no effect on percentage plasma calcium, but plasma phosphorus was reduced in the low calcium, low phosphorus group (Table IV and Figures 2 and 3). Although some difficulty was encountered in inducing the male chickens to eat the purified experimental diets in place of the commercial ration fed originally, the changeover was successfully accomplished and maintenance of weight and adequate feed intake were obtained throughout the

experimental period (Tables V and VI). Although feed intake appeared to be related to the experimental plan, the standard deviations showed that the slight increases were of no significance. This is supported by the small increases in weight obtained over the weight of the males at the start of the experiment.

No changes in the percentage of fat-free dry tibiae or bone ash content were obtained during the experiment. These weights were almost identical with the percentage of the fat-free dry tibiae and bone ash content of males, fed the commercial ration, that were sacrificed when the experiment was started. Percentage bone phosphorus was also unchanged but the males appeared to have slightly greater bone calcium at the end than at the start; however, the standard deviations indicate that the increases were not significant. Bone magnesium also was unchanged. (For results of tibiae analyses see Table VII and VIII).

Since the percentage mineral composition of the fat-free dry tibiae was not affected by the levels of calicum and phosphorus supplied on the experimental diets, it appeared possible that by relating the average weight of fat-free dry tibiae during the last half of the experiment to the average weight of the males during this time, an indication of the effect of low calcium intake might be obtained. The results after making the necessary calculations indicated that the

males fed the low-calcium, low-phosphorus diet had slightly smaller bones than the males supplied the higher levels of calcium and phosphorus. The values expressed in grams of fat-free dry tibiae per kilogram of body weight for the diets containing 0.02 per cent calcium/0.03 per cent phosphorus, 0.2 per cent calcium/0.133 per cent phosphorus, and 0.4 per cent calcium/0.267 per cent phosphorus were respectively 7.0 gm., 7.9 gm., and 7.5 gm. (Table IX and Figure 4). The differences observed, however, were not statistically significant presumably because of the small number of individuals sacrificed during the last half of the experiment. The slightly higher average for the hydroxyproline content of the blood of the males receiving the highest levels of calcium and phosphorus also was not significant (Table X). Thus, no evidence of loss of bone matrix, presumed to accompany loss of bone calcium and phosphorus, was obtained.

However, under the experimental conditions, it is obvious that loss of fat-free dry bone weight must eventually be obtained in adult White Leghorn males fed a diet practically devoid of calcium and phosphorus, since plasma calcium and phosphorus were maintained at normal levels throughout the experiment. This is also in accord with known facts about the effect of low-calcium phosphorus intake on maintenance of normal bone weight or mass. If the depletion period is sufficiently long, osteoporosis (loss of bone weight or mass) with no change in percentage mineral content

must occur. This is due to reduction in bone matrix in osteoporosis attending loss of bone mineral, a phenomenon first reported by Koelliker (1873).

In addition to the research on the requirements of the adult male White Leghorn chicken for calcium and phosphorus and the effect of intake of these minerals on the rate of development of osteoporosis, studies on the effect of graded levels of calcium and phosphorus on the rate of fracture healing have been initiated. Results at this time are not complete.

Summary

Graded levels of calcium and phosphorus have been fed in a purified diet to adult White Leghorn male chickens to determine the quantity of these minerals required for maintenance and the effect of graded levels on the rate of development of osteoporosis.

The results indicated that the requirement for calcium and phosphorus was not greater than 0.2 per cent calcium and 0.133 per cent phosphorus over a period of twelve weeks. Slight evidence was also obtained that the bones of the males fed the diet containing 0.02 per cent calcium and 0.03 per cent phosphorus were beginning to develop osteoporosis during the last six weeks of the experiment.

Except for calcium and phosphorus the purified diet was found to be nutritionally adequate for maintaining the weight

of males during the course of the experiments; measurement of blood constituents and leukocytes also indicated that they were maintained in a hea thy state.

Work in Progress

A second experiment dealing with the effect of low and adequate calcium levels, similar to the experiment reported herein except for duration, has been initiated. Simultaneously the effect of calcium and phosphorus levels on the rate of wound healing following an osteotomy has been started. The levels of calcium and phosphorus supplied are 0.02 per cent and 0.03 per cent, respectively, and 0.4 per cent and 0.247 per cent, respectively. The duration of the experiment has been set at twenty-four weeks. Osteotomies on three adult male chickens of each group were made at the start of the experiment and are to be made again at eight, sixteen, and twenty-four weeks. All osteotomies will be allowed to heal four weeks.

The diet fed the male chickens has been modified in an effort to increase pelleting characteristics. In order to accomplish this the starch in the diet fed in the previous experiment was replaced with glucose monohydrate and one per cent of gelatin was added. The formula of the new diet is given in Table XI.

In order to eliminate the powder-like consistency of the diet, a small amount of water is added to the diet before feeding and the moist mixture is then placed on stainless steel trays for drying. When the mixture is almost dry, it is passed through a screen to render the particle size suitable for feeding. Final drying makes the diet into a crumbly preparation, which the male chickens eat more readily.

The modified diet has been found to pellet readily and the pellets are relatively dust free. Moreover, the inclusion of five per cent powdered nickel made the pellets magnetic to a sufficient extent that they could be picked up readily by means of a small magnet. This characteristic is essential in order to feed the males in space by means of a magnetized belt or other device of this nature.

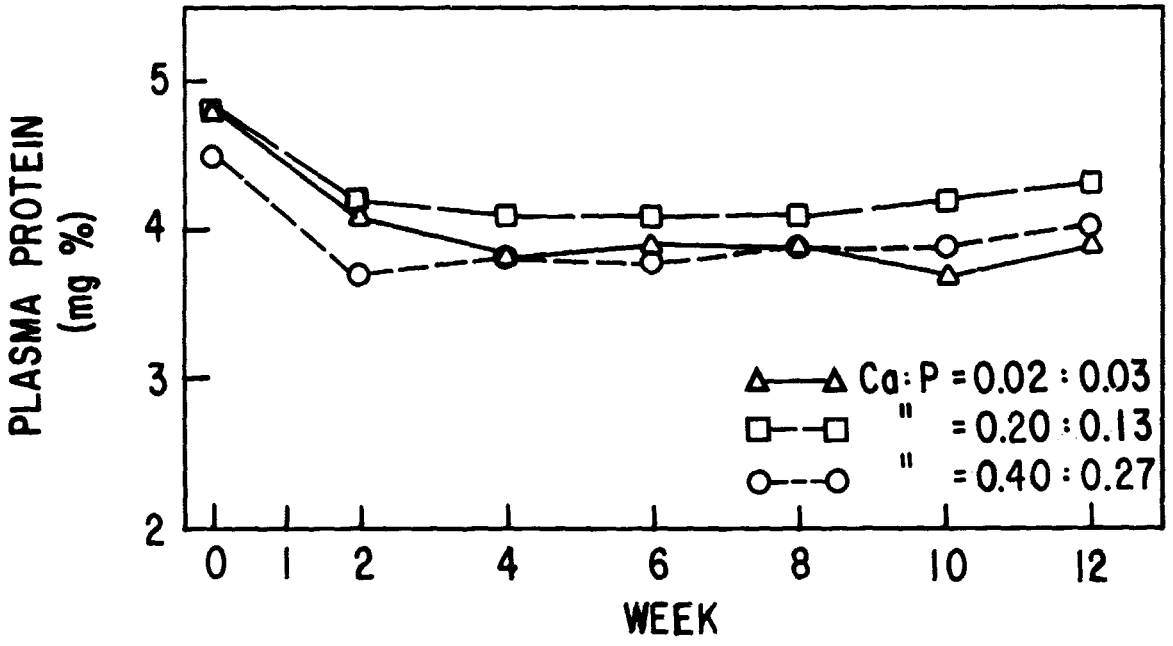

Figure 1: Plasma protein expressed as grams per cent. Each data point reflects the mean of all animals on the diets at that time.

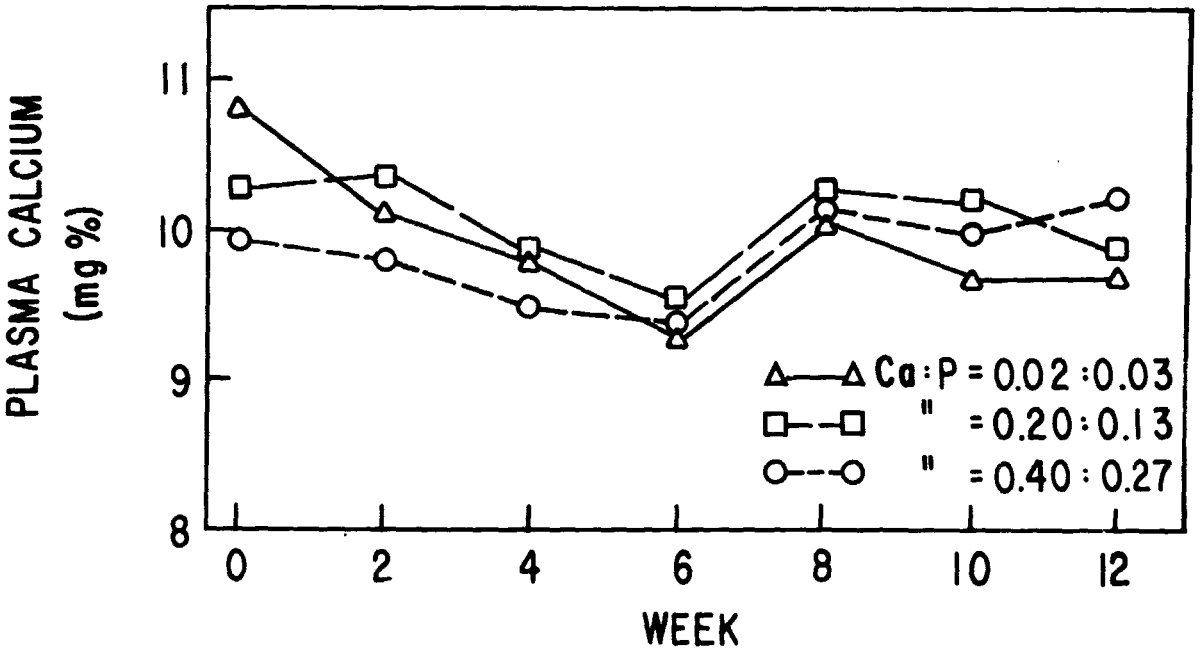

Figure 2: Plasma calcium expressed as milligram per cent. Data points are means of all animals on the diets at that time.

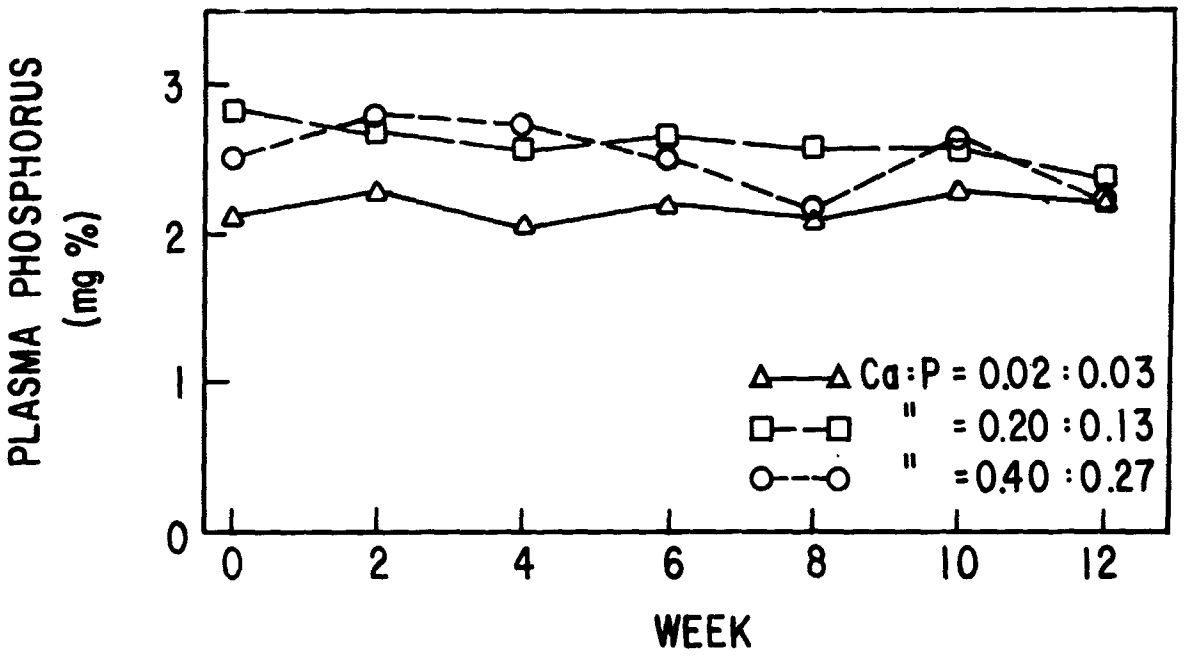

Figure 3: Plasma phosphorus expressed as milligrams per cent. Data points are means of all animals on the diets at that time.

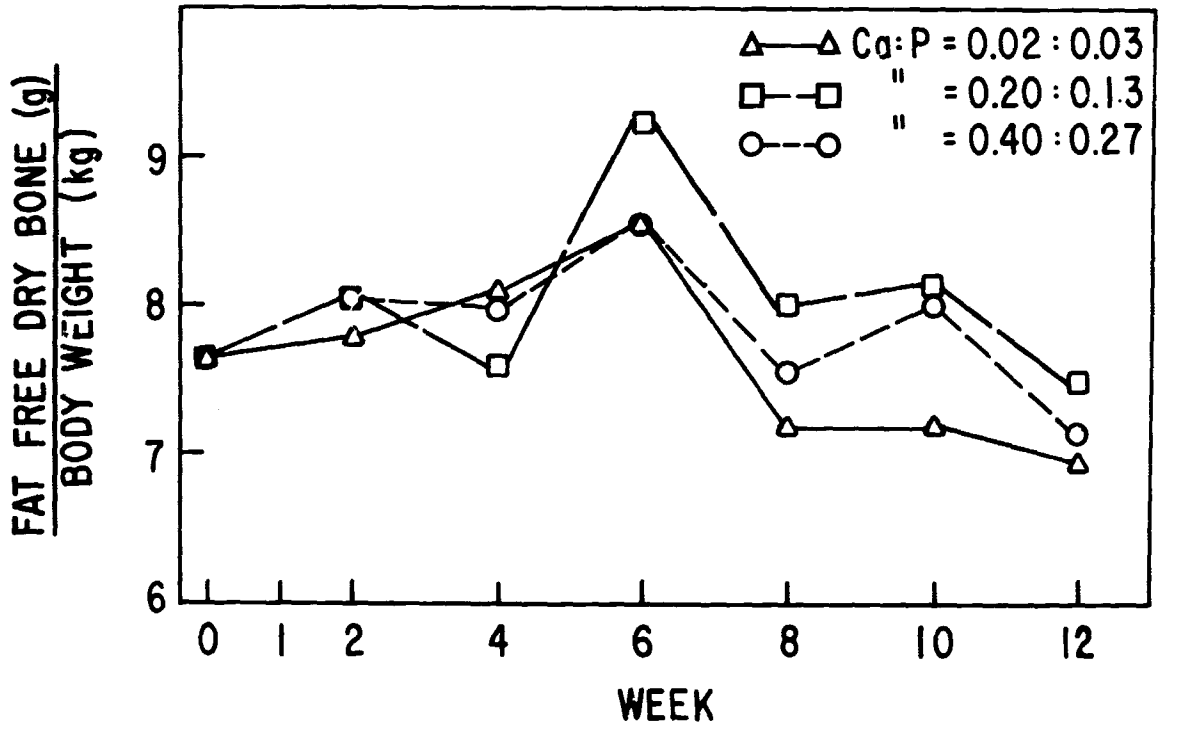

Figure 4: Ratio of grams of fat free dry bone to body weight in kilograms. Results of two animals are averaged for each data point.

TABLE I

COMPOSITION OF THE BASAL DIET

Ingredients	Per cent of total diet
Corn Starch	73.250
Blood Fibrin	14.500
Soybean Oil	4.000
Cellulose	3.000
Mineral Mixture (1)	1.970
Vitamin Mixture (2)	3.250
Butylated hydroxytoluene	0.025

1) Provides in mg./Kg. diet: K, 2,344; Mg, 592; Na, 2,376; Fe, 100; Mn, 98; Al, 20; Zn, 56; Cu, 6.4; Co, 1.2; I, 1.52; F, 1.36; Mo, 0.79; Si, 0.40; Se, 0.16.

2) Provides in mg./Kg. diet: Thiamine-HCl, 10.0; riboflavin, 10.0; pyridoxine-HCl, 10.0; calcium pantothenate, 30; niacin, 100; folic acid, 5; biotin, 0.2; vitamin B_{12} (3 mg./g.), 6.67; 2-methyl-1, 4-naphothoquinone 10.0; vitamin A (325,000 IU/g.), 1000.0; vitamin D_3 (32,5000 IU/g.), 1000.0; vitamin E (44 IU/g.), 2,000.0 and choline (50%) 3,410.0. The vitamins were mixed with glucose to make up the required percentage.

TABLE II

PACKED CELL VOLUME, BUFFY COAT, AND PLASMA PROTEIN AS PERCENTAGE OF WHOLE BLOOD

	Packed Cell Volume			Buffy Coat			Plasma Protein		
[Ca] WK	0.02	0.2	0.4	0.02	0.2	0.4	0.02	0.2	0.4
0	42.0	41.0	42.0	1.0	0.8	0.5	4.8	4.8	4.5
2	41.0	40.2	41.5	0.8	0.7	0.7	4.1	4.2	3.7
4	39.9	39.0	39.2	---	---	---	3.8	4.1	3.8
6	39.8	38.3	39.8	0.6	0.5	0.7	3.9	4.1	3.8
8	37.3	39.0	39.4	0.7	0.8	0.5	3.9	4.1	3.9
10	39.5	40.6	40.3	0.5	0.6	0.6	3.7	4.2	3.9
12	38.7	37.1	39.7	0.5	0.5	0.6	3.9	4.3	4.0
$\bar{X}$	39.7	39.3	40.3	0.6	0.6	0.5	4.01	4.26	3.94
S.D.	1.52	1.38	1.08	0.32	0.28	0.24	0.37	0.24	0.26

TABLE III

RELATIVE LEUKOCYTE COUNTS

	Lymphocyte			Heterophil		
[Ca] WK	0.02	0.2	0.4	0.02	0.2	0.4
0	59.4	58.5	57.8	33.3	31.9	30.5
2	58.2	57.9	59.0	32.5	32.4	30.1
4	65.3	65.6	67.1	24.2	24.2	24.4
6	58.1	60.5	59.1	32.8	30.0	29.1
8	69.5	58.3	56.8	23.7	31.1	33.6
10	64.3	60.2	60.5	24.3	28.5	31.3
12	65.5	62.5	60.5	23.0	29.3	29.3
$\bar{X}$	62.9	60.5	59.2	27.7	29.6	29.8
S.D.	4.39	2.74	4.06	4.77	2.76	2.81

	Monocyte			Eosinophil			Basophil		
[Ca] WK	0.02	0.2	0.4	0.02	0.2	0.4	0.02	0.2	0.4
0	5.6	7.9	9.5	0.2	0.2	0.0	1.5	0.5	1.6
2	6.5	7.9	8.1	0.6	0.3	0.3	2.1	1.5	2.4
4	7.7	7.9	7.0	1.0	0.3	0.3	1.7	1.1	0.9
6	7.9	8.1	10.1	0.4	0.1	0.1	0.8	1.3	1.5
8	5.8	8.3	7.9	0.0	0.4	0.0	1.4	2.0	1.8
10	10.2	8.3	11.0	0.0	1.5	0.7	1.7	2.5	3.0
12	9.5	6.8	8.5	1.3	0.8	0.0	0.5	1.3	1.8
$\bar{X}$	7.6	7.9	8.9	0.5	0.4	0.2	1.39	1.31	1.86
S.D.	1.77	0.51	1.39	0.50	0.22	0.26	0.56	0.94	0.67

Commercial:

Lymphocyte	Heterophil	Monocyte	Eosinophil	Basophil
61.8±14.9	30.2±14.5	6.3±4.0	0.2±0.41	10.±0.63

TABLE IV

PLASMA CALCIUM AND PHOSPHORUS IN MG. PER CENT

	Calcium				Phosphorus		
[Ca] WK	0.02	0.2	0.4	[Ca] WK	0.02	0.2	0.4
0	10.08	10.28	9.94	0	2.10	2.57	2.51
2	10.10	10.36	9.82	2	2.29	2.72	2.75
4	9.80	9.86	9.50	4	2.03	2.57	2.73
6	9.29	9.59	9.37	6	2.53	2.13	2.67
8	10.13	10.28	10.16	8	2.10	2.58	2.21
10	9.70	10.21	10.00	10	2.00	2.47	2.29
12	9.74	9.89	10.23	12	2.21	2.38	2.36
$\bar{X}$	9.82	10.10	9.86	$\bar{X}$	2.19	2.49	2.50
S.D.	0.31	0.28	0.31	S.D.	0.17	0.20	0.22

TABLE V

BODY WEIGHT CHANGES IN GRAMS/WEEK/BIRD

[Ca] WK	0.02	0.2	0.4
1	+12.0	-31.0	-34.0
2	- 7.5	- 0.9	- 5.1
3	- 5.0	+37.0	+22.6
4	- 2.0	- 9.0	+ 8.0
5	+16.0	+ 1.5	+ 7.2
6	+11.0	+16.0	+16.0
7	- 9.0	-11.0	-13.0
8	+23.0	+ 3.0	+14.0
9	+19.0	-14.0	+11.0
10	+13.0	+ 0.2	+18.0
11	+15.0	+11.0	+18.0
12	-24.0	+63.0	-16.0
$\bar{X}$	+ 4.15	+ 5.48	+ 3.89
S.D.	±14.05	±24.72	±17.24
Initial Weight:	1806 ± 170.1	1799 ± 166.8	1801 ± 161.5

TABLE VI

FEED INTAKE IN GRAM/DAY/BIRD

[Ca] WK	0.02	0.2	0.4
1	48	49	41
2	48	48	49
3	44	53	54
4	49	54	52
5	50	47	54
6	52	52	52
7	50	45	45
8	50	50	50
9	53	51	55
10	53	51	59
11	52	53	59
12	52	63	53
$\bar{X}$	50.1	51.3	51.9
S.D.	8.77	13.9	17.29

TABLE VII

PER CENT FAT-FREE DRY BONE AND BONE ASH CONTENT

	Dry Matter			Ash		
[Ca] WK	0.02	0.2	0.4	0.02	0.2	0.4
2	89	89	89	59.8	58.6	60.8
4	90	90	90	59.9	60.3	57.7
6	89	89	89	60.6	60.6	60.1
8	90	90	90	58.8	60.2	61.4
10	89	89	89	61.8	59.3	59.1
12	90	90	89	60.7	61.0	60.6
$\bar{X}$	89.5	89.5	89.3	59.9	60.0	60.0
S.D.	0.55	0.55	0.52	1.41	0.89	1.34

Commercial:

Dry Matter	Ash
90.0±1.30	60.4±1.31

TABLE VIII

PER CENT CALCIUM, PHOSPHORUS AND MAGNESIUM CONTENT OF FAT-FREE DRY BONE

	Calcium			Phosphorus		
[Ca] WK	0.02	0.2	0.4	0.02	0.2	0.4
2	20.9	19.3	21.3	10.6	10.6	11.3
4	21.6	22.6	20.8	10.5	11.0	10.6
6	19.9	23.0	20.3	10.3	11.0	9.8
8	21.6	21.9	22.1	10.8	11.0	10.7
10	22.8	21.4	21.4	10.4	10.7	11.1
12	22.3	22.6	22.3	10.8	10.5	10.9
$\bar{X}$	21.52	21.80	21.37	10.57	10.80	10.73
S.D.	1.02	1.35	0.75	0.20	0.22	0.52

	Magnesium		
[Ca] WK	0.02	0.2	0.4
2	0.32	0.32	0.32
4	0.31	0.34	0.31
6	0.31	0.31	0.31
8	0.33	0.33	0.32
10	0.32	0.33	0.32
12	0.32	0.31	0.34
$\bar{X}$	0.32	0.32	0.32
S.D.	0.41	0.71	0.41

Commercial:

Calcium	Phosphorus
20.8±1.58	10.4±0.52

TABLE IX

RATIO OF DRY BONE WEIGHT TO BODY WEIGHT

	[Ca]	Dry Bone Weight (gm.)	Body Weight (Kg.)
Weeks 0-6	.02	14.9±1.3	1.79±0.017
	.2	14.9±1.4	1.77±0.012
	.4	14.6±1.4	1.78±0.014
Weeks 7-12	.02	13.2±0.6	1.89±0.036
	.2	14.2±0.8	1.80±0.041
	.4	14.1±1.1	1.88±0.038

	[Ca]	Dry Bone/Body Weight
Weeks 0-6	.02	8.03
	.2	8.41
	.4	8.20
Weeks 7-12	.02	7.00
	.2	7.90
	.4	7.50

Commercial:

Dry Bone	15.0
Body Weight	1.99
Dry Bone/Body Weight	7.54

TABLE X

PLASMA HYDROXYPROLINE CONTENT IN MG. PER CENT

[Ca] WK	0.02	0.2	0.4
2	0.93	0.95	0.92
4	0.86	0.89	1.04
6	0.85	0.85	0.90
8	0.86	0.82	0.90
10	0.91	0.90	0.95
12	0.95	0.85	1.04
$\bar{X}$	0.89	0.88	0.96
S.D.	0.04	0.04	0.06

TABLE XI

COMPOSITION OF THE BASAL DIET

Ingredients	Per cent of total diet
Glucose monohydrate	72.500
Blood Fibrin	14.500
Gelatin	1.000
Soybean Oil	4.000
Cellulose	3.000
Mineral Mixture (1)*	2.000
Vitamin Mixture (2)*	3.000
Butylated hydroxytoluene	0.025

1) Provides in mg./Kg. diet: K, 2,344; Mg, 592; Na, 2,376; Fe, 100; Mn, 98; Al, 20; Zn, 56; Cu, 6.4; Co, 1.2; I, 1.52; F, 1.36; Mo, 0.79; Si, 0.40; Se, 0.16.

2) Provides in mg./Kg. diet: Thiamine-HCl, 10.0; riboflavin, 10.0; pyridoxine-HCl, 10.0; calcium pantothenate, 30; niacin, 100; folic acid, 5; biotin, 0.2; vitamin B_{12} (3 mg./g.), 6.67; 2-methyl-1, 4-naphothoquinone 10.0; vitamin A (325,000 IU/g.), 1,000.0; vitamin D_3 (325,000 IU/g.), 1000.0; vitamin E (44 IU/g.), 2,000.0 and choline (50%) 3,410.0.

* Mixed with corn starch to make up the required percentage.

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APPENDIX A: ANIMAL RESTRAINT

A. H. Smith*

The pathophysiological responses of animals to restraint are of dual importance to Gravitational Biology. The consequences of such limitation in voluntary activity -- like chronic recumbency in humans -- may be at least partially related to those of subgravity and weightlessness exposure. As such they may furnish a basis for anticipating physiological changes in space flight. Also, preparation of animals for space flight experiments will require application of some degree of restraint to replace the orienting influence of gravity. Obviously, it will be quite important to understand the purely restraint-induced phenomena, so that they will not become confused with those arising from weightlessness. On this basis, fairly detailed discussions of animal restraint and restraint stress have been included herein.

Limitation of an animal's mobility -- which is analogous to forced bed rest in humans -- may become harmful. The initial responses to such restraint are largely emotional, and involve epinephrine secretion. In natural situations,

*A. H. Smith. Extracted from Monograph 9, Space Biology and Medicine Series, NASA Contract NSR-09-010-027.

this response may be useful as an emergency procedure -- preparing the animal for "fight or flight" (Cannon, 1929). However, in constrained wild animals the reaction may be quite severe, inducing shock, and even "fright death" (Thomas, 1961; Guthrie et al., 1967). The psychogenic nature of the response is indicated by the direct relationship of its degree to the position of the animal in its social hierarchy.

Adrenocortical participation also occurs soon after the application of restraint. This early syndrome has been called the "Alarm response" by Selye (1950), and its presence indicated by an increase in circulating adrenocorticoids. With continued restraint, the influence of catecholamines diminishes and only the effects of corticosteroids persist. The patho-physiology of restrained animals has come to be called "restraint stress", but with little distinction regarding its duration or hormonal mechanisms.

The degree of restriction of an animal is quite important to the patho-physiological changes induced. Confinement may vary from a more-or-less permissive limitation in the range of movement ("restraint"; Besch et al., 1967). The latter may proceed in severity to an abolishment of movement -- "immobilization." Much of the variability in reports of restraint stress arises from differences in the method and degree of restraint, as well as its duration and frequency.

The simplest and least severe procedure for reducing an animal's mobility is confinement within a small space (cage restraint) -- with a volume perhaps only several-fold that of the body. The volumetric relationships of animals so restricted is important, since simple isolation in quite large volumes also may induce physiological changes (Sackler et al., 1967; Weltman et al., 1967). Restraint (as distinguished from restriction) may be arranged by direct attachments to the body. The attachments may be more-or-less slack or elastic, permitting defined movement, or resistance to movement. In the extreme situation, the animal may be immobilized -- perhaps by a rigid encasement of the body (e.g., Plaster of Paris cast). However, there also are variations in the conditions of immobility. It may be arranged in such a way as to eliminate muscular activity related to posture or attempted locomotion -- or the potential for isometric activity of antigravity muscles may remain. For example, Bartlett and Altland (1959a,b) found that encasing rats in wire-mesh cylinders permitted a substantial activity of leg muscles, and the results of such restraint were not typical of hypokinesia. Consequently, in studies of animal restraint, definition of the physiological status of the experimental animals is important.

The physiological responses to restraint have been known for 75 years (Löwit, 1892, discussed by Goldscheider and Jacob, 1894). Since then, "restraint stress" has been

reported periodically -- principally as an unanticipated and complicating response in control animals. Generally, such observations are without concern for mechanism, or distinction between treatments involved. Only recently, with the necessity of rigorous restraint of animals for space flight, has the subject become systematically investigated.

Chronic Restraint Syndrome:

There are some similarities in response of various animals subject to long-term continuous restraint. The most obvious changes are behavioral. Initially there may be a period of anxiety and enhanced activity as the animal seeks to escape its confinement. However, the animal eventually becomes quiet, and progressively lethargic. A similar sequence has been reported in immobilized humans (Gerd, 1963)-- an initial condition of pain and anxiety giving way to a prolonged depression.

In restrained animals, feed intake may be temporarily or permanently decreased, with a comparable degree and period of wasting. Differences are apparent between individuals and between species in the nature and rate of development of sequellae. In mice and rats subject to cage restraint the period of increased activity is about one day (Portugalov et al., [illegible]7). As they become more inactive, they also become [illegible]cid to the touch (Gass, 1960).

Dogs restrained by harness have an immediate emotional response, which is quite variable in degree and duration among individuals (Gerd and Gurovskiy, 1962). Hyperventilation generally developed and was persistent. Anorexia and diminished urinary output develop rather rapidly. After 10 hours of rigid restraint, the appearance and behavior of restrained dogs gave evidence of malaise. Some became disoriented after a few days, and had signs of "other disorders" of the nervous system.

Monkeys have been restrained in a chair by "tables" -- pillory attachments at the neck and waist (Mason, 1958; Lilly, 1958). After an initial few days, they tolerated this treatment peaceably for 3-4 weeks. The positioning of the monkey in the restraint device was critical, and if improper, pressure sores and leg edema developed. Four weeks was considered the maximum period of such restraint -- and with longer retention there was a loss of muscle tone -- especially of the abdominal wall. Bogina et al., (1965 reported a more permissive restraint system, with a belt and collar on the animal connected by flexible attachments to two vertical bars. A transient emotional period (2-4 days) was noted, after which the monkey appeared normal for periods up to four months. Muscular weakness, edema of the extremities, and decubitus were avoided. However, with restraint in excess of four months, signs of uncoordination and even disorientation appeared. Upon release, the animal

was often ataxic -- however, perfectly capable of sexual intercourse when offered the opportunity.

A definitive chronic restraint syndrome in chickens has been reported by Besch et al., (1967). This condition is induced in most (75-90%) birds subjected to immobility. More liberal restraint procedures were developed through a series of trials to those utilizing a light-weight nylon "vest" (Burton et al., 1969) -- Figure VIII-11. Merely wearing the vest causes no observable physiological or behavioral change. It is readily applied to animals of varying size providing standardized degrees of restriction of mobility.

When birds are suspended by the vest (sling restraint), or subjected to a "4-point" restraint (2 ties each in the vertical and lateral axes) they soon exhibit a marked behavioral change (Besch et al., 1967). The legs, if free, are retracted against the body, the head hangs, and the animal becomes progressively lethargic (Figure VIII-12). Consistent with this behavior is an almost immediate cessation of drinking and eating, and a progressive loss of body mass.

Hematology:

Restraint stress was first recognized by hematological changes (Löwit, 1892 -- cited by Goldscheider and Jacob, 1894) -- although Goldscheider believed the leucocytic

response was secondarily induced by the accompanying hypothermia. As with other stressors, these reflect endocrine activity, and furnish convenient and reliable indexes of the animal's physiological status (vide, Acceleration Stress, Chapter VI).

The hematology of prolonged restraint, (1 hour to several days duration), has been studied by several investigators with various species of laboratory animals (Table VIII-2). Generally, restraint produces changes in the relative and/or absolute leucocytic hematology which are indicative of physiological stress. A recent review of the hematology of stress in various species of animals is available (Schalm, 1965). It appears that a relative and absolute lymphopenia and neutrophilia usually occur during stress, or with ACTH or cortisone injections, in all species examined -- horses, cows, dogs, cats, rats, mice, and rabbits. The recent work of Chapman (1968), with adrenalectomized mice indicates that adrenal hormones are a major factor in controlling the frequency of circulating leucocytes, even in nonstressed animals. The effect of stress upon the total leucocyte count, however, is variable among species, and is dependent upon the normal relative leucocyte distribution. For example, mice, rats, rabbits, and chickens, with normally high percentages of lymphocytes respond to stress with a relative lymphopenia and neutrophilia and with a <u>decrease</u> in total leucocytes. Dogs, cats,

horses, and man, with low relative lymphocyte frequencies, exhibit an increase in leucocytes during stress.

The role of the adrenal cortex in the restraint hematology of mice was investigated by Elmadjian and Pincus (1945). They reported a severe lymphopenia (20-50% less than control values) within 2 hours after being tied to a wire grid, and persisted in 4 of the 5 mice over 10 hours of restraint (Figure VIII-13). The recovery of lymphocyte count in the one mouse is indicative of physiological adaptation to stress. Unilateral adrenalectomy decreased the degree of lymphopenia by 50% and bilateral adrenalectomy abolished it. However, it is quite significant that adrenalectomized animals became prostrate and died soon after the development of restraint stress.

Newcomer (1958) compared restraint to cold (4°C) both dry and wet, acute anoxia, and injections of epinephrine, histamine, formaldehyde, cortisone, DCA, hydrocortisone, aldosterone, or ACTH in chickens. He concluded from the degree of acidophilia induced, that restraint produced the "severest state of stress of any of the conditions utilized." Since hematological changes induced by restraint were not prevented by the administration of antihistamines (Phenergan or Pyribenzamine) and only partially prevented by hypophysectomy, Newcomer concluded that a nonpituitary factor was involved in the acidophilia -- which could be epinephrine but not histamine. Colfer et al. (1950), however, found that

hypophysectomy completely abolished the restraint lymphopenia of rabbits, although it did not affect the neutrophilia. Denervation of the adrenal glands did not alter the lymphopenia, and administration of epinephrine (i.v.) did not produce a lymphopenia. They concluded that contrary to Newcomer's earlier report (1958) in chickens, the adrenal medulla probably had little effect upon the restraint hematology of rabbits.

Farris (1938) restrained rats briefly (5 minutes) by tying them to a cork board, which produced considerable excitement. This treatment elicited a marked relative lymphocytosis which was maintained for 1 hour following the restraint. This hematological response was prevented by either splenectomy or adrenalectomy, suggesting that it was caused by an activation of the adrenals (epinephrine) and subsequent splenic contraction. This relatively bizarre finding, however, is not typical of restraint-induced hematological changes (Table VIII-2).

Marked individual variation in restraint-response (like that reported in mice by Elmadjian and Pincus, 1945) is evident among chickens. Birds subjected to severe restraint (two lateral and a dorsal tie) are resolvable, after five days, into three groups (Figure VIII-14):

Asymptomatic (50% of group); maintaining a lymphocyte frequency greater than 45%; and not exhibiting signs of stress.

Recoverable stress (31%); developing a moderate lymphopenia (>25%), with some individuals exhibiting signs of stress.

Following release from restraint, most of those suffering irreversible stress died. With longer restraint, individuals of the more tolerant groups may develop irreversible stress -- the nature of the response being quite dependent upon the duration of exposure.

Body Mass:

Where restraint is continued for weeks or months there is a marked decline in body mass, resulting from diminished feed intake and increased utilization of body substance.

In cage-restrained mice (Portugalov et al., 1967), body mass loss was maximal after 15 days, and was substantially less after 30 days. In cage-restrained rats, the loss in body mass was progressive (Gass, 1960; Sullivan, 1967) and was not affected by increasing the caloric content of the diet (Sullivan, 1968).

Harness restrained chickens (Besch et al., 1967) ceased to eat almost immediately, and the fecal portion of the excreta soon disappeared. Such birds lose about 3% of their body mass per day -- as compared to 5% per day for those deprived of feed and water. Forced hydration (adding water directly to the crop) has little effect upon maintenance of body mass or survival. Severe anorexia also

develops rapidly in harness-restrained dogs (Gerd and Gurovskiy, 1962).

Anorexia and progressive loss in body mass also has been reported for monkeys subject to 3-week harness restraint (Rokotova et al., 1962) and to 5-weeks couch restraint (Hoffman et al., 1968). In the latter study those on a lower protein (although iso-caloric) diet had a somewhat better (+10%), but highly significant maintenance, of body mass. Recovery of body mass after relaxation of both kinds of restraint was slow, and incomplete even after 3-5 weeks.

Increased excretion rates of nitrogenous compounds have been reported for cage-restrained rats (Federov et al., 1967) immobilized in body casts (Federov and Grishanina, 1967), and couch-restrained monkeys (Hoffman et al., 1968). In these studies, the enhanced excretion of creatine and creatinine was pronounced.

In restrained chickens, the rate of loss of body mass is proportional to the degree of stress induced. Regression of body mass loss upon change in lymphocyte frequency during five days of restraint indicates a rectilinear relationship:

$$\text{body mass loss}_{(\Delta\%)} = 0.16 + 0.12\ \text{lymphocyte decrease}_{(\Delta\%)}$$

$$(r = 0.69;\ \text{random probability} < 0.01)$$

The physiological effects of these two treatments (chronic

acceleration and chronic restraint) are, of course, broadly dissimilar. This particular similarity in response merely indicates the presence of a common stress mechanism. The two phenomena are not coupled, a 5-day fast decreasing body mass approximately 12% (similar to the loss during 5 days of stress) but without any appreciable effect upon lymphocyte frequency.

The changes in body mass are not equally divided among the various organs. Besch et al., (1967) compared relative organ sizes (gms per kg body mass) between two groups of chickens -- one subject to harness restraint for 9 days and the other fasted for an equivalent period. Since the influence of these treatments on body mass was similar, the effect on organ size (as ± Δ%) was compared directly between the two groups -- Table VIII-3. Increases in adrenal size, and decreases in pancreas, thymus, and spleen with restraint are consistent with the hematological indications of stress (lymphopenia). The lesser loss of pectoral muscle mass in restrained chickens is not consistent with the enhanced nitrogenous excretion accompanying restraint stress in mammals. There is no apparent rationale for the lesser effect of restraint upon liver, lung, and heart size.

Similar results were obtained by Gabel and Clay (1961) for adrenal, heart and kidney in rats subject to cage restraint for 16 days. Rosecrans and DeFeo (1965) found a decrease in thymus, and an increase in adrenal size in rats

subjected to a daily 3-hour tie-down for 32 days. Sullivan (1967) found no selective change in kidney size in rats subjected to cage restraint for 63 days.

Kravchuck and Ovechkin (1968) observed a loss of body mass and an enhanced sensitivity to barbiturates in mice after 35 days of cage restraint. With a standard dosage, control mice remained unconscious for 4 ± 1.7 minutes, whereas, those subject to 35 days hypokinesia were unconscious 10 ± 2.2 minutes. Such a result would be expected with a selective loss of body fat -- however, changes in body composition resulting from restraint-stress have not been systematically reported.

Skeleton/Mineral Metabolism:

Examinations of restraint-induced changes in skeleton and mineral metabolism of animals have been prompted by the marked changes reported in humans subjected to chronic recumbency.

Sullivan (1967) found no effect of 12 weeks cage restraint in rat femur size. However, there was a minor but statistically significant decrease (-7.5%) in femur ash content after 6 weeks restraint -- but it was transient, and not apparent at 12 weeks. After 12 weeks restraint, there were minor decreases in the retention of intraperitoneally administered ^{45}Ca, which were not statistically

significant. At no time during the restraint were differences in blood calcium content evident. Consequently,in rats, restraint appears to have but little influence upon skeletal physiology.

With chickens, which are substantially larger than rats, restraint often has pronounced effects upon the skeleton. Restriction of mobility (not involving direct restraint) became a husbandry practice in California in the 1940's, with the adoption of "laying cages" for hens. This procedure involved confinement of layers to cages 2-3x their body volume -- approximately the ratio employed for restraint studies with rats (Gass, 1960; Sullivan, 1967). This practice spread rapidly through poultry operations in temperate climates. Shortly thereafter a disease, "Cage-layer Fatigue", was described (Couch, 1955; Grumbles, 1956), characterized by some type of "leg trouble", which was readily reversible when affected birds were placed in larger enclosures, such as "floor pens". A substantial variation has been reported in the incidence of cage-layer fatigue among strains (0.7-4.0%; Francis, 1957), which is consistent with the variability in response to restraint that is noted among individuals and species.

The cage-layer fatigue syndrome has been studied by Urist (1960) and by Urist and Deutsch (1960a,b) as a model for human osteoporosis. In this disease there is a thinning

of the cortex and an enlargement of the Haversian canals of the long bones. This bone erosion is not due to enhanced osteoclastic activity, and the decalcification mechanism is not apparent. The adrenal glands in affected hens become atrophic, similarly as in aged humans with osteoporosis. The relationship of this avian disease to restraint stress is indicated by the response to cortisone administration. In females so treated, the cage-layer fatigue syndrome is essentially duplicated in quite severe form; however, males are unresponsive, and castrate males only moderately so.

There also are skeletal effects in restrained chickens of both sexes that are not limited to the cage-layer fatigue syndrome (manifest leg weakness). Bone breaking strength of nonrestricted pen-raised birds is 28-40% greater, and bone mineralization 5% greater than in equivalent cage-raised (restricted) birds (Rowland et al., 1968) -- both differences are statistically significant.

The influence of 35 days couch restraint upon the skeleton (Mack et al., 1968) and upon bone mineral excretion (Pyke et al., 1968) has been measured in male Nemestrina monkeys. Bone density was measured radiographically at 17 points in the skeleton. There was a marked decrease in bone mass, which was not affected by the dietary mineral content. There also were differences in bone demineralization at the various sites, and these were proportional to the local degree of restraint. There was some recovery in bone density

upon release from restraint, and this was related in degree to dietary calcium concentration. Restraint also increased the calcium excretion rate and its fecal:urinary partition.

The variation of skeletal response to restraint stress evident in these various studies indicates that such changes may be size related -- large animals being more responsive.

Digestive Tract:

The production of erosions of the upper digestive tract in restrained animals was first reported by Rossi et al. (1956), and the earlier studies have been reviewed by Brodie (1962). Subsequently, this technique became a common technique for the production of gastric ulcers. Lahtiharju and Rytömaa (1967) and Kim et al. (1967), found that the erosions resulted from a failure of physiological regeneration with normally sloughed epithelial cells not being replaced. Imondi et al. (1968), examined DNA and RNA metabolism in mice restrained 16-40 hours. DNA synthesis was diminished along the entire tract -- but RNA was affected only in the stomach (coinciding with the occurrence of mucosal erosions). No similar response was elicited by equal periods of fasting.

A particularly interesting observation was reported by Sullivan (1967) -- that no interaction was apparent between chronic cage restraint (2-3 weeks) and 600r abdominal x-irradiation. In view of the marked effect of both treatments upon

gastrointestinal mucosa an additive response may have been anticipated. This apparent independence is quite in contrast to the enhancing effect of chronic acceleration upon radiosensitivity (Casey et al., 1967).

Edlich et al. (1969), found that gastric mucosal perfusion was significantly reduced (about -45%) in dogs after 3 days of restraint -- although concomitant ulceration or focal ischemic regions were not reported. It was concluded that this decreased gastric perfusion might be the initial pathological change in the development of gastric ulcers reported in smaller mammals upon restraint.

Brodie and Hanson (1960) compared the influence of restraint upon gastric ulceration in several species -- Table VIII-4. The effect appears to be restricted to small animals -- none appearing in rabbits or monkeys, or chickens (Besch et al., 1967), or dogs (Edlich et al., 1969). Among rats, Brodie and Hanson (1960) found a greater tendency for restraint-induced ulceration in smaller (and younger) animals. The incidence and severity of gastric lesions is proportional to the duration of treatment -- Figure VIII-15. Probit analysis of the induction kinetics reveals a typical sigmoidal dose-response curve -- indicating that restraint is the single effective factor and that its effect is continuous with duration of restraint. Induction rate of gastric ulcers is about five-fold the recovery rate.

The influence of long-term cage-restraint upon gastrointestinal function has been examined by Gass (1960) and Sullivan (1967, 1968). No significant influences were found upon the rate of intestinal passage, nor upon the absorption rates of water, ions (Ca^{++}, Na^{+}, $^{-}l^{-}$) sugars (glucose, fructose) or amino acids (phenylalanine, glycine).

Circulation and Respiration:

Brief periods of restraint tend to enhance respiratory and circulatory frequencies -- a typical emotional response. In chickens subject to 2-3 hours "tie-down" restraint (Whittow et. al.,1965), heart rate, stroke volume, and cardiac output are increased. Blood pressure, and estimated peripheral resistance are decreased, but respiratory frequency is unaffected. These restraint-induced changes persist in chickens for several hours after their release. Rabbits restrained in a small box respond similarly with panting, tachycardia, and peripheral vasodilation (Grant, 1950; Cragg, 1961). These effects become maximal with 30 minutes restraint, and are ameliorated with treatment continued for 3 hours. Dogs subject to rigorous harness restraint (Gerd and Gerovskiy, 1962) also exhibit hyperpnea, and presumably a decreased blood pressure (e.g., urinary output is reduced). Monkeys immobilized in a "straight-jacket" (Berendt, 1968) also developed pronounced hyperpnea and tachycardia.

The combined effects of peripheral vasodilatation and enhanced cardio-pulmonary function frequently result in a decreased body temperature. Rabbits restrained in a mesh cylinder may have a 2.7°C decrease in body temperature in 30-90 minutes (Grant, 1950). Goldscheider and Jacob (1894) believed that this hypothermia was the primary response to restraint. They found that leucocytic changes in restrained rabbits could be prevented if they also were "warmed" (in a sand bath heated with a gas flame!).

Bartlett and Miller (1956) examined restraint hypothermia in rats and Bartlett and Quimby, (1958) found it to be due to an enhanced heat loss, rather than a diminished heat production. Restraint also limits thermoregulation of monkeys in hyperthermal environments (Frankel et al., 1958). In chickens restraint does not lead to an immediate decrease in body temperature, presumably a result of greater insulation (Whittow et al., 1965). However, protracted restriction from confinement in laying cages does lead to a lower body temperature (Hutchison, 1954).

With longer periods of restraint, the emotionally associated changes disappear -- and with chronic restraint other changes are evident. Rats subject to 16 days cage restraint (Gabel and Clay, 1961) have normal heart rates and an increased systolic pressure (+ 40 mm). Three days cage restraint in dogs lowered the cardiac output - 25% (Edlich et al., 1969). However, dogs immobilized in a body cast for

two weeks did not show any increased tendency towards orthostasis (Asymaolov and Voskresenskiy, 1968) -- which was interpreted as an artifact, the cast not permitting a reduction in the hydrostatic aspects of blood pressure. Cage restraint in monkeys (Banerjee, 1969) leads to a progressive increase in the arterial-alveolar oxygen tension gradient (A - a DO_2), from an initial 2 mm, to 21 mm after 10 months. This is largely the result of a decreasing arterial saturation (P_aO_2), and was interpreted as resulting from a progressive uneveness of ventilation and perfusion, induced by physical inactivity.

Metabolism:

Changes in metabolic function are induced by restraint, reflecting altered hormone secretion -- largely of the adrenal cortex. Macho et al. (1968) subjected rats to repeated 2.5 hour periods of immobility ("tie-down"), after which the animals were sacrificed and the oxygen consumption rates of several tissue homogenates were measured -- Table VIII-5. Such restraint caused very little change in tissue respiration but rather marked effects were evident in the utilization of various substrates. They attributed this influence of restraint upon tissue respiration to d[illegible]nd upon adrenocortical hormones, since it was abolished in animals that were adrenalectomized 14 days prior to the treatment. Physiological adaptation also was evident, the influences of restraint being ameliorated after 40 episodes.

Federov et al., examined the nitrogenous excretion (1967) and tissue protein synthesis (1968) in rats subject to 15 days of restraint, and for a subsequent six days of recovery. Such treatment rapidly reduced protein synthesis in heart, kidney, spleen, small intestine, and skeletal muscle (Table VIII-6). The only tissue to recover during the treatment or within six days thereafter was skeletal muscle. The early restraint was accompanied by a negative nitrogen balance including a marked creatinuria. These results were interpreted as representing enhanced adrenocorticoid and diminished somatotropin activity.

Similar studies were reported by Portugalov et al.(1967) for mice during 30 days cage restraint. Some effects of restraint stress were apparent after one day of treatment, and these became maximal during the fifteenth day of treatment. Atrophic changes in skeletal muscle were accompanied by diminished activity of enzymes related to oxidative metabolism. There was a loss of myofibrilar elements and changes in mitochondrial morphology. These changes appeared to be more pronounced in the extensor (antigravity muscle). Other organs also appeared to be affected by restraint: intestine, liver, endocrines, and nervous tissue. There was evidence of diminished intestinal motility and thyroid function, and of enhanced adrenocortical activity. By 30 days of restraint, many of the induced changes were restored to normal levels, indicating a physiological adaptation of mice to chronic restraint.

Neurological Changes:

One of the commonest debilities of long-term restraint is a disorientation which has been reported in chickens (Besch et al., 1967; Burton, et al., 1969), dogs (Gerd and Gurovskiy, 1962), and monkeys (Bogina et al., 1965). This appears to be related to body size, since it has not been reported for mice and rats. Such disabilities persist for some time after release from restraint. No explanation of the mechanism of this disorientation has been proposed.

Prolonged restraint also is accompanied by a muscular weakness -- which has been reported in rats (Gass, 1960) and monkeys (Mason, 1958). A similar progressive decrease in muscle tone has been reported for chronically recumbent humans (Cherepakhin, 1968) -- however, it is not accompanied by any change in proprioceptive reflexes (knee and ankle jerk), which would tend to minimize a neurological basis.

Pathology:

The pathology of restraint is largely dependent upon its severity. Sullivan (1967) found no mortality in rats subject to cage restraint for 9 weeks. With similarly restrained rats, Gass (1960) obtained 2.5% mortality for a 25 week period, all of it occurring in the first 13 weeks. With more severely restrained rats -- 18 hours daily tie-down for 5 days -- Brodie and Hanson (1960) encountered 13% mortality,

all of it on the 4th and 5th days. Severe restraint was 100% lethal to chickens (Besch et al., 1967), with the survival time being inversely related to the degree of immobility -- Table VIII-7. Rabbits are equally intolerant to immobilization, suffering an 80% mortality in 4 days of such treatment (Knize et al., 1969). When immobilization was combined with limb fracture, all rabbits died within 48 hours (Knize, personal communication).

At autopsy, birds dying from restraint exhibit no specific lesions -- Table VII-8. General atrophic changes are more-or-less proportional to the duration of the treatment. Some organ regression (e.g., spleen, testes) is typical of stress -- of adrenocortical activity.

Etiology of Restraint Stress:

The mechanism of restraint stress appears to be generally similar to other "nonspecific" stresses -- in which increased adrenocorticoid secretion is prominent. Evidence for this is found in the increased secretion of cortisol or corticosterone in restrained animals (Mason et al., 1957; Mason, 1958; Rosecrans and DeFeo, 1965); as well as adrenal hypertrophy (Rosecrans and DeFeo, 1965; Federov et al.,1967) and increases in adrenal ascorbic acid and cholesterol contents (Bartlet and Miller, 1956). There also is indirect evidence of hyperadrenocortical activity with restraint, such as involution of lymphoid tissue (Rosecrans and DeFeo,1965);

development of lymphopenia (Besch et al., 1967); and the lack of metabolic changes in restrained adrenalectomized animals (Macho et al.,1968). Also, in some instances, symptoms of restraint stress have been elicited by the administration of adrenocorticoids (Rossi et al., 1956; Bartlett and Miller, 1956; Urist and Deutsch, 1960).

Restraint stress, like other stresses, is generally ameliorated by a physiological adaptation. This has been observed with serial acute immobilizations of rats (Rosecrans and DeFeo, 1965; Macho et al., 1968) as well as with continued restraint of monkeys (Mason, 1958; Bogina et al., 1965). However, such adaptation appears to require a sympathetic accommodation, since it is prevented by Reserpine treatment (Rosecrans and DeFeo, 1965). Similarly, the viability of restraint-stressed chickens is reduced by Reserpine administration (Besch et al., 1957) -- Table VIII-7.

Not all animals are equally susceptible to restraint stress. The effects appear to be more severe with larger animals. Unfortunately, information on the consequences of restraint is unavailable for large animals -- goats, horses, etc. Although they do tolerate restraint as provided by a stanchion,current veterinary practice does not include immobilization. It may be inferred that the response of large animals to such treatment prevents its use. It is known that leg muscular activity is essential for adequate venous

return in large herbivores -- such as cattle and horses (Dukes, 1955). Also deep body temperature, and body heat loss rate of sheep (Brockway, 1965) is greatly affected by posture -- lying vs.standing. There also are some doubts regarding human tolerance to immobility. Prior to the development of more sophisticated methods, this was a common form of punishment (stocks, pillory) and even execution (crucifixion). It is perhaps of physiological significance that punishment in stocks and pillories was limited to periods of 1 to 3 hours (Whitmore, 1889; Rankin, 1965). Where more severe punishment was merited, it was achieved by combining the restraint with a different form of chastisement (e.g., flogging; and in Virginia, an aggravated offense might additionally warrant nailing the ears to the pillory).

However, some restraint pathologies, such as gastric ulceration, are restricted to small animals (rats and mice), which may be related to "temperament". Wild animals, and those in the dominant parts of a hierarchy are more affected by restraint. Within a species, the susceptibility to restraint stress is a highly individualized character. This has been tested in chickens which were subjected repeatedly (five times at 2-3 week intervals) to five days of severe restraint (lateral and dorsal ties). During each treatment the birds were examined for signs of restraint stress, loss of body mass, and changes in hematological parameters. An analysis of variance of the results (Table VIII-9) indicated

an individually characteristic response with regard to symptomology and degree of lymphopenia -- but not for changes in body mass, hematocrit value, or plasma proteins. Consequently, the basis for development of restraint stress is inherent, rather than circumstantial.

The mediation of restraint stress appears to be complex. Recent examinations of biological stresses (i.e., treatments inducing an adrenocortical response) indicate that they may be divided into two broad categories. Some (e.g., audiogenic; Feldman et al., 1968) can be abolished by hypothalamic deafferentation, and are considered "Neurogenic." Others (e.g., ether anaesthesia; Matsuda et al., 1964) are unaffected by hypothalamic deafferentation, and are considered of "Systemic" origin. The adrenocortical response to restraint is somewhat reduced, but not abolished, by hypothalamic deafferentation (Palka et al., 1968). From this, Feldman et al. (1968), conclude that restraint stress has both neurogenic and systemic elements.

Investigations with chickens indicate that the most serious consequences of restraint result from a dorsal suspension -- removing the support of body weight from the legs. Counterweighting experiments (Figure VIII-16) indicated that there is a critical equivalent of the body weight which can be compensated before restraint stress becomes evident. Presumably a certain level of leg afferent stimuli is necessary

for the continued health of the bird -- and a lesser input initiates the restraint syndrome. There is a substantial variation among individuals in the degree of counterweighting that is tolerated (Figure VIII-17). For some, removing even a few % of the body weight stimulates a lymphopenia ("maximum nonstressing load") -- and removing 40% is universally effective. There is another increment of deweighting in which the lymphopenia is transient ("maximum load for recoverable stress"). There also is a limit to the degree of counterweighting which leads to a serious stress syndrome ("minimum load causing stress failure"). Repeated experiments indicate that the "tolerable deweighting" is individually characteristic.

SELECTION AND TRAINING OF ANIMALS FOR CHRONIC RESTRAINT

Restraint, like other stressors, is subject to modification of response (physiological adaptation) with repeated or continued application. Mikulaj et al. (1963 -- cited by Macho et al., 1968), found a lesser corticoid response in rats after 40 periods of restraint (although adrenal hypertrophy persisted unchanged). Rosecrans and DeFeo (1965) found a similar decrease of corticosterone secretion in rats with repeated daily (3 hours) dorsal tie-down. However, Reserpine treatment prevented this adaptation, resulting in a 50% mortality with continued restraint. Consequently, a suitable program of repeated, or increasingly severe restraints

should substantially modify the response, and train (or physiologically adapt) animals to tolerate chronic restraint.

Gerd and Gurovskiy (1962) reported a three-stage restraint training program for dogs in preparation for rocket flights. Initially the animals were confined in small containers (with dimensions 10-15 cm greater than those of the animal) for periods of 1-3 days. Later they were subject to rather loose harness restraint in a small capsule (equivalent in size to that used for rocket flight). The response of all animals was not equally satisfactory to each stage of restraint training (and especially to the second stage), and on this basis some were eliminated from the program. For the suborbital flights, the more excitable dogs were retained -- whereas those that were less excitable were selected for orbital flights.

Satisfactory restraint of monkeys over long periods also was found to require selection and training -- with periodic release at early stages (Bogina et al., 1965).

A similar method of restraint training (and selection) has been developed for chickens. Initially, birds wearing "vests" (Figure VIII-11) are placed in small cages (layer cages, 10" x 12" x 16") and subject to lateral ties. They are periodically examined for maintenance of posture, body mass, and a normal lymphocyte frequency -- which appear to be reliable indexes of restraint stress in chickens. As

individuals become moderately affected (exhibiting some postural instability, 2-300 gms body mass loss, and lymphocyte frequencies less than 40%), the lateral ties are released until recovery. This degree of restraint is repeated (usually 3-4 times) until the birds tolerate it for 20 days. About 60% of the initial population can be brought to this level of training. Subsequently, dorsal and ventral restraints are applied, and the procedure repeated. Approximately 40% of the initial population can be trained to tolerate such severe restraint -- and maintained in this condition for approximately 6 months, and some for over a year.

Although restraint-trained birds will recover their body mass, normal hematology, and appear normal after 60 days, they quite characteristically lack tolerance for other environmental extremes -- e.g., hyperthermia. A similar impairment of thermoregulation with continued restraint has been reported in monkeys by Frankel et al. (1958). This is perhaps indicative of a more general principle -- the inverse relation between physiological adaptation and physiological reserve ("homeostatic capacity"; Pace, 1959). For example, in modern breeds of livestock, the metabolic demands for increased production (eggs, milk, etc.) decrease the physiological reserve, and they are particularly susceptible to environmental extremes. Also, rats physiologically

adapted to either low temperature or low barometric pressure become more susceptible to the other stressor (Fregly, 1954). Consequently, the management of animals trained for chronic restraint will have more precise husbandry requirements than those ordinarily accepted for the species.

It is apparent that individuals of at least several species can be prepared to tolerate chronic restraint. Such preparation involves both selection and training. An attractive hypothesis is that restraint training is related to the neurogenic aspects, and the selection, to the systemic aspects of restraint stress.

Restraint Stress and Weightlessness:

There are important implications of the chronic restraint syndrome with respect to orbital experiments with animals -- as noted by Salisbury (1969). From the literature it appears that restraint stress may be a universal response, at least of subhuman animals. The lesser experience with training and selection of animals for toleration of some restraint procedures indicates that the untoward response to restraint can be avoided.

From the counterweighting experiments with chickens, it appears that the critical factor in the induction of the chronic restraint syndrome may be a deficiency of proprioceptor input from the legs. In all probability, a similar

lack of leg afferents would occur in weightlessness -- inducing a response quite like chronic restraint stress. The rather dramatic nature of this response would complicate, if not completely mask, systemic responses to weightlessness. A possible solution for avoiding such untoward events would involve ventral spring-loading the orbiting animal to provide a suitable level of leg proprioceptor stimuli. From the counterweighting experiments a tension equivalent to 70% of the body weight at Earth gravity would be satisfactory for chickens. However, determinations of the appropriate loading for a particular animal in Earth-orbit would be a desirable initial approach.

It is perhaps significant that the restraint systems applied to the dogs in Cosmos 110 (Figure VIII-2) appear to have a ("downward") leg loading component -- which was not discussed. Also, the dogs in Sputnik 5 developed a similar effect by working against the instrumentation cables so that they were able to "stand in place" (Zhuravlev, 1962).

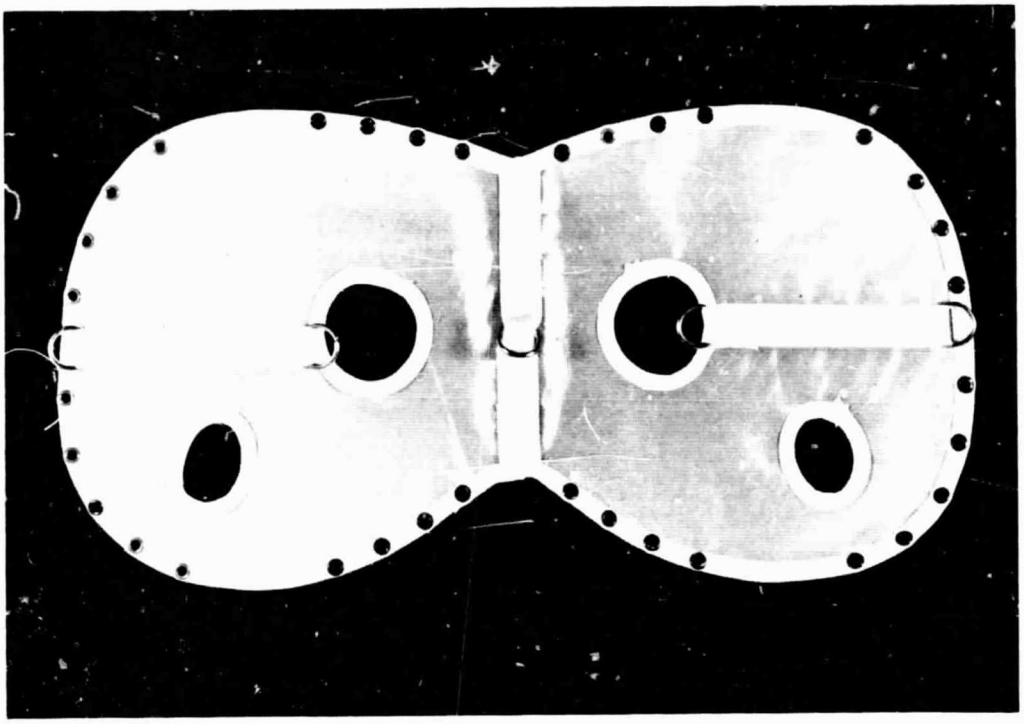

Figure VIII-11: Nylon vest for restraint of chickens. This devise has the overall dimensions 8 in x 17 in and weighs 60 gms. It can be readily fitted to adult birds 0f 1.5-2 kg body mass.

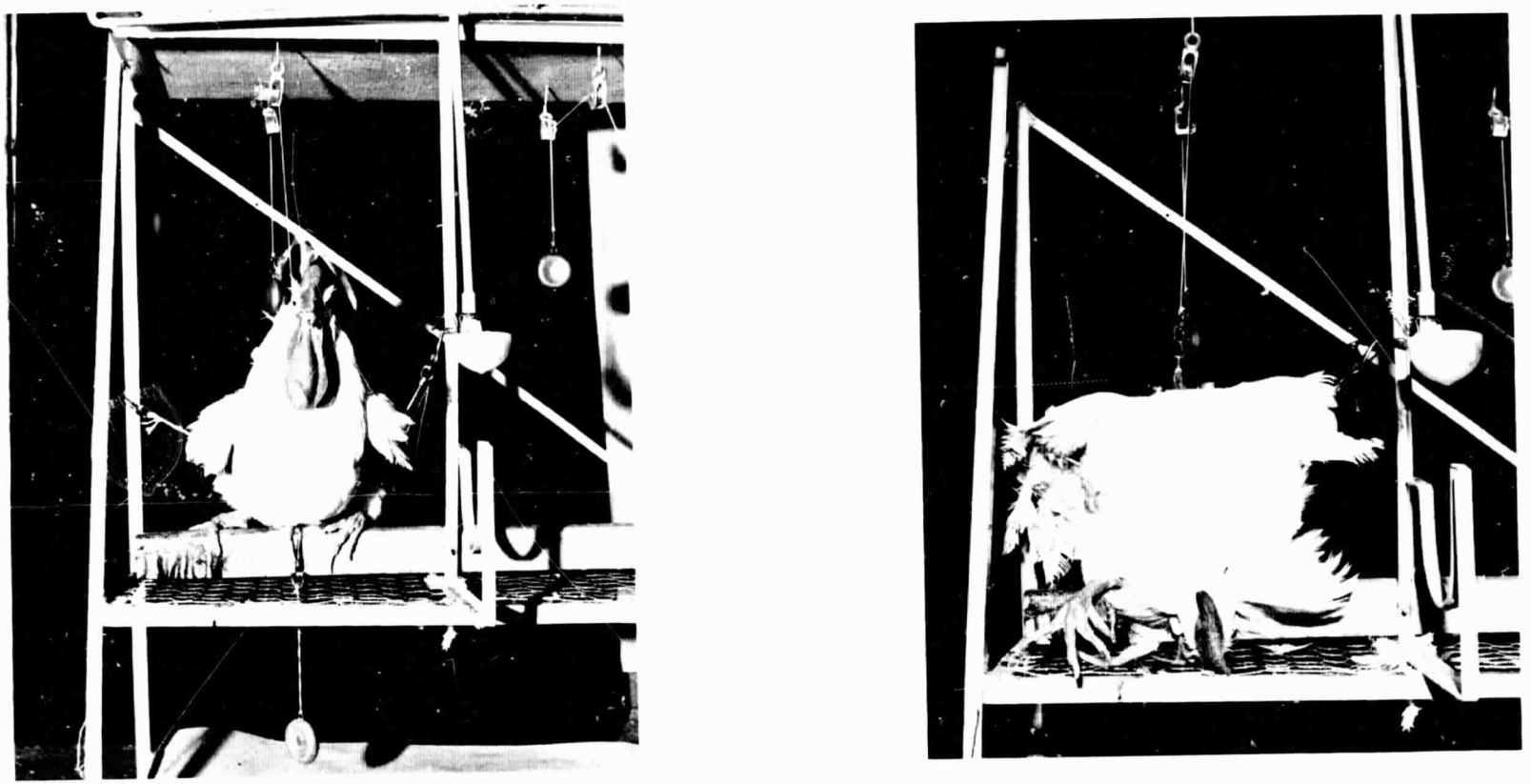

Figure VIII-12: Chronic restraint syndrome in chickens. The bird on the left is tolerating the treatment, whereas the one on the right has become disoriented and lethargic.

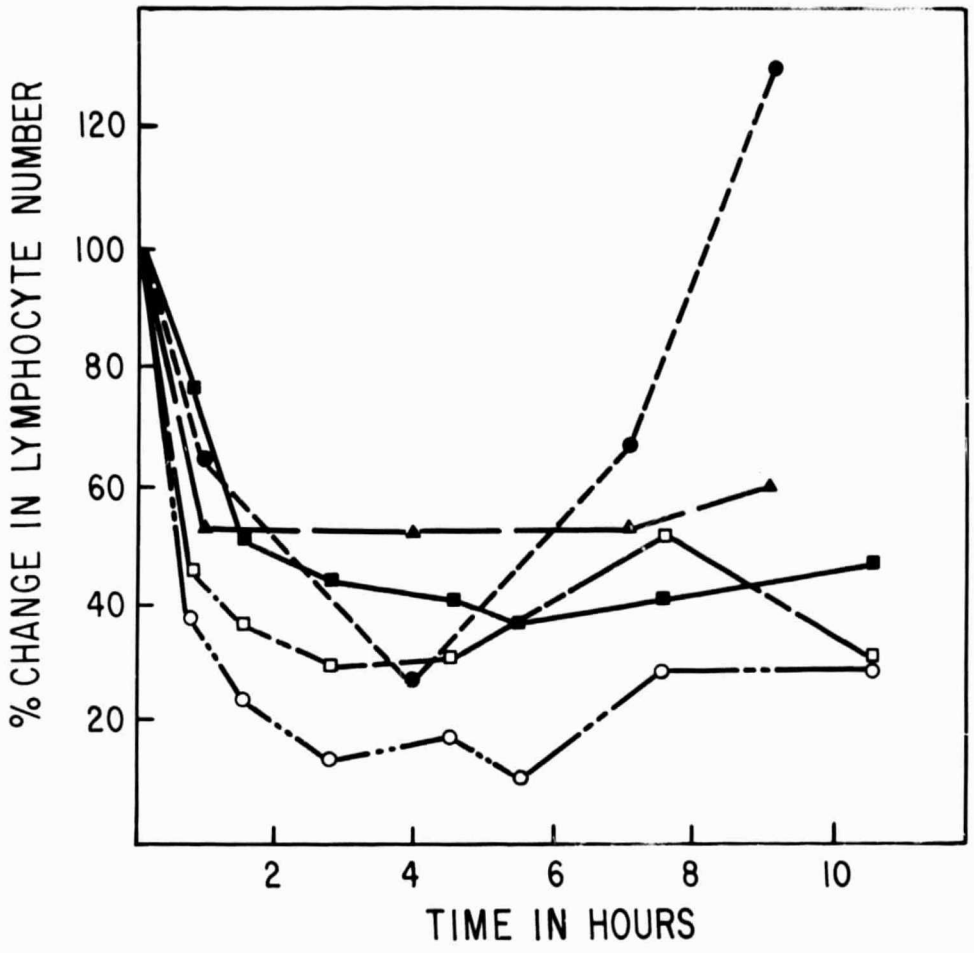

Figure VIII-13: The lymphocyte response of five normal male mice at various intervals, after being tied to a wire grid. The lymphocyte count at 0 time is taken as 100% (Elmadjian and Pincus, 1945).

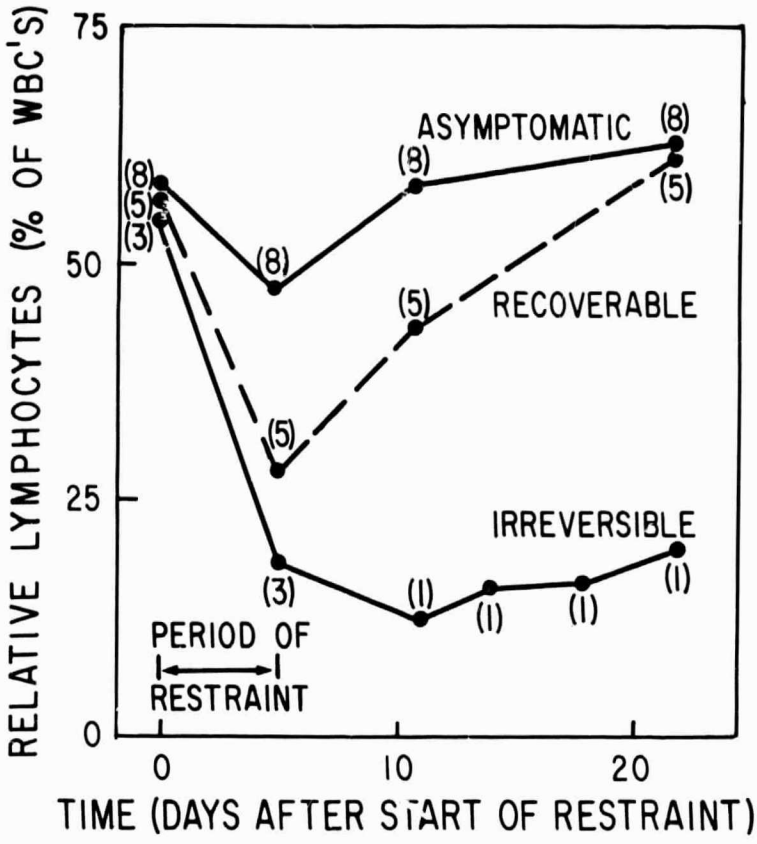

Figure VIII-14: Variation in lymphocytic response of chickens to severe restraint.

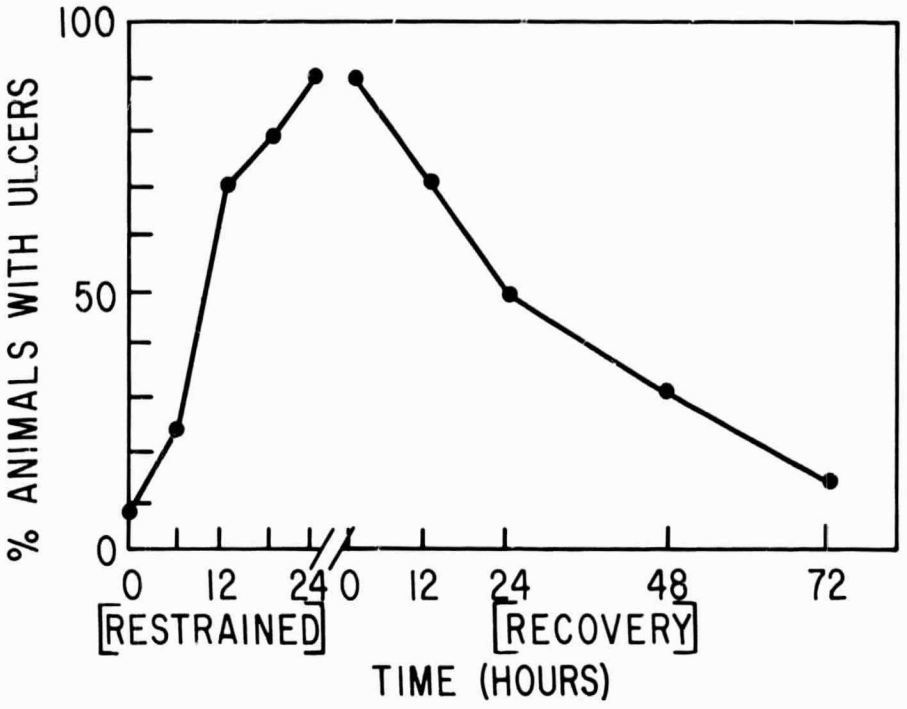

Figure VIII-15: Restraint induction of gastric ulcers, and recovery upon release (Brodie and Hanson, 1960).

Both induction and recovery have exponential kinetics (t in hours):

Induction (6 hours and later);

$$\% \text{ ulceration} = 100 - 145e^{-0.12t}$$
$$(r = 0.989,\ p < 0.05)$$

Recovery:

$$\% \text{ ulceration} = 93.8e^{-0.024t}$$
$$(r = 0.995,\ p < 0.01)$$

Figure VIII-16: Shown is an apparatus for animal restraint with lateral ties and variable counter-weighting.

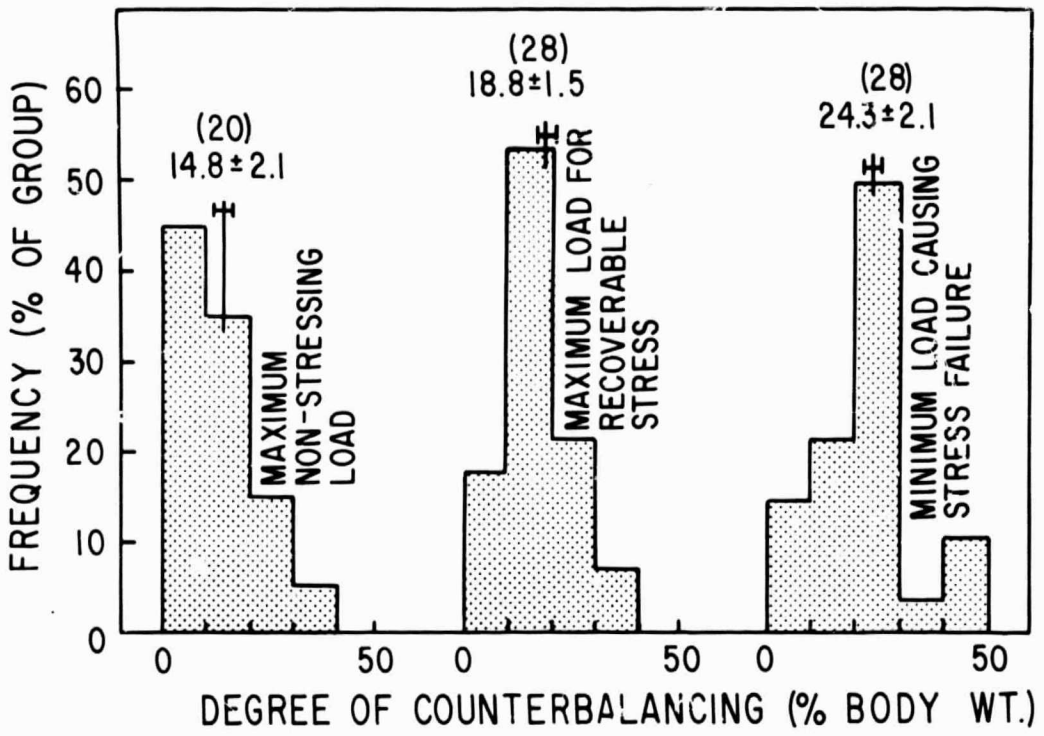

Figure VIII-17: Distribution of counterweighting tolerances. Means and standard errors are indicated for each group and group size. Numbers of individuals are indicated in parentheses.

RESTRAINT HEMATOLOGY TABLE VIII-2

Reference	Species	Sex	Age	Type Restraint	Duration of Restraint	Differential findings (WBC)	Other Hematology
Nye and Barrs (1932)	Rabbits	-	-	Strapped down	4 1/4 hrs	-	WBC ↓ followed wild WBC ↑
Latta and Nelson (1948)	Rats	F	Gms	-	6 1/2 hrs	Lymphopenia Neutrophilia	WBC ↑
Gutherie et al., (1967)	Squirrels	M F	-	Confined in live traps	12-48 hrs	Lymphopenia Neutorphilia	Hct ↑ WBC ↑
Besch et al., (1967)	Chickens	M F	Adults	Harness tie-down	Several days	Lymphopenia Neutrophilia	WBC ↓
Elmadjian and Pincus (1945)	Mice	-	40-60 days	Tie-down to grid	1-10 hrs	Lymphopenia	-
Newcomer (1958)	Chickens	M	16 days	-	4-6 hrs	Heterophilia*	-
Goldscheider and Jacob (1894)	Rabbits	-	-	Tie-down to grid	hr	-	Leucopenia
Katsura (1930)	Rabbits	-	-	Tie-down	1 hr	-	Leucopenia
Colfer et al., (1950)	Rabbits	M F	Adults	Restraining clamps	1-2 hrs	Lymphopenia Neutrophilia	RBC = n.c.
Katz and Nice (1936)	Rabbits	-	Adults	Animal holder and shocking	Few min.	-	Leucopenia
Cheng (1930)	Rats	-	-	Restrained "belly up"	-	Lymphopenia	Leucopenia

* In the chicken this indicates probably a relative lymphopenia and/or neutrophilia.

Table VIII-3: Comparison of 9-days restraint (R) versus 9-days fast (F) upon organ size (Besch et al., 1967).

	Change in Relative Organ Size $\frac{\Delta R - \Delta F}{\Delta F} \times 100 = \pm\Delta\%$	(p)	
Testes	+96%	-	↑
Liver	+39%	<.01	greater restraint effect
Adrenal	+37%	<.05	
Lung	+31%	<.02	
Heart	+21%	<.01	
Kidney	+16%	-	
Small Intestine	-6%	-	↓
Thyroid	-20%	-	
Pancreas	-21%	<.05	greater fasting effect
Pectoral Muscle	-23%	<.02	
Spleen	-52%	<.001	

Table VIII-4: Incidence of gastric ulceration following 24 hours restraint (Brodie and Hanson,1960).

Species	Number Animals	Mean Body Mass	Frequency of Gastric Ulcers	
			No.	%
Mouse	50	20 gm	46	92%
Rat	50	190 gm	43	86%
Guinea Pig	50	250 gm	23	46%
Hamster	50	110 gm	2	4%
Rabbit	6	1.5 kg	0	0%
Monkey	5	2.0 kg	0	0%

Table VIII-5: Influence of repeated periods of restraint upon tissue homogenate metabolism (Macho et al., 1968)

Organ and Substrate	Control Tissue Resp. ($ml\ O_2/gm/hr$)	Ratio: Restraint resp. / control resp. For indicated periods of restraint		
		1x	10x	40x
LIVER				
Saline	5.6	1.25	0.96	0.89
Glucose	6.0	1.00	0.90	0.83
Pyruvate	10.2	0.84*	0.82**	0.96
α-keto glutarate	21.8	0.64**	0.82	0.88
Succinate	60.0	0.89**	0.66**	1.00
HEART				
Saline	3.0	1.13	0.87	0.80
Pyruvate	5.0	2.92**	2.00*	1.60*
α-keto glutarate	11.0	5.91**	4.71**	2.22**
Succinate	44.5	1.64**	1.01	0.88
SKELETAL MUSCLE				
Saline	2.4	0.83	1.00	0.88
Glucose	2.9	0.56*	0.82	0.78
Pyruvate	4.3	0.73	0.64*	0.90
α-keto glutarate	6.4	0.56**	0.73**	0.91
Succinate	13.9	0.60**	0.78	1.02

Random Probability { * < 0.05, ** < 0.01

Table VIII-6: Incorporation of radio-methionine into tissue proteins of restrained rats (Federov et al., 1968)

The incorporation of radio-methionine, administered intraperitoneally two hours prior to sacrifice, into tissue protein is presented as the ratio of restrained:control animals. The figures in parentheses are for S^{35} labeled, and the others for 1-C^{14} labeled methionine.

	Period of Restraint			Period of Recovery
	3 days	10 days	15 days	6 days
Heart	0.94 (0.72)	0.76 (0.74)**	0.65* (0.79)	0.68* (0.70)**
Kidney	1.08 (0.80)*	0.76** (0.80)	0.66*** (0.68)**	0.65** (0.55)***
Liver	1.01 (1.00)	0.76* (0.94)	0.60** (0.87)	0.54** (0.59)***
Spleen	0.88 (0.66)	0.79 (0.69)**	0.65*** (0.88)	0.82 (0.86)
Small Intestine	0.85* (0.82)*	0.75* (0.84)	0.64*** (0.79)*	0.65** (0.71)**
Skeletal Muscle	0.52*** (0.42)***	0.75 (0.60)*	0.80 (0.90)	1.18 (1.25)

Random Probability:

*** $p < 0.001$

** $p < 0.01$

* $p < 0.05$

Table VIII-7: Summary of mortality and survival times for domestic fowl exposed to various restraint procedures of 21 days minimum duration (Besch et al., 1967)

Group	Sex	No. of Animals	No. of Deaths	% Mortality	Survival Time of Those Dying (Days)
Bird Box	Male	5	5	100.0	7.4±1.6[1]
Harness-Sling					
Non-reserpine	Male	24	22	91.7	11.5±1.3
Non-reserpine	Female	6	3	50.0	16.3±2.9
Reserpine[2]	Female	17	11	64.7	6.3±0.8
Harness-Cage					
Non-trained[3]	Male	12	8	66.7	8.5±1.1
Trained	Male	12	9	75.0	14.3±1.7
Feed Deprivation	Mixed Group	16	16	100.0	12.8±0.4

[1]Mean ± Standard Error.
[2]Female Reserpine trial only 14 days duration.
[3]All animals in this group were dead after 30 days of restraint.

Table VIII-8: Summary of results of necropsy examinations conducted on 29 animals exposed to harness and bird box types of restraint (Besch et al., 1967).

Observation	Percentage	Remarks
Condition of animal		
Emaciated	38.4	Catabolic process
Thin	30.8	
Fair to good	30.8	
Liver (small)	69.2	Catabolic process
Gizzard (small)	42.3	Bird not eating
Crop or gut (empty)	53.8	Bird not eating
Spleen (small)	65.4	Stress factor
Testes (medium sized)	23.1	Stress factor
(small sized)	34.6	Stress factor
Dehydration	80.8	Negative fluid balance
Urates in cloaca	76.9	Negative fluid balance

Table VIII-9: Analysis of variance of restraint-induced changes in chickens with repeated (5x) exposures to 5 days of severe restraint.

	Variance Ratio (F)	Probability of Significance
Restraint syndrome (+ or -)	4.22	0.99
Lymphocyte frequency (Δ%)	2.67	0.98
Body mass (Δ%)	1.75	-
Packed cell volume (Δ%)	0.78	-
Plasma protein conc. (Δ%)	0.49	-
Buffy coat (Δ%)	0.78	-

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