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To the Graduate Council:

I am submitting herewith a thesis written by Gurujala N. Subbasastry entitled "Tongue, Heart, and Liver Tissues as Sources of Calcium and Total Vitamins for Rats." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Husbandry.

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TONGUE, HEART, AND LIVER TISSUES AS SOURCES OF CALCIUM AND TOTAL VITAMINS FOR RATS

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Gurujala N. Subbasastry

December 1965

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CHAPTER I

INTRODUCTION

Nutrition is one of the most fertile fields in biological research. Because of the public's growing health consciousness, the role of adequate nutrition in maintaining the well-being and health of populations has been accentuated. The science of nutrition has, therefore, been under increasing scrutiny. This interest has fostered a vigorous revival in the science and has led to re-evaluations of many of the commonly accepted concepts in nutrition (Siedler, 1963). It also has led to increased understanding of the role of nutrition in the continuing health of man.

Throughout the ages the meat group has played a significant role in the adequate nutrition of man. The science of meat is one of the most important branches in the field of nutrition, embracing the application of many basic scientific disciplines. Most of the problems relating to meat and meat products are solved by a co-ordinated investigation which utilizes several areas of knowledge. The literature relating to meat science today is voluminous and research quite enormous.

A marked upsurge in inquisitiveness concerning the composition and dietary significance of meat and meat products occurred during the decade immediately preceding 1925. Excellent research, including studies on the chemistry of proteins and amino acids, on the nutritional quality of meat protein, and especially on the dietary value of liver

and other meats as sources of B vitamins and trace minerals, was conducted in leading laboratories in several parts of the world. In the early 1920's too, private and governmental groups in the United States developed programs that throughout the subsequent period have had a continuously stimulating effect on the force and direction of research on meat.

Meat has long been recognized as an excellent source of high quality protein, of vitamins of the B complex group, and of certain minerals.

The importance of having a variety of foods in the ordinary mixed diet, to provide ample quantities of each of the nutrients needed without an excessive caloric intake, requires continuing emphasis.

The meat group significantly contributes to four of the basic nutritional components of man's diet, namely, protein, fat, vitamins, and minerals. The carbohydrate contribution of the meat group is relatively insignificant.

The human appetite cannot ordinarily discern which foods are best for the individual, or which are needed to complete the daily requirements. Many individuals tend to eat what they like when given a choice of foods, and what they like may not be what they need or can best use. Others are restricted in their food intake by dislikes and lack of tolerance for some or many foods.

The vitamin content of fresh organ meats compares well with that of fresh muscle meats, on an over-all basis. Both muscle and organ

meats provide nutritionally important amounts of a large number of vitamins of B group required by man, but vitamins A and C are present in significant amounts only in the organ meats.

The biological process of nutrition is like a symphony. The interrelationship of many of the constituent portions of a diet for a normal living organism is well established. But yet the concept of balanced diet is sometimes intriguing. While on the one hand, new fields of discoveries are being explored, the accumulated knowledge, on the other hand, makes it necessary for science to frequently re-examine established opinions and to re-open issues supposedly closed. This is particularly true of minerals and vitamins in the science of nutrition.

The present work was undertaken to study the effects of feeding three organ meats, namely, heart, liver and tongue. Since meat is known to be deficient in calcium, it was proposed to find whether mere supplementation with calcium was adequate to correct the deficiency or any inter-action of vitamins was also involved in the process.

The main point of interest in this work was one aspect of metabolism of calcium and phosphorus, namely, the deposition of these minerals in bone. The technique of employing radioactive isotopes (Ca^{45} and P^{32}) has been made use of in this study to measure the state of calcium and phosphorus absorption and deposition.

The observations and results of this work form the basis of this presentation.

CHAPTER II

LITERATURE REVIEW

I. MEAT AS A FOOD

Meat has long been recognized as a nutritious food, and a good deal of literature has accumulated over the years concerning the various aspects of this important food item. What stands out conspicuously in most of the work reviewed deals with the unquestionably superior quality and quantity of the protein and the availability of several other nutrients. The protein of organ meat is excellent in nutritional quality and is contained in amounts equivalent to muscle (Siedler, 1963).

One of the most dramatic changes in nutritional concepts and in medical opinion regarding meat in the diet resulted from an experiment conducted in 1928 by the Russel Sage Foundation. The arctic explorers, Vilhjalmer Stefansson and Karsten Anderson, lived for a year on a diet consisting exclusively of meat. These studies provided proof of the value of meat in the diet of man and disproved many beliefs commonly accepted by physicians at that time that certain disease conditions resulted from meat in the diet. This experiment and the several other encouraging factors greatly stimulated interest in the nutritional value and nutritive content of meat and their relationship to the nutritional requirements of man (Schweigert and Armstrong, 1960).

A brief but an excellent account of the nutritive content and nutritional value of meat and meat products is given by Lushbough and Schweigert (1960). Their review indicates that the protein in meat is of a high biologic value. It is also pointed out that while the differences observed between the various organs from a single species are frequently large, the differences between species in the vitamin content of a specific organ generally are not large.

The nutritive value of liver is shown to be of special interest because of its relatively high content of a number of vitamins (Lushbough and Schweigert, 1960). Liver is a good source of vitamins A, C, D, B_6, B_{12}, nicotinic acid, pantothenic acid, and biotin.

Discussing the subject of meat as a food, Ziegler (1964) surveyed the several important constituents of meat and pointed out that muscle itself is a poor source of calcium. However, muscle meats, and more particularly glandular meats, are exceptionally rich in iron and phosphorus.

The mineral content of phosphorus is generally higher in the fresh organ meats than in the fresh muscle meats, and the calcium level in pork heart is substantially higher than in any of the other muscle and organ meats (Lushbough and Schweigert, 1960). It is recognized that meat contains additional vitamin-like factors not as yet identified (Schweigert and Payne, 1956).

The organ meats have much higher polyunsaturated fatty acids and cholesterol than muscle meats (Siedler, 1963).

II. EXPERIMENTAL AND OTHER STUDIES

As early as 1912, the Nobel Prize winner, Sir Frederick Gowland Hopkins, announced:

> No general or widespread belief in the view that an adequate diet must contain indispensable constituents other than adequate calories, a minimum of protein, and a proper mineral supply, could be said to exist till the years 1911-12 (Wagner and Folkers, 1964).

In 1913, Funk made the statement that vitamins are found in meat and are especially abundant in heart muscle, brain, and probably in most animal tissues.

Osborne and Mendel (1917), reviewing the work of several investigators on nutritive factors in animal tissues, mention Suzuki, Shimanura, and Odake, who reported that an alcoholic extract of horse meat did not prevent the death of mice fed on a diet of polished rice, and that dogs fed exclusively on boiled rice and boiled horse meat became greatly emaciated and ill in three to four weeks. Osborne and Mendel, from experiments on rats fed beef muscle and pork liver, also demonstrated that both the meat powder and meat residue were suitable as sources of protein in the diet when the other essential ingredients were present in sufficient amounts, and that dried pig liver contained both adequate protein and a large proportion of water soluble vitamins.

Monkhouse et al. (1952) made a study of hemorrhagic conditions brought about by irradiation of rats, rabbits, and guinea-pigs, and also of the release of heparin in anaphylactic shock in irradiated and non-irradiated animals. They were unable to find heparin in the blood

of rats, though they were able to find varying amounts of heparin in the other animals.

Blackiston (1949) defined heparin as a substance found in the liver and other tissues which is used in blood transfusion for inhibiting coagulation of the blood platelets. It is a polysaccharide material which, on hydrolysis, yields sulfuric acid, acetic acid, 2-amino-glucose (glycosamine), and an unidentified uronic acid.

Riley and West (1953) pointed out that a strong positive correlation was found between the histamine and mast cell contents of a variety of normal and pathological tissues; that mast cells are particularly numerous in the liver capsule of ox and sheep and in ox pleura; and that these tissues contain considerably more histamine than found in the underlying parenchyma.

Monkhouse (1956) demonstrated that large amounts of heparin are present in dog liver, beef bung and certain tumors and in lesser amounts in many other tissues. He developed a method for the extraction of heparin from small quantities of tissue and assayed the heparin content of livers of dog, rat, rabbit, guinea pig, and other animals.

Evidence of osteoporosis, associated with flesh-eating habits, was noticed by Greeves et al. (1959) in young captive lions and foxes, and in those domestic cats which were fed unsupplemented raw heart. They refuted an earlier contention that this condition, referred to as "osteogenesis imperfecta" was of genetic origin in dogs and cats fed on "good meat diets."

In their experiment of feeding rations containing codfish and whale products to breeding minks, Friend and Crampton (1960) observed adverse effects of raw whale liver on the breeding performance of female mink.

Moore and Sharman (1960) noted that rats fed on diets consisting exclusively of meat developed severe fragility of the bones. The lesions were worse when the meat was mixed with fat than when lean alone was given. They opined that this was presumably because fat was even lower in calcium than lean meat. With fat in the diet, the skeletal lesions could be completely prevented and growth maintained by the addition of calcium carbonate to the diet. Dosing with vitamin D without calcium was ineffective in preventing the lesions.

Ilan and Guggenheim (1962) reported that young mice maintained with a diet of muscle meat of pork, beef or chicken developed severe anemia. When the diet consisted of heart muscle of beef or of fish muscle no anemia accurred. Livers of various animals exhibited different anemia-preventing potencies. The most potent were beef and sheep followed by rat, pig, human, and chicken livers. Severe heat treatment or drying of the liver did not destroy its anemia-preventing potency.

Guggenheim et al. (1962) found that the severe type of anemia, which developed in mice fed for six weeks on a diet of muscle meat, could be prevented by supplementing the meat with one milligram of copper as copper sulfate. Smaller amounts were less effective.

A diet of raw, minced beef steak caused the development of copper deficiency in young rats, as evidenced by hypochromotrichia, a slight anemia, and a reduction in the rate of weight increase (Moore, 1962). The deficiency was prevented by the addition to meat of copper sulfate or of impure ferrous carbonate containing copper. Chemical analysis showed that the meat contained the expected amounts of copper. According to this investigator, a defect in its absorption, probably due to the high phosphate content of the meat, seemed probable. He also observed that in adult rats hypochromotrichia could be prevented and in young rats partially prevented by the addition of calcium carbonate to the diet. The calcium carbonate contained only traces of copper and presumably acted by counteracting the high intake of phosphorus.

Using raw minced beef in their experiments on young male rats, Moore et al. (1962) confirmed and amplified the earlier claims that the metabolism of calcium and iron is interrelated.

When twelve male rats were fed irradiated beef, 75 per cent of them died of hemorrhage, while rats fed non-irradiated beef were normal (Reber and Malhotra, 1961).

Metta et al. (1959) reported that consumption of diets containing gamma-irradiated beef resulted in internal hemorrhages and prolonged prothrombin times in growing male rats. Generally the female rats did not show this syndrome. They found that supplementation with vitamin K prevented the hemorrhagic diathesis in rats consuming irradiated beef.

In their experiment with rats, Malhotra and Reber (1963) found that feeding irradiated beef decreased the prothrombin rate and supplementation with methionine increased the rate.

Doisy and Matschiner (1961), in elucidating some of the mechanisms operating to cause hemorrhagic diathesis in rats fed irradiated beef, recognized the prothrombin-lowering action of vitamin A in rats with limited intake of vitamin K, the hemorrhagenecity of vitamin A acid, and of squalene and oxidized squalene. They also showed that extraction by lipid solvents of certain proteins before feeding increased the severity of hypoprothrombinemia. The authors also point out that intact females are more resistant than spayed females which in turn are more resistant than males to nutritional hypoprothrombinemia and that the level of vitamin A in maternal diets is of importance. This is a possible explanation for some of the differences between strains of rats.

Richardson (1961) carried out further studies to determine the mechanism for the production of the hemorrhagic syndrome which developed in male rats receiving irradiated beef and also rats receiving soybean protein, and concluded that the irradiation process by itself was not responsible for the production of the syndrome; that high protein diets increase the vitamin K requirement of male rats; and that there was a probable action of some amino acids like L glutamic acid, L cystine and glycine in preventing or reducing the incidence of hemorrhage. However, he could not determine the mechanism of their action.

The incidence of hemorrhage was higher in rats receiving 60,000 I.U. of vitamin A/kg. than those receiving 30,000 I.U. The incidence of hemorrhage occurred in the offspring when the diet of the mother was low in both vitamin K and vitamin A.

III. MINERAL METABOLISM AND BONE CALCIUM AND PHOSPHORUS

In the field of nutrition mineral elements have aroused a considerable interest. Calcium and phosphorus have been studied very extensively because of their importance in mixed diets. However, this can be said for all of the required elements. It looks as though the most important mineral element is the one that is missing or that is present in the diet in suboptimal amounts or in an improper ratio to others. This can be especially true for calcium and phosphorus.

Over 70 per cent of the ash of the body consists of calcium and phosphorus (Maynard and Loosli, 1962). Approximately 99 per cent of the calcium and 80 per cent of the phosphorus of the body are present in the bones and teeth. Though somewhat variable according to age, state of nutrition, and species, normal adult bone may be considered to have the following approximate composition: water, 45 per cent; ash, 25 per cent; protein, 20 per cent; and fat, 10 per cent. The water content of bone decreases with age. In mammals the ash is made up approximately as follows: calcium, 35 per cent; phosphorus, 17 per cent; and magnesium, 0.8 per cent. There is little variation in

the elementary composition of bone ash. The calcium and phosphorus always occur in approximately a 2:1 ratio. Since bone ash consists almost entirely of calcium and phosphorus salts and since the relative amounts of these elements show very little variation, the ash content of bone is commonly used as the measure of its state of calcium and phosphorus nutrition. Further, one bone may be selected for analysis as being representative of the skeleton as a whole. The nature of the diet, however, can affect somewhat the mineral relationships in bone, even though the ash content is not appreciably changed.

Sherman and McLeod (1925), in their study on composition of the rat bone, concluded that, without questioning the importance of the vitamin factor in certain relations, it was plain that in such cases as studied by them, the factor of prime importance to the optimal storage of calcium in the growing animal body was the presence of a sufficiently liberal allowance of calcium in the food.

Hammett (1925), who made a biochemical study of bone growth in albino rats, demonstrated that the composition of ash of humerus and femur of male and female rats with respect to calcium, magnesium and phosphorus changes during the growth period from 23 to 150 days. The changes consisted of an increase in the calcium and decrease in the magnesium and phosphorus per cent of the ash.

Hammett (1925) also showed that the percentage of ash increases as the animals grow older, and the percentage of ash is higher in females than in males, as the degree of ossification is relatively greater

in females than in males.

Moore et al. (1963) explain that the higher femur ash content in females is attributable to their lower growth rate, and opine that in females growth would exert a lesser force than in males in the competition between growth and bone mineralization, each to maintain its normal level.

IV. RADIOACTIVE ISOTOPES

The availability of radioactive isotopes has provided the investigators of metabolism in animals with techniques of unusual scope and flexibility. An element which occurs in the diet at a level of 0.05 p.p.m. or less can still be followed from feed to ultimate location within a specific tissue of an animal without increasing the amount of the element in the diet above the physiological level.

Marcus and Lengemann (1962) demonstrated that in rats the total absorption of Ca^{45} and Sr^{85} from a liquid dose averaged 45 and 28 per cent, respectively; from solid dose it averaged 19 and 13 per cent. They also showed that absorption occurred rapidly at first, but then tapered off and essentially ceased after about two hours for the liquid dose and six hours for the solid dose.

The several aspects of the principles of tracer methodology, procedures, and other pertinent details concerning the radioisotopes are provided by Comar (1955).

CHAPTER III

EXPERIMENTAL MATERIALS AND METHOD

In view of the great importance of meat as an article of diet and the widespread use of different kinds of meat for food, it has seemed desirable to undertake this experiment of studying the nutritive adequacy of three organ meats, namely the heart, liver, and tongue.

The study has been limited to finding the calcium and phosphorus levels in the experimental animals as influenced by feeding each of the three organ meats as the sole diet with and without supplementations.

I. ANIMALS

The rat, as an omnivorous mammal, whose general development has been well studied in connection with many other investigations and which is being widely and increasingly used in nutrition research and in testing food values, was chosen as the experimental animal for this work.

The albino rats used for the study were those raised in the animal science colony of The University of Tennessee, and were randomly allotted.

The rats were kept in all-metal cages without bedding and without giving chance for coprophagia.

Sex and weight groups. The rats were grouped into four categories as light females, light males, heavy females, and heavy males on the basis of their initial body weight. Each cage consisted of two rats of the same sex and weight group, which served as two replications.

Groups under basic diet. The rats under each of the four groups mentioned above were allotted to three diet groups for the feeding trial with three organ meats, namely, pork heart, pork liver, and pork tongue as the exclusive basic ration.

Groups under supplementation. Each of the three basic diet groups was in turn allocated into four different sub-treatments, namely, (1) supplementation with complete vitamin mixture, (2) supplementation with calcium, (3) supplementation with vitamin mixture plus calcium, and (4) without any supplementation.

There were thus, in all, four sex and weight groups, and a total of 4 by 3 by 4 forty-eight different treatment combinations.

In addition, under each sex and weight group there was one cage with two rats, to serve as control on normal routine rat feed.

A diagrammatic representation of the arrangement of the cages, according to the feed treatment, is shown in Figure 1.

II. THE FEED

The organ meats required for feeding were obtained partly from hogs slaughtered in the University Meat Laboratory and partly purchased from the East Tennessee Packing House in Knoxville.

Light Females	H+V	L+V	T+V	H+V	L+V	T+V	Light Males
	H+Ca	L+Ca	T+Ca	H+Ca	L+Ca	T+Ca	
	H+V+Ca	L+V+Ca	T+V+Ca	H+V+Ca	L+V+Ca	T+V+Ca	
	H	L	T	H	L	T	
			Control	Control			
Heavy Females	H+V	L+V	T+V	H+V	L+V	T+V	Heavy Males
	H+Ca	L+Ca	T+Ca	H+Ca	L+Ca	T+Ca	
	H+V+Ca	L+V+Ca	T+V+Ca	H+V+Ca	L+V+Ca	T+V+Ca	
	H	L	T	H	L	T	
			Control	Control			

NOTE: H = Heart; L = Liver; T = Tongue; V = Vitamin Mix Supplementation; Ca = Calcium Supplementation; V+Ca = Vitamin + Calcium Supplementation.

FIGURE 1

EXPERIMENTAL DESIGN

The organ meats were ground, separately, made into patties of sixty grams each and frozen, at start, for feeding. Each patty, weighing about sixty grams, was meant for meeting the daily feed requirement of a rat, on the basis of an assumed consumption of fifteen to twenty grams of dry matter per day. One such patty in the morning and one in the evening were fed to the two rats in each cage.

Supplements. The vitamin mixture used was Vitamin Diet Fortification mixture of the Nutritional Biochemical Corporation, containing the following formulation:

Vitamin A concentrate (200,000 units per gram)	4.5 grams
Vitamin D concentrate (400,000 units per gram)	0.25 grams
Alpha tocopherol	5.0 grams
Ascorbic acid	45.0 grams
Inositol	5.0 grams
Choline Chloride	75.0 grams
Menadione	2.25 grams
P Aminobenzoic acid	5.0 grams
Niacin	4.5 grams
Riboflavin	1.0 gram
Pyridoxine hydrochloride	1.0 gram
Thiamine hydrochloride	1.0 gram
Calcium pantothenate	3.0 grams

Biotin	20 milligrams
Vitamin B-12	1.35 milligrams

Each rat in the vitamin supplement group was provided with 0.44 grams of the vitamin mixture with 1 gram of corn meal. This quantity was added to the meat patty at each feeding.

Thirty milligrams of calcium, representing the approximate daily requirement of a weaned growing rat, was provided to each rat as calcium carbonate with 1 gram of corn meal. This quantity was added to the meat patty at each feeding, for the rats under the calcium supplement group.

To provide both calcium and the vitamins, a mixture of both of these calculated on the basis as above was given, along with the meat patties at each feeding, to the group of rats receiving both vitamin and calcium supplementation.

One gram of corn meal, without vitamin or calcium additive, was added to those lots receiving neither vitamin nor calcium.

Ration for control rats. This consisted of the normal routine rat feed of the laboratory with the guaranteed analysis of crude protein, not less than 20 per cent; crude fat, not less than 0.5 per cent; and crude fibre, not more than 5.5 per cent. Free access to this ration was allowed for the control rats.

Drinking water. In order to avoid the possibility of calcium source through ordinary tap water, distilled water was provided, ad

libitum, to all the rats, including the controls.

The rats were maintained on the experimental diet for a period of seven weeks, during which weekly weights were taken to study the gain in weight. Daily observations were also being made regarding the general condition of the rats.

During the course of the experiment, certain rats on liver diet showed abnormal symptoms and eventually died. A test for blood clotting time was conducted on some rats to study the nature of the syndrome, and is discussed later.

III. DOSING WITH ISOTOPE MATERIAL

At the end of the feeding period, the rats were dosed with Ca^{45} and P^{32}. The supply of radioisotope material was obtained from New England Nuclear Corporation, Boston, Massachusetts. The technical particulars of the isotope were as in Table I.

The isotope materials were diluted with distilled water so that each milliliter of the mixed solution contained 27.27 microcuries of Calcium-45 and 90.90 microcuries of Phosphorus-32, to be administered orally to each rat. The dose of P^{32} was made heavier in view of its much shorter half-life of 14 days, as compared to 152 days for Calcium 45.

All the care and protective precautions were scrupulously followed in handling the radioactive materials, both at the time of dosing and in all the subsequent operations, as outlined in the Health

TABLE I

TECHNICAL DATA

	Radio-Isotope Solutions	
	1	2
Isotope	Ca^{45}	P^{32}
Amount	3 mc.	10 mc.
Assay Date	9-2-65	9-2-65
Chemical Form	$CaCl_2$ in HCl	H_3PO_4 in water
Specific Activity	11.05 mc./mg.	CF
Concentration	21.2 mc./ml.	10 mc./ml.
Volume	0.142 ml.	1.0 ml.
Radioactive Purity	99%	99%
Total Solids	1.85 mg./ml.	$<$0.1 mg./ml.
Method of Preparation	Ca^{44} (n, r) Ca^{45}	Synthesis from P^{32}

Physics and Radiation Protection Procedures (Comar, 1955).

The rats were individually anaesthetized with ether and dosed orally with 1 milliliter of the isotope mixture, using a special dosing syringe and needle.

Collection of materials. Twenty-four hours after dosing, the rats were killed by severing the head with scissors, and the femur and tibia from both the hind limbs of each rat were collected and labeled. The bones were cleared of the adhering flesh by boiling in water. After recording the weight of the green bones, separately as femur and tibia from each rat, in tared crucibles, they were ashed in the muffle furnace at 500°C. for 12 hours. The ash was completely dissolved in 5 milliliters of 6 N hydrochloric acid solution in the crucible, transferred quantitatively to a 25 milliliter flask, made up to 25 milliliters with distilled water and used for further analysis.

The ash solution of tibia was used for radiochemical analysis, while that of femur was used for chemical analysis of calcium and phosphorus.

Radiochemical analysis. Using infra-red lamps, 0.5 milliliter of the ash solution of tibia was evaporated to dryness in the center of stainless steel planchets. Radio-activity was measured using the Geiger-Muller counting apparatus. From the original dosing solution, a standard was prepared, identical to the tibia samples and counted

at 3 to 4 hour intervals, along with the tibia samples. As is the practice at the UT-AEC laboratories, the counts were reduced to per cent of the dose administered for purposes of calculation.

Phosphorus-32. In contrast to the low energy of Ca^{45} beta (0.26 Mev.), that from p^{32} has high energy emission (1.71 Mev.). This difference in energy provides a convenient basis for measuring the activity of p^{32} in the presence of Ca^{45}. An aluminum absorber having a surface density of 55 milligrams per square centimeter would absorb all the beta particles from the Ca^{45} but would reduce p^{32} contribution by a factor of only 1.5. All samples and standards were therefore counted for p^{32} activity through such an absorber.

Calcium-45. The beta emissions from Ca^{45} are of low energy and this results in high self-absorption. All the measurements of Ca^{45} were made against standards prepared from aliquots of the original dosing solutions which were suitably diluted and evaporated to dryness in metal planchets. The counts due to Ca^{45} were obtained from the difference between the counts taken with and without the absorber. In all the cases, two counts were made for two minutes each and the average was taken.

Chemical analysis. Phosphorus was determined by a photometric method using an Evelyn Photoelectric colorimeter at 660 mu (Hawk et al., 1947). Calcium was estimated by the method of Clark-Collip (1925),

by precipitation of calcium in ash solution as oxalate and then titrating with standard potassium permanganate solution.

CHAPTER IV

RESULTS AND DISCUSSION

Statistical analysis was made using analysis of variance and Duncan's multiple range techniques as described by Snedecor (1962) for the different aspects of study. Data for fifteen of the rats were incomplete in one or more respects due to such causes as death of the animal during the feeding period or accidental loss of the material during the chemical analysis or otherwise. Therefore, the data for the eighty-nine rats remaining out of a total of 104 at the start of the experiment was subjected to statistical analysis. The results and discussion are presented below.

I. GAIN IN WEIGHT

There was a difference ($p < 0.01$) between the sex groups with regard to gain in the weight, with the females gaining significantly more than the males. The light weight rats showed a highly significant increase in weight gain compared to the heavy rats. The treatments were significantly different ($p < 0.01$) with regard to their effects. There was no difference between the rats fed with tongue supplemented with vitamin and calcium, tongue alone, and tongue with vitamin alone, but these three treatments showed significantly higher gains than did the rest of the treatments. While no difference was observed among the

treatments with liver alone, liver plus vitamin, liver plus calcium, and liver combined with both calcium and vitamin, they had significantly lower gains than did the other treatments. The rest of the treatments were intermediate in their effects.

No interaction between the size and sex groups, or treatment and size groups was evident. However, an interaction between treatment and sex was present. This indicated a probable effect of the treatment due to sex.

The results thus indicated that tongue was probably a more balanced diet and induced more gain in weight than either liver or heart. The model of the analysis of variance used is shown in Table II. Additional measures of significance were made using Duncan Multiple Range Test (Table III).

II. WEIGHT OF FEMUR ASH

The weight of femur ash obtained from individual treatments is furnished in Appendix I. The analysis of variance data are presented in Table IV. There was no significant difference in the weight of ash obtained from the different sexes. However, the heavy rats gave significantly higher femur ash than the light weight rats, as would be expected. No significant difference was observed with regard to the different treatments of meat sources, either with or without supplements. No interactions were present.

TABLE II

ANALYSIS OF VARIANCE--VARIABLE 1, GAIN IN WEIGHT, GRAMS

Source	DF	SS	MS	F Cal.
Total	88	291,947.92		
Sex Group	1	28,709.72	28,709.72	76.64**
Size Group	1	34,707.84	34,707.84	92.65**
Treatments	12	49,935.64	4,161.30	11.11*
Size x Sex	1	397.83	397.83	1.06
Size x Treatments	12	3,951.49	329.29	0.879
Sex x Treatments	10	14,880.51	1,488.05	3.972**
(Error)	51	159,364.89	374.60	

*$P < 0.05$.

**$P < 0.01$.

TABLE III

MEANS OF TREATMENTS OF DIFFERENT SOURCES OF MEAT, WITH OR WITHOUT SUPPLEMENT--VARIABLE 1, GAIN IN BODY WEIGHT IN GRAMS

Treatment[a]	Treatment Number	No. of Rats	Mean (Gm.)	Least Squares Constant
T+V+Ca	12	6	126.5 [b]	36.32
T	13	8	117.9	43.33
T+V	10	8	106.0	31.45
T+Ca	11	8	88.0	13.45
H+V	2	7	74.0	0.48
H+V+Ca	4	8	70.9	-3.68
H+Ca	3	8	62.0	-12.55
H	5	7	61.6	-7.54
Control	1	6	50.3	-11.99
L+V+Ca	8	8	44.6	-29.93
L+V	6	8	42.4	-32.18
L+Ca	7	4	39.2	-15.58
L	9	3	37.7	-11.58

[a]H = Heart; L = Liver; T = Tongue; V = Vitamin Mix; Ca = Calcium; V+Ca = Vitamin Mix + Calcium.

[b]The line indicates that the treatments covered by it are not significantly different.

TABLE IV

ANALYSIS OF VARIANCE--VARIABLE 2, WEIGHT OF FEMUR ASH, GRAMS

Source	DF	SS	MS	F Cal.
Total	88	4.51238		
Sex Group	1	0.01458	0.0146	0.373
Size Group	1	0.5068	0.5068	12.97**
Treatments	12	0.8123	0.0677	1.73
Size x Sex	1	0.0264	0.0264	0.677
Size x Treatments	12	0.3756	0.0313	0.802
Sex x Treatments	10	0.4577	0.0458	1.17
Within (Error)	51	2.319	0.0391	

**$P < 0.01$.

III. STABLE CALCIUM PERCENTAGE IN FEMUR ASH

The heavy rats had a higher calcium percentage in the femur ash than the lighter ones. This difference was not observed in sex groups. The different meat sources with or without supplemental diets did not show differential response with respect to calcium percentage. An interaction between size and sex was present. Inasmuch as there was no difference between the sex groups there is the probability that the size groups might have influenced the interaction more than the sex group. The analysis of variance for the data are presented in Table V. The higher percentage of calcium in the heavy groups might be explainable in terms of the lower body requirements of the element for this group, and a greater storage of the element in the bone.

IV. STABLE PHOSPHORUS PERCENTAGE IN FEMUR ASH

The female rats had a significantly higher per cent of phosphorus in the femur than did males. There was no difference in phosphorus content either between the size groups, or among the different diet sources. However, an interaction between the size and treatments was present. This might have been due to different initial weights of the rats which might have influenced the phosphorus absorption. These data are presented in Table VI.

V. THE WEIGHT OF ASH FROM TIBIA

There was a significantly higher ash weight in the males than in the females, and in heavy weight rats compared to the lighter ones.

TABLE V

ANALYSIS IN VARIANCE--VARIABLE 3, STABLE CALCIUM PERCENTAGE IN FEMUR ASH

Source	DF	SS	MS	F Cal.
Total	88	1685.766		
Sex Group	1	9.132	9.132	0.659
Size Group	1	192.871	192.871	13.93**
Treatments	12	221.601	18.467	1.33
Size x Sex	1	149.072	149.072	10.76**
Size x Treatments	12	206.630	17.219	1.243
Sex x Treatments	10	134.51	13.451	0.971
Within (Error)	51	771.95	13.85	

**$P < 0.01$.

TABLE VI

ANALYSIS OF VARIANCE--VARIABLE 4, STABLE PHOSPHORUS PERCENTAGE IN FEMUR ASH

Source	DF	SS	SS	F Cal.
Total	88	1073.1462		
Sex Group	1	65.426	65.426	5.96*
Size Group	1	15.780	15.780	1.44
Treatments	12	145.400	12.117	1.104
Size x Sex	1	0.0712	0.0712	0.006
Size x Treatments	12	270.939	22.578	2.057*
Sex x Treatments	10	42.260	4.226	0.385
Within (Error)	51	533.27	10.98	

*$P < 0.05$.

The ash weights of tibia of rats fed the different meat sources were significantly different. The heaviest bone ash was from the control rats. Rats receiving liver alone had lightest ash weight of the tibia. Treatments with heart supplemented with calcium and vitamin, the tongue with calcium alone and the tongue with calcium and vitamin induced greater weight for tibia ash than the rest of the treatments except the control, but no difference was found among these treatments. The rest of the treatments were intermediate in effect. The rats on the control treatment had higher bone ash probably due to the balance of nutrients in the diet while the other rats had a diet restricted in one or more nutrients. Heart and tongue with calcium or with calcium and vitamin might have proved to be better sources of body metabolic requirements, but not to such an extent as the control diet. Liver was a poor source of diet, even with the different supplements, so far as ash weight of the tibia was concerned. Interaction between size and treatments was present. This indicated that the bone ash is influenced jointly by the treatment effects and the initial weight of the rats. The requisite data are presented in Tables VII and VIII.

VI. RADIOISOTOPE RETENTION

The radioisotope usage and interpretation of results were based on two major assumptions: (1) that the radioisotope behaved in the animal body similar to the stable element, and (2) that an increase in retention of the isotope indicates a low level of metabolic activity or a

TABLE VII

ANALYSIS OF VARIANCE--VARIABLE 5, WEIGHT OF TIBIA ASH, GRAMS

Source	DF	SS	MS	F Cal.
Total	88	1.8727196		
Sex Group	1	0.0584	0.0584	44.01**
Size Group	1	0.3544	0.3544	267.25**
Treatments	12	0.3913	0.0326	24.59*
Size x Sex	1	0.0000196	0.0000196	0.015
Size x Treatments	12	0.0662	0.0055	4.163*
Sex x Treatments	10	0.0073	0.00073	0.551
Within (Error)	51	0.9951	0.00133	

*$P < 0.05$.

**$P < 0.01$.

TABLE VIII

MEANS OF TREATMENTS OF DIFFERENT SOURCES OF MEAT, WITH OR WITHOUT SUPPLEMENT--VARIABLE 5, TIBIA ASH IN GRAMS

Treatment[a]	Treatment Number	No. of Rats	Mean (Gm.)		Least Squares Constant
Control	1	6	0.53203		0.1415
T+V+Ca	12	6	0.47598	[b]	0.0790
T+Ca	11	8	0.47146		0.0852
H+V+Ca	4	8	0.43431		0.0606
H+Ca	3	8	0.42375		0.0375
L+V+Ca	8	8	0.40923		0.0230
H	5	7	0.35491		-0.0128
T	13	8	0.34666		-0.0683
L+Ca	7	4	0.34175		-0.0163
H+V	2	7	0.33757		-0.0685
L+V	6	8	0.32757		-0.0585
T+V	10	8	0.30305		-0.0831
L	9	3	0.26850		-0.1193

[a]H = Heart; L = Liver; T = Tongue; V = Vitamin Mix; Ca = Calcium; V+Ca = Vitamin Mix + Calcium.

[b]The line indicates that the treatments covered by it are not significantly different.

deficiency of the element in the tissue studied.

Stored Ca^{45} as per cent of dosage administered. There was no significant difference between sexes with respect to Ca^{45} retention. The lighter group of rats had a significantly higher retention of Ca^{45} that did the heavier ones. The treatments of different meat sources showed significant differences in stored Ca^{45}. The treatments consisting of tongue and tongue with vitamin had the highest retention of Ca^{45}. The treatment of tongue with vitamin did not differ significantly from the treatments consisting of liver with vitamin, heart alone, and tongue with vitamin and calcium. The control group indicated the lowest retention of Ca^{45} but did not differ significantly from the treatments liver alone, or heart and calcium, or liver and calcium. The other treatments were intermediate in their effects.

Calcium deficiency was also influenced by the size and treatment interaction and by sex and treatment interaction as indicated by the statistical significance (Table IX). The deficiency did occur irrespective of the meat diet and the vitamin supplied but only when calcium was not supplied.

The analysis of variance and the treatment comparison are shown in Tables IX and X.

Stored p^{32} as per cent of dosage administered. The statistical analysis of the data with regard to percentages of p^{32} stored indicated differential response between sex groups that was significant. The

TABLE IX

ANALYSIS OF VARIANCE--VARIABLE 6A, STORED RADIOACTIVE CALCIUM AS PERCENTAGE OF ADMINISTERED DOSE

Source	DF	SS	SS	F Cal.
Total	88	23.6783		
Sex Group	1	0.0790	0.0790	1.035
Size Group	1	0.8054	0.8054	40.55**
Treatments	12	5.538	0.4615	6.04*
Size x Sex	1	0.1559	0.1559	2.04
Size x Treatments	12	2.376	0.1981	2.594**
Sex x Treatments	10	2.094	0.2094	2.743**
Within (Error)	51	12.63	0.0764	

*$P < 0.05$.

**$P < 0.01$.

TABLE X

MEANS OF TREATMENTS OF DIFFERENT SOURCES OF MEAT, WITH OR WITHOUT SUPPLEMENT--VARIABLE 6A, STORED RADIOACTIVE CALCIUM AS PERCENTAGE OF ADMINISTERED DOSE

Treatment[a]	Treatment Number	No. of Rats	Mean (%)	Least Squares Constant
T	13	8	2.3894[b]	0.6528
T+V	10	8	2.1148	0.3429
L+V	6	8	1.9574	0.1856
H	5	7	1.8530	0.0888
T+V+Ca	12	6	1.8487	0.0158
H+V+Ca	4	8	1.7844	-0.0126
H+V	2	7	1.7710	-0.0428
T+Ca	11	8	1.7556	-0.0161
L+V+Ca	8	8	1.6936	-0.0781
L	9	3	1.6520	-0.2173
H+Ca	3	8	1.5886	-0.1831
L+Ca	7	4	1.4572	-0.2818
Control	1	6	1.3117	0.4441

[a]H = Heart; L = Liver; T = Tongue; V = Vitamin Mix; Ca = Calcium; V+Ca = Vitamin Mix plus Calcium.

[b]The line indicates that the treatments covered by it are not significantly different.

male rats retained more p^{32} than the female rats. The lighter group of rats had a highly significant increase in p^{32} retention in bone than the heavier ones.

There was a significant difference among the different treatments. The treatment effects with liver, immaterial of the supply of calcium and/or vitamin, had significantly lesser absorption of p^{32} (or lesser deficiency) than all but two of the rest of the treatments. In general the treatments with heart or tongue, supplemented with calcium and/or vitamin had greater retention of phosphorus 32 than the treatments with liver supplemented with calcium and/or vitamin.

Since the liver diet is higher in phosphorus than either tongue or heart meat, the results indicated greater deficiency of phosphorus in bone with the latter two treatments. This fact of the liver being richer in phosphorus than the tongue or heart is in agreement with the data furnished by Lushbough and Schweigert (1960). As in the case of calcium, a size, treatment interaction was present. The relevant data are presented in Tables XI and XII.

TABLE XI

ANALYSIS OF VARIANCE--VARIABLE 6B, STORED P^{32} AS PERCENTAGE OF DOSAGE ADMINISTERED

Source	DF	SS	MS	F Cal.
Total	88	19.1079		
Sex Group	1	0.4884	0.4884	6.605*
Size Group	1	1.732	1.732	23.43**
Treatments	12	3.678	0.3065	4.14*
Size x Sex	1	0.0348	0.0348	0.470
Size x Treatments	12	1.885	0.1571	2.12*
Sex x Treatments	10	1.2647	0.1265	1.71
Within (Error)	51	10.025	0.0740	

*$P < 0.05$.

**$P < 0.01$.

TABLE XII

MEANS OF TREATMENTS OF DIFFERENT SOURCES OF MEAT, WITH OR WITHOUT SUPPLEMENT--VARIABLE 6B, STORED P^{32} AS PERCENTAGE OF DOSAGE ADMINISTERED

Treatment[a]	Treatment Number	No. of Rats	Mean (%)	[b]	Least Squares Constant
T+V	10	8	1.6318		0.3418
T+V+Ca	12	6	1.5448		0.1944
T	13	8	1.5254		0.1082
T+Ca	11	8	1.4860		0.1960
H+Ca	3	8	1.3903		0.1004
H+V+Ca	4	8	1.3879		0.0979
H	5	7	1.3151		0.0429
H+V	2	7	1.2220		-0.1705
Control	1	6	1.0913		0.1470
L+V	6	8	1.0401		-0.2498
L+Ca	7	4	1.0400		-0.1686
L+V+Ca	8	8	1.0346		-0.2553
L	9	3	0.8757		-0.3844

[a]H = Heart; L = Liver; T = Tongue; V = Vitamin Mix Supplement; Ca = Calcium Supplement; V+Ca = Both Calcium and Vitamin Supplement.

[b]The line indicates that the treatments covered by it are not significantly different.

CHAPTER V

HEMORRHAGIC SYNDROME IN SOME EXPERIMENTAL RATS

Three weeks after the start of the experiment, one of the male rats in the heavy groups on liver diet with calcium was observed to be listless, dull, and off feed. In about thirty-six to forty-eight hours, it became unable to move about. There was a general swelling all over the body. Gradually, the ears, tail and feet became chalky white in color, typical of an anemic condition. Four days from the onset of first symptoms, the rat died, giving an appearance suggestive of vitamin K deficiency. Post mortem examination revealed that the bladder was filled with sanguineous fluid.

Meanwhile, another male rat, receiving plain liver also developed similar symptoms, showed epistaxis, in addition, and died in three days. Post-mortem examination revealed hemorrhages in the testes and in the subcutaneous areas, and the lungs were found to be highly congested.

This was followed by seven more rats, in rapid succession, presenting a similar condition. These rats were: three males receiving liver plus calcium, and three males and a female rat receiving plain liver. Incidentally, it may be pointed out here that this was the only female that developed this condition. All seven rats showed chalky white extremities and died in the course of three to four days. Four of these had epistaxis and one had ocular hemorrhage (Figure 2).



Left: Hemorrhage in the nose and fresh blood from the pin-prick made the previous day at the tip of the tail, seen on the legs (male rat, No. 20a).

Right: Ocular hemorrhage (female rat, No. 8b).

FIGURE 2

PHOTOGRAPH OF TWO RATS SHOWING HEMORRHAGIC SYNDROME

In all the cases, post-mortem examination revealed internal hemorrhages in the subcutaneous region, on testes, bladder, or liver tissue and even in some intramuscular situations.

After the occurrence of the first two cases, it was decided to make a study of the blood-clotting time in the ailing and normal cases. The method used in this study was as described by Wintrobe (1949). A drop of blood got by pricking the tip of the tail with a short, sharp hypodermic needle was collected into a capillary tube, and small lengths of the tube were broken at intervals of 15 to 20 seconds to find out at what time a strand of fibrin formed as the two broken pieces of the capillary tube were pulled apart.

Out of a total of sixty rats of different treatments in which blood clotting time was studied, four were ailing cases and the rest normal. The clotting time in normal rats ranged from 1-¼ minutes to 4 minutes, a majority of them being 2 to 3 minutes. This compared well with the average clotting time of 2-½ minutes for normal rats (Creskoff et al., 1949). A prolonged clotting time of 15 to 16-½ minutes was observed in the ailing cases. It was interesting to notice that one of the ailing rats, whose tail was pricked lightly at the tip for collection of a drop of blood, continuously bled from this tiny pricked wound for more than 24 hours until death.

This development of an unexpected morbid condition in the rats receiving a sole diet of liver or liver with supplemental calcium was of great interest, because the condition was not noticed in any of the

rats that had received liver with vitamin supplementation. Further, all except one of the eight affected were males. All aspects of this unexpected problem could not be investigated owing to the fact that the program of the experiment was a different one. However, this is a matter for a separate study involving several questions: (1) whether this syndrome was due to actual vitamin K deficiency or any other factor having an antivitamin-K effect; (2) whether the prothrombin level of blood was altered; (3) whether supplementation with menadione or vitamin K would prevent the occurrence of such a condition. The peculiar susceptibility of the male rats for this condition further suggests the possibility of a sex difference with possible hormonal interaction. Which of the nutrients led to this hemorrhagic condition may also require to be investigated. A study of any of the above factors might yield some interesting information.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In feeding trial experiment with organ meats to study the nutritive adequacy of raw heart muscle, tongue muscle, and liver, as sole diets, a seven-week feed treatment of forty-eight combinations was conducted using white rats. Emphasis was on calcium and phosphorus metabolism. Four control groups were simultaneously maintained on the regular rat diet used in routine feeding of rats in the animal science laboratory. All rats were dosed at the end with Ca^{45} and p^{32} to aid in the analysis. Under each treatment, including the four controls, there were two rats serving as two replications. There were thus 104 rats in all at the start but for final analysis some or all particulars were not obtainable for fifteen rats, due to various causes such as death of the rats during the experimental period or accidental loss of analytical tissues. The complete data on the remaining eighty-nine rats has been analyzed. The following is a summary of the conclusions on seven aspects obtained from a statistical analysis of results of the experiment.

1. Tongue meat appeared to be superior to heart meat or liver in inducing gain in body weight.

2. The ash weight of femur was significantly higher in the heavy group of rats than the light weight group. No significant difference was observed with regard to the different meat sources, either

with or without supplementation.

3. Calcium percentage was highest in the femur ash of the heavy group.

4. Female rats showed a significantly higher per cent of phosphorus in the femur ash than the males.

5. Ash weight obtained from tibia was significantly higher in the males than in the females. Heavy rats also yielded higher ash from tibia. Rats fed with the normal rat diet, yielded highest ash weight, those fed with liver without supplements were the least in this respect. Heart meat supplemented with calcium and vitamin, and the tongue meat with calcium alone or with added vitamin induced greater weight of tibia ash. The control diet which contained a balance of all known nutrient requirements for the white rat and which included animal and vegetable proteins, carbohydrates, vitamins, and minerals probably could explain the higher ash content of bones.

6. Tongue, without added calcium indicated the maximum absorbed Ca^{45} showing a corresponding maximum deficiency of calcium.

7. Absorbed p^{32} was highest in male rats, indicating that the males were more deficient in phosphorus. The lighter rats had a higher rate of absorption of p^{32}. Among the treatments, liver induced less absorption than tongue or heart meat.

8. The post-mortem examination of the rats which died of hemorrhagic syndrome revealed internal hemorrhages either in subcutaneous region or in various internal organs. It was seen that the clotting

time was much prolonged in the affected cases, while the normal rats had a clotting time ranging from 1-¼ to 4 minutes.

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APPENDIX