RECEIVED BY DTIC NOV 12 1970

Animal Metabolic Studies of Strontium and
Calcium with Specific Reference to Fallout Radionuclides

MASTER

Seventh Progress Report

August 16, 1969 - August 15, 1970

Submitted by the
Department of Veterinary Physiology and Pharmacology
College of Veterinary Medicine
University of Illinois at Urbana-Champaign

Contract NO-AT(11-1)-1339

Supported by: Division of Biology and Medicine
United States Atomic Energy Commission
Washington, D. C.

LEGAL NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

	<u>PAGE</u>
1. SUMMARY: SCOPE OF INVESTIGATION AND SIGNIFICANT RESULTS OBTAINED	1
a. Kinetic Studies of Calcium Metabolism in Cows	1
b. Kinetic and Balance Studies of Calcium Metabolism in Goats	6
c. 17 B -Estradiol Radioimmunoassay	9
d. Preliminary Studies on Effects of Estrogen on Calcium Metabolism in Rats	13
e. Retention of Strontium in Swine	15
2. TECHNICAL REPORTS PREPARED FOR PUBLICATION	16
3. REPRINTS OF PUBLICATIONS	17
4. OTHER ACTIVITIES	18

1. SUMMARY: SCOPE OF INVESTIGATION AND SIGNIFICANT RESULTS RETAINED

1.

a. Kinetic Studies of Calcium Metabolism in Cows

^{47}Ca kinetics have been investigated in about eight cows during successive periods of gestation, parturition and lactation. However, only in a couple of cows are estimates of kinetic parameters obtained at parturition, because of the availability of ^{47}Ca only at certain times of the calendar year. The findings which are summarized here, are to be presented in greater detail at the forth coming McLean Conference on Cellular Mechanisms for calcium Transfer and Homeostasis (see Abstract, COO-1339-44).

The method used for estimating the kinetic parameters of ^{47}Ca is the same as previously reported, namely: injection of ^{47}Ca via the jugular vein, serial withdrawal of blood through four days post injection; determination of plasma specific activity curve; and calculation of size (E) in grams, half-life ($T_{\frac{1}{2}}$) in hours and turnover rate (TR) in grams per hour of a readily exchangeable calcium pool (RECaP) as determined from the best-fit of the linear portion of the plot between 20 and 64 hours post-injection. This method is based on an arbitrary selection of only a part of the curve and thus the measured pool really represents only a part of the so called exchangeable pools. In our studies the short-term pool or pools observed within the first 20 hours or so post-injection are ignored. However, the method employed permits comparisons of the kinetic parameter as a function of time and during the various stages of reproduction in the same

animal, which thus serves as its own control. For this reason, then, the method is of considerable value in determining what role the RECaP has in serving as a readily available source of calcium during calcium stress at the onset of lactation around the time of parturition. These kinetic studies are not attempting to determine what role the deep so-called non-exchangeable calcium of bone plays, and the hormonal interactions involved in response to the stress. The ultimate objective of these studies continues to be the elucidation of the etiology of parturient hypocalcemia in cows.

Summary of the kinetic data during the periods of pregnancy, parturition and lactation are as follows:

1.) During pregnancy (3 cows):

E - large increase within approximately a month prepartum

$T_{\frac{1}{2}}$ - slight increase near the end of gestation

TR - moderate increase near the end of gestation

2.) At parturition and onset of lactation (2 cows):

E - remained elevated as during late gestation

$T_{\frac{1}{2}}$ - fell to about two-thirds of prepartum value

TR - increased to about one-and-a-half times prepartum value

3.) During lactation (3 cows)

a.) Moderate to heavy lactation (2 cows)

E - continued to increase as milk production increased and
fell off as production decreased

$T_{\frac{1}{2}}$ - remained depressed through most of lactation and then
increased toward "normal" value as production fell off

TR - remained elevated when production was high and subsequently
decreased

a.) Light lactation (3 cows)

E - decreased moderately relative to value at partum

$T_{\frac{1}{2}}$ - continued to fall and remained low during production

TR - remained slightly elevated during production.

The actual data for these 3 cows (2 Jerseys and 1 Holstein) are presented in Table I along with the data of a comparator cow (Jersey) which was not pregnant or lactating over a period of about a year. The conclusions reached from these data are: (1) the turnover of the RECaP as indicated by TR, is markedly increased only at parturition and the onset of lactation, but not during pregnancy itself; and (2) the turnover remains at a high level only during moderate to heavy lactation, subsequently declining as milk production falls off. The non-pregnant, non-lactating cow demonstrated relatively constant kinetic values for her RECaP, from these limited data it, thus, appears that the onset of lactation exerts a more severe demand for calcium from the RECaP than pregnancy itself, but none of the cows exhibited severe hypocalcemia peripartum. Data on the same animals undergoing several successive calvings, thus, are highly desirable for a more conclusive definition of the role of the RECaP as a source of calcium "on demand" at the onset of lactation.

Several ionic calcium measurements have been made of serum obtained from cows peri-partum. Although an insufficient number of determinations are available, it appears as though the ionic calcium concentration is little changed by parturition as whereas the bound calcium fraction and hence the total calcium may be affected.

Table I

⁴⁷ Ca Inj. Date		Jersey 361* (heavy lactater)				(Jersey 364*) (moderate lactater)			(not pregnantory)		
		E	T $\frac{1}{2}$	TR		E	T $\frac{1}{2}$	TR	E	T $\frac{1}{2}$	TR
4-27-69	2-4 begin Preg.	53.8	23.5	1.59	1-31 Begin Preg.	51.3	22.0	1.62	54.1	24.4	1.54
5-21-69		69.0	32.0	1.49		87.0	37.0	1.63	69.0	34.5	1.39
6-23-69		41.7	21.5	1.34		54.1	25.5	1.47	41.7	20.5	1.41
8-13-69		38.5	24.0	1.11		40.8	22.0	1.29	34.5	22.0	1.09
9-12-69		43.5	20.5	1.47		41.7	18.0	1.61			
9-24-69		33.3	22.0	1.05		40.0	21.0	1.32			
10-8-69		70.4	31.0	1.57		83.3	30.0	1.92			
10-31-69		(P) _____				(P) _____					
11-4-69											
11-5-69		69.0	18.5	2.59		48.1	20.0	1.67	53.2	24.2	1.52
11-19-69		51.3	14.0	2.54		45.5	17.0	1.86	41.7	26.0	1.11
12-31-69		66.7	14.0	3.30		83.3	18.0	3.21	33.3	26.0	0.89
1-14-70		74.1	15.0	3.42		82.0	16.5	3.44	43.5	20.4	1.48
1-28-70		79.4	14.6	3.77		76.9	15.7	3.39			
2-25-70		90.9	15.0	4.20		78.7	18.5	2.95			
3-11-70		80.0	17.0	3.26		100.0	28.0	2.48	48.8	22.0	1.54
3-30-70		128.2	19.0	4.68		106.4	25.0	2.95			
									$\bar{X}$ =46.6	24.4	1.30

*First calving

E = pool size

P = parturition

L = lactating

T $\frac{1}{2}$ = half life of pool in hr

TR = turnover rate of pool in gm/hr

Table I continued

⁴⁷ Ca Inj Date	Begin Preg. 1-29-69	Holstein: 634** (light lactator)		
		<u>E</u>	<u>T$\frac{1}{2}$</u>	<u>TR</u>

9-12-69				
9-24-69				
10-8-69				
11-5-69				
11-18-69 (PM)	Eve	(P) 57.1	32.2	1.23
11-19-69		62.5	26.5	1.63
12-31-69		39.2	19.0	1.43
1-14-70		54.1	17.0	2.21
1-28-70				
2-25-70				
3-11-70				
3-30-70				
4-23-70				
5-5-70				
5-8-70				
5-19-70				

**Third Calving

b. Kinetic and Balance Studies of Calcium Metabolism in Goats

This study of four-months duration involved 18 mature goats which were first placed on a control ration for two months and then divided into three groups of six animals each according to dietary calcium and phosphorus intake. After a month adaptation to their ration three goats from each group were placed in individual metabolic units, one under each feeding regimen, for four or five day balance trials for calcium and phosphorus. Unconsumed feed was weighed back and urine and feces were measured for calcium and phosphorus output. Determination of these minerals in feed was also made. In addition six ^{47}Ca experiments, three under each dietary regimen, were conducted during the three-months period in order to estimate any effects of dietary Ca and P intake on the kinetic parameters of the RECaP.

The data obtained from these experiments is summarized in Table II. The metabolic balance trials indicated that only goats ingesting the high calcium ration were in positive calcium balance, whereas all goats were in positive phosphorus balance. Dietary phosphorus was considered to be adequate as was the calcium in the control ration, ration 1. Data from the ^{47}Ca kinetic studies demonstrated that the turnover rate (TR) of all goats decreased during the second trial period, even in the goats on ration 1 which was not changed in any way between trials I and II. This finding is difficult to understand and indicates that more extensive studies on the dietary effects of calcium and phosphorus on the kinetic parameters of the readily exchangeable calcium pool are needed. These are planned in the proposal for 1970-1971 with the addition of new metabolic units.

Additional studies employing goats have been conducted during the past year but all of the data have not yet been analyzed. Briefly, three more female goats have been thyropara thyroidectomized and ^{47}Ca kinetic measurements have been made on these animals. Preliminary inspection of the data indicates, in agreement with earlier findings on two similarly operated animals, that removal of the parathyroids has little effect on the kinetic parameters of the RECaP although blood calcium and phosphorus concentrations are indeed affected in time.

The effect of subcutaneously injected 2 mg of 17 B-estradiol per day for five days of the ^{47}Ca kinetic parameters is being being studied in six female goats neither pregnant nor lactating. No statements can be made yet in comparing the behavior of the kinetic parameters of goats in two pre-treatment experiments and two post-treatment experiments with the three treatment experiments, each animal serving as its own control.

Table II

<u>Ration</u>	<u>Metabolic Trial</u>	<u>No. of Goats</u>	<u>Mean Calcium Balance (gm/day)</u>	<u>Mean Phosphorus Balance (gm/day)</u>	<u>No. of Goats</u>	<u>Mean TR of RECaP in gm Ca/hr</u>
1	I	9	-0.5	+0.9*	18	.154
1	II	3	-0.4	+0.4	6	.123
2	II	3	-0.6	+0.9	6	.124
3	III	3	+6.3	+0.8	6	.113

Ration 1 contained 1.20% Ca and 0.72% P by analysis.

Ration 2 contained 0.45% Ca and 0.68% P by analysis.

Ration 3 contained 2.70% Ca and 0.66% P by analysis.

*Only data from 8 animals included.

C. 17 B-Estradiol Radioimmunoassay

Our laboratory is investigating certain factors which may be important in precipitating parturient paresis in dairy cows. Some of the more recent literature, namely Bach and Messervy (Vet. Rec. 84:210, 1969), Payne (Vet. Rec. 76:1275, 1964) and Stott (Fed. Proc. 27:156, 1968), has indicated that estrogens may play an important role in the etiology of parturient hypocalcemia. Therefore, it was considered of value to measure the plasma estrogen concentration during gestation and parturition and to correlate these values with the changes in the plasma calcium and phosphorus concentrations. Until recently it had been practically impossible to perform such a task since the examination of the changes in estrogen concentration during pregnancy required large amounts of plasma. However, an estrogen radioimmunoassay method has now, been developed by Abraham (J. Clin. Invest. 29:866, 1969). This method requires only two to five ml. of plasma to analyze the estrogen concentrations.

This technique was considered to be satisfactory for measuring estrogen concentrations during gestation and, thus, a two phase program to perfect this technique was set up in our laboratory.

The first phase was to perfect a technique such that we could obtain a reproducible standard curve.

However, an allotment of anti-estradiol serum was received earlier from the National Institutes of Health and we then elected to use the technique of G. Mikhail, C. H. Wu, M. Ferin and R. L. Vande Wiele (J. Clin-Endocrinol, 1969). This technique involved the use of polymerized antibodies. We attempted to polymerize our antibodies using ethyl chloroformate, but had very little success

with this method. Later where we contacted Dr. Vande Wiele's Laboratory, we learned that other investigators were having the same problems. The laboratory of Dr. Vande Wiele had abandoned the technique and reverted back to the method of Abraham. As a result of this failure we had nearly depleted our allotment of anti-estradiol serum and had to write directly to Dr. Guy Abraham who graciously sent us more antibody and a copy of his procedure. Upon receiving Dr. Abraham's antibodies we set up a dilution curve to determine where our maximum binding would occur. We found as shown in figure 1 that maximum binding (65%) occurred at a 1/2,500 dilution instead of the 1/10,000 dilution recommended by Dr. Abraham. Using this dilution we found that we got an excellent reproducible standard curve. An example of a typical standard curve obtained by this method is shown in figure 2.

The method of Abraham is fairly simple but time consuming and is as follows: Antibodies are coated to polystyrene test tubes by means of a barbitol coating solution. After 18 hours this coating solution is removed and a phosphate buffer is added to the tubes. The tubes are then stored at 4° C until needed. When performing the assay, varying amounts of estradiol are added along with a constant amount of ^3H -estradiol (double labeled). The tubes are then incubated at 4° C for 6 hours. The solution is then counted. This gives the amount of free estrogen. Seven different concentrations of estrogen are used, ranging from 10-400 picograms. Each tube is counted for 10 min. on a Packard Tri-Carb and plotted as CPM vs. cold standard concentration as shown in figure 2.

With the success of phase one we were ready to proceed to the second phase of our program, extraction and separation of the estrogens from the blood plasma of pregnant cows. The reason for this procedure was that Abraham (1969) had shown that there was some cross reaction between estradiol-17 B, estrone, and estriol. Steroids tested other than C-18 showed very little cross reaction with these antibodies.

In his procedure Abraham recommended the use of ether extraction to remove the estrogens from the plasma and of thin-layer chromatography to separate the estrogens. We felt, however, that we could get just as good a separation of the estrogens using a sephadex LH-20 column. This method of separation was sent to us by Dr. Vande Wiele: "glass columns 300 x 10 mm filled with teflon stopcocks and removable stoppered reservoirs were used. Three grams of sephadex LH-20 were suspended in a mixture of benzene: methanol 85:15 and transferred to the column. The same benzene: methanol mixture was used throughout the procedure for transfer of extracts, elution of columns and preparation of sample aliquots. Each column was usually eluted with 20-30 ml. solvent between sample applications." The chromatographic behavior of a mixture of estradiol and estrone was tested and the results of a typical column are illustrated in figure 3.

With this extraction and separation procedure we found that our water blanks had exceptionally high values even when all the glassware used was washed in an HCl bath. Through trial and error, however, we think we were able to determine the cause. It seems to be in our ether extractions. For some unknown reason when we extract our water blanks with ether we obtain

estrogen values which vary from 10-200pg. These values seem to be lower when fresh ether is used and may result from some of the peroxides which are formed in ether.

This problem is now being investigated. Once this problem can be solved we will be able to measure biological samples. With this procedure we have found that we can recover between 70-90% of H^3 -estradiol extracted from water and we have hopes of measuring many of the stored plasma sample obtained from pregnant cows during the next year.

d. Preliminary Studies on Effects of Estrogens on Calcium Metabolism in Rats

These studies were undertaken to investigate the possibility that estrogen influences calcium metabolism at several anatomic or physiologic loci in the mammalian body. Rats were chosen for these preliminary studies because they are available in larger numbers more inexpensively than goats or even cows. If feasible, it is hoped that some of these experiments can be repeated using ruminants since effects in these animals of interest may differ from the observed effects in the monogastric rats.

Intestinal calcium transfer is considered to be influenced mainly by vitamin D and parathyroid hormone. However, few studies have examined the effect of estrogen on calcium transfer. The purpose of this experiment was to do this by using an in vivo gut sac technique. The major finding was that 17 B-estradiol at either dose level used (3 or 30 μg) resulted in a stimulated ^{47}Ca and ^{85}Sr transfer in the females only and the increase was up to the level observed in males, which did not respond to the estrogen treatment. A manuscript based in this study appears as COO-1339-45.

A second preliminary study was designed to determine if non-radioactive estrogen priming for four days had an effect on radioactive estrogen uptake by bone, namely the femur. A dose level of 0.1 μg . of 17 B-estradiol was administered in 0.2 ml. of ethanol subcutaneously to 5 rats and the uptake of ^3H -2, 4, 6, 7 - 17 B-estradiol at four hours post-injection was compared to the uptake in 5 non-treated rats injected with the labeled estrogen. From an earlier study the four-hour uptake was found to be maximal in bone, a so-called "non-target" tissue of estrogen. In order to check on our newly devised

method of separation of the radio-label from the femur of two other groups rats, five in each, one treated with cold estrogen and the other not, were injected with ¹⁴C-proline. It is hoped, after further corroborative evidence that the new method can be submitted as a manuscript for publication.

The summarized data are as follows (also see COO-1339-46 for an abstract of this work);

<u>Group</u>	<u>Number of Femurs</u>	<u>Mean DPM/gm Fresh Tissue</u>
Treated	10	6021.5
Control	10	2881.5

The difference between the groups was only significant at the 5% level of confidence because of large variations, especially in the treated group which was primed with estrogen. The results indicate that circulating estrogen is influencing bone tissue so that it is more sensitive to additional estrogen being presented to it. It is hoped to compare these findings to those from uptake studies in pregnant animals near full term when peripheral estrogen concentrations are surging to high levels is binding sufficient amounts of the hormone so that some cellular antagonism to parathyroid hormone results and concomitantly hypocalcemia ensues. Further experiments are obviously needed to test this hypothesis vigorously.

e. Retention of Strontium in Swine

Construction of a new facility for holding swine at the whole-body counter is nearly completed and the extension of these studies should be completed within the next nine months.

2. TECHNICAL REPORTS PREPARED FOR PUBLICATION

- COO-1339-44 ^{47}Ca Turnover of a Readily Exchangeable Calcium Pool in Pregnant and Lactating Cows. J. J. B. Anderson and W. C. Crackel. Abstract to be Presented at the Franklin C. McLean Commemorative Workshop Conference on Cellular Mechanisms for Calcium Transfer and Homeostasis, September 1970.
- COO-1339-45 Intestinal Absorption of ^{47}Ca and ^{85}Sr in Estradiol Treated Rats. R. E. Borchard and J. J. B. Anderson.
- COO-1339-46 Estrogen Uptake by Rat Bone. J. J. B. Anderson and J. M. Floeckher. Abstract to be Presented at Annual Meeting of American Society of Zoologist, December, 1970.
- COO-1339-47 Summary and Conclusions. A Comparative Study of Calcium and Strontium Metabolism in the Pack Rat (Neotoma albigula) and White Rat (Rattus albus). Ph.D. Thesis, University of Illinois, 1970 Gaylord E. Shaw.
- COO-1339-48 Distribution of Injected ^{85}Sr in the Pig. J. Boomgaardt, L. L. Krusemark, J. J. B. Anderson and B. G. Harmon. Abstract to be Presented at Winter Meeting of American Society of Animal Science.

3. REPRINTS OF PUBLICATIONS

"None"

4. OTHER ACTIVITIES

- a. Parturient Hypocalcemia, edited by J. J. B. Anderson, was published early in 1970. Plans are being made to hold a second conference on the subject in 1972, tentatively to be held at the Institute for Research on Animal Diseases, Compton, England.
- b. The Thesis work of Arthur Baeder, a graduate student is being directed by J. J. B. Anderson. Baeder is working on the estrogen radioimmunoassay.
- c. J. J. B. Anderson is writing two chapters on physiological topics which are to be included in a textbook entitled Human Ecology, edited by Norman D. Levine of the College of Veterinary Medicine.

Dilution Curve for Abrahams' antibodies

Batch #A-1 July 7, 1970

10 min. maximum binding - 1/2500 65.0%

FIG. 2

Fig. 3