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	TITLE Toxin Product Metabolism and Response in Laboratory and Domestic Animals

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FISSION PRODUCT METABOLISM AND RESPONSE
IN LABORATORY AND DOMESTIC ANIMALS
and
PLANNING STUDY FOR EVALUATION OF RADIOACTIVE
CONTAMINATION OF THE FOOD CHAIN

MASTER

Progress Report

January 1, 1967 - December 31, 1967

Submitted by:

C. L. Comar
and Staff

This document is
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I. GENERAL SUMMARY

The overall program supported by this contract with the Division of Biology and Medicine can be summarized briefly as a co-ordinated research effort to obtain fundamental information that will be helpful in understanding and coping with problems of environmental radiocontamination; in addition, basic data are obtained that are generally useful in the biological and health sciences. An important strength of the present program is the competence of the staff in subject areas that include agricultural production and distribution, food technology, dietary habits and nutrition, mineral metabolism, physiology, radiation biology, and computer technology. We feel strongly that in order to be able to focus on critical experimental studies and to be able to carry them out, it is necessary to have an understanding or at least an accurate recognition of all links in the chain of events between the release of radioactivity to the environment and finally the harm that may be caused to human beings.

For reasons of administrative convenience the work is considered in three major categories: Phase I, Fission Product Metabolism and Response in Laboratory and Domestic Animals; Phase II, Planning Study for Evaluation of Radioactive Contamination of the Food Chain; Phase III, Neural and Behavioral Response to Radiation. It is emphasized, however, that all studies are closely integrated and in operation little distinction can usually be made between the work in the various phases; there has always been a flow of ideas among all staff members about which problems are likely to be important, what sort of information is most needed, and how best to obtain it.

It is not the intention nor is it possible in this statement to summarize all the progress that has been made. Such information is available from scanning the list of recent and pending publications and from reading the abstracts of the experimental studies themselves. Rather, some comments are offered on the broad accomplishments particularly in view of the direction of future work.

As the culmination of a great deal of experimental work in our own laboratories and elsewhere and from understanding of metabolic processes and their kinetics, reliable models have been developed for prediction of the total projected intakes of I^{131} , Cs^{137} and Sr^{90} by human consumers of milk from knowledge of levels of these radionuclides in milk at known times after a contaminating event. These models are most useful for practical monitoring assessments after an event has occurred and for determining the time-dependent effects of such remedial measures as removing the cows from contaminating pasture or stopping the consumption of contaminated milk. The total projected intakes can be converted to estimated radiation doses to the population by the usual calculations, however, in some instances the effects of age of the individual and time pattern of exposure may be complicating factors. It is also necessary to increase the reliability of the link-up between the release of the radioactivity and the levels that are predicted to be found in milk.

Interest has continued in methods to reduce the transfer to milk of chronically ingested Sr^{90} . The best treatment is that which causes the maximum reduction in the value of OR milk/diet $\div$ % dietary calcium. Individual experiments in various species have indicated that reductions can be obtained by: (1) high dietary calcium (2) high dietary phosphorus (3) medium to high dietary magnesium (4) alginate (5) phospho-gel preparations. Studies are planned on the additivity of these factors in the dairy cow to determine the maximum reductions of Sr^{90} in milk that are practicable.

In studies directly related to the food chain, major progress has been along three lines: (a) use of metabolic integrating samples for evaluation of dietary contamination (b) role of non-milk foods as contributors of I^{131} to the population and (c) the link-up of meteorological distribution of radioactivity to plant and soil contamination. Carefully controlled studies with individuals in various groups, arranged by school authorities and local physicians, are leading to more reliable methods for estimating dietary contamination from measurements of pooled urine samples. Preliminary studies have shown empirical relationships between climatological factors and the radionuclide contents of milk that appear interesting enough to warrant further studies, which are being proposed.

The fundamental studies of alkaline earth metabolism have centered upon the properties and role of a substance discovered in our laboratories called the Calcium Binding Protein (CaPB). Attention was given mainly to: (a) demonstration of CaPB in various species, (b) improved assay and purification procedures, (c) determination of physical and chemical properties (d) correlation of CaPB with efficiency of calcium absorption.

A relatively new area of work just started and to be emphasized is the effect of Sr^{90} on reproductive function and embryological development. It is hoped that a systematic approach and the use of recently developed techniques of in vitro embryo culture will permit resolution of some of the controversial literature.

Intensive studies are being started on retention patterns of radiostrontium in the dog skeleton particularly in relation to known sites of tumor formation. The experiments are planned on the basis of tumor experience, knowledge of Sr kinetics, and the use of special whole body counting, scanning, and autoradiographic techniques.

The application of atomic energy techniques to problems of animal health has continued. The following have been studied: (a) diagnosis of pancreatic dysfunction, (b) improvement of thyroid function tests, (c) hemokinetics in equine infectious anemia, (d) ferro-kinetics in hookworm infection.

Careful consideration has been given to studies of the neural and behavioral response to radiation. Although there is no general agreement on the mechanism by means of which rats can detect X-radiation or in regard to the nature and time course of induction of aversion by radiation it is strongly felt that we are in position to clarify certain aspects of this important area. Evidence to date on the basis of ozone

masking, interchangeability of ozone and X-ray as conditional stimuli, and effects of water vapor levels, support the idea that X-ray detection is by way of a gaseous odorant rather than by direct action in the sensory cell. In regard to the study of radiation-induced gustatory avoidance behavior a most useful technique has been developed which offers considerable advantages over previous methods. It has now become possible to offer and to simultaneously monitor the response of an animal to a flavor cue during and subsequent to radiation exposure. Thus one can watch avoidance develop almost minute by minute and can also test the strength of avoidance.

Other aspects of the program during the past year include: (a) completion of building remodifications to provide more laboratory space, (b) cooperation on and off campus on problems such as body composition of women, leukemia induction in calves, and on-line computer applications, (c) increasing activities in summer research and graduate student research; the theses of the latter contribute greatly to the research output and therefore titles have been listed in the next section on Publications.

II. PUBLICATION LIST

This section lists the titles of papers appearing during the year 1967 and papers in press at the end of this period. These papers present the details of studies that have either been completed or reached a state that publication in the open literature was meaningful.

A. Papers Appearing During 1967

Early Developments in the Use of Radioisotopes in Agriculture.

Comar, C. L.

Isotopes and Radiation Technology 4, 53-58 (1966).

General Principles of the Distribution and Movement of Artificial Fallout through the Biosphere to Man.

Comar, C. L. and F. W. Lengemann

International Symposium on Radio-ecological Concentration Processes, Stockholm, April (1966).

Predicting the Total Intake of Radioiodine of Humans Consuming Goats Milk.

Lengemann, F. W. and R. A. Wentworth

Health Physics 12, 1655-1659 (1966).

Effect of Various Substances on Secretion of Radiostrontium into Milk.

Comar, C. L., R. H. Wasserman, A. R. Twardock and F. W. Lengemann
Health Physics 12, 1661-1669 (1966).

Natural Radioactivity in the Biosphere and Foodstuffs.

Comar, C. L.

In, "Toxicants Occurring Naturally in Foods."

National Academy of Sciences Publication 1354 (1966).

Laboratory Training Manual on the Use of Isotopes and Radiation in Animal Research.

Technical Report Series No. 60

Joint FAO/IAEA, Vienna (1966)

Estimation of Dietary Strontium-90 by Urinary Assay. Applied and Theoretical Aspects.

Thompson, Jr., J. C. and C. L. Comar

Health Physics 13, 5-13 (1967).

Terminal Properties of a Vibro-Tactile Sensor.

Lindblom, U. and D. N. Tapper

Exptl. Neurol. 17, 1-15 (1967).

A Theory of Integumental Penetration.

Buerger, A. A.

J. Theoret. Biol. 14, 66-73 (1967).

In vivo Effects of Canine Lymphosarcoma Deoxyribonucleic Acid.

Prier, J. E., J. Roszel, P. H. Craig and D. F. Cain

Am. J. Vet. Res. 28, 183-189 (1967).

Erythrocyte Loss and Restitution in Ovine Haemonchosis. Estimation of Erythrocyte Loss in Lambs Following Natural Exposure.

Georgi, J. R. and J. H. Whitlock
Cornell Veterinarian 57, 43-53 (1967).

Dietary ^{90}Sr Reductions through Food Substitutions in the Fruit and Vegetable Category.

Thompson, Jr., J. C., R. R. Alexander and C. L. Comar
J. Nutrition 91, 375-382 (1967).

Phosphate (in Animal Nutrition and Metabolism).

Wasserman, R. H.
In, "The Encyclopedia of Biochemistry," R. H. Williams and E. M. Lansford, Eds., Reinhold Publishing Corp., New York, (1967).

Vitamin D -Induced Calcium-Binding Protein: Partial Purification, Electrophoretic Visualization, and Tissue Distribution.

Taylor, A. N. and R. H. Wasserman
Arch. Biochem. Biophys. 119, 536-540 (1967)

Predicting the Total Projected Intake of Radioiodine from Milk by Man: Modification of the Original Equation.

Lengemann, F. W.
Health Physics 13, 521-522 (1967).

The Relationship between Hematocrit, Erythrocyte Volume, and Weight in Sheep.

Whitlock, J. H., R. A. Menzies and J. R. Georgi
Cornell Veterinarian 57: 276-291 (1967).

Absorption of Certain Iron Compounds by Sheep, Dogs and Rats.

Djurdjevic, D. and J. R. Georgi
Am. J. Vet. Res. 28, 787-791 (1967).

Vitamin D-Induced Calcium Binding Factor in Rat Intestinal Mucosa.

Kallfelz, F. A., A. N. Taylor and R. H. Wasserman
Proc. Soc. Exptl. Biol. Med. 125, 54-58 (1967).

Some Principles of Strontium Metabolism: Implications, Applications Limitations.

Comar, C. L.
In, "Strontium Metabolism," Eds., J. M. Lenihan, J. F. Loutit, and J. H. Martin, Academic Press, New York, (1967).

Reconsideration of the ^{131}I Iodine Contribution from Fruits and Vegetables.

Thompson, Jr., J. C.
Health Physics 13, 883-887 (1967).

Demand-Supply Relationships for Veterinarians. 1960-1980.

Thompson, Jr., J. C.
JAVMA 151, 1033-1040 (1967)

B. Papers in Press

Comparison of Radiation-Induced Chromatid Aberrations in the Testes and Bone Marrow of Chinese Hamster.

Brooks, A. L. and F. W. Lengemann
Rad. Res.

Predicting the $^{137}\text{Cesium}$ Intake from Milk of a Human Population after a Single, Short-term Deposition of the Radionuclide.

Lengemann, F. W., R. A. Wentworth and F. L. Hiltz
Health Physics

Comparison of ^{131}I Intake from Milk and Non-Milk Foods.

Thompson, Jr., J. C.
Health Physics

Intestinal Calcium Absorption, Vitamin D and a Mucosal Calcium-Binding Protein.

Wasserman, R. H., A. N. Taylor, R. A. Corradino and F. A. Kallfelz
Symposium of the Fifth European Calcification Conference, (G. M. Milhaud, Ed.).

Osteochondritis Dissecans in the Dog.

Craig, P. H.
In, "Kirk's Current Veterinary Therapy."

Some Aspects of the Physiology of Intestinal Absorption.

Wasserman, R. H.
Symposium on Gastrointestinal Radiation Injury.

Membrane Transport of Nutrients.

Wasserman, R. H.
In, "Metabolism"

Escape of the Extensor Monosynaptic Reflex from Presynaptic Inhibition.

DeCandia, M., E. L. Gasteiger and M. Mann
Brain Research

Spinal Electrogram of the Cat I. Study of Origin by Degeneration and Ischemia.

Brust-Carmona, H., H. Levitan, H. Kasprzak and E. L. Gasteiger
Electroencephalography and Clin. Neurophysiol.

Spinal Electrogram of the Cat II. Super-Spinal Influences.

Brust-Carmona, H., H. Levitan, H. Kasprzak and E. L. Gasteiger
Electroencephalography and Clin. Neurophysiol.

Puerperal Tetany.

Kallfelz, F. A.
In, "Kirk's Current Veterinary Therapy."

Radioactivity in Foods.

Comar, C. L. and J. C. Thompson, Jr.
In, "Modern Nutrition in Health and Disease."

Radiation Biology Text.

Casarett, A. P.
Prentice-Hall, New Jersey

Characterization of Electrical Activity of the Spinal Cord by
Pattern Recognition Techniques.

Rothmeier, J. J. and E. L. Gasteiger

Symposium on Data Acquisition and Processing in Biology and
Medicine.

The Physiology of Bone

Wasserman, R. H.

In, "Physiology of Domestic Animals," Ed., H. H. Dukes

Erythrocyte Loss and Restitution in Ovine Haemonchosis. III. Re-
lation between Erythrocyte Volume and Erythrocyte Loss and Related
Phenotype Displays.

J. H. Whitlock and J. R. Georgi

Cornell Veterinarian

Strontium Retention in Dogs as a Function of Age.

Anderson, J. J. B. and C. L. Comar

Radiation Research

C. Published Abstracts of Papers Presented at Meetings

Use of a Specific Skin Mechanoreceptor in Classical Conditioning.

Tapper, D. N. and R. Van Scoy

Fed. Proc. Vol. 26, no. 2 (March-April 1967).

Cinematographic Animation of Brain Structures.

Gasteiger, E. L., D. Moltzau and J. Leukowicz

Fed. Proc. Vol. 26, no. (March-April 1967).

Adrenalectomy and Bone Calcium Kinetics in Rachitic and Vitamin D-
Treated Rats.

Kallfelz, F. A. and R. H. Wasserman

Fed. Proc. Vol. 26, no. 2 (March-April 1967).

Development and Extinction of Radiation-Induced Saccharin-Avoidance
Behavior.

Tapper, D. N. and R. Van Scoy

Rad. Res. Vol. 31, no. 3 (July 1967).

Behavioral Evidence that Ozone Mediates X-ray Detection.

Helling, S. A. and E. L. Gasteiger

Rad. Res. Vol. 31, no. 3 (July 1967)

Radiosensitivity of Neonatal Rats.

Casarett, A. P. and T. R. Noonan

Rad. Res. Vol. 31, no. 3 (July 1967)

Actinomycin D Inhibition of a Vitamin D -Induced Calcium-Binding
Protein (CaBP) in Chick Duodenal Mucosa³.

Corradino, R. A. and R. H. Wasserman

The Physiologist Vol. 10, no. 3 (August 1967)

Purification and some Properties of a Vitamin D₃ -Induced Intestinal
Calcium Binding Protein.

Wasserman, R. H. and R. A. Corradino

The Physiologist Vol. 10, no. 3 (August 1967)

D. Published Theses

This section lists theses published as a result of research conducted under the support of the Atomic Energy Commission. It is here included for the first time and contains titles of all theses published on subject matter relevant to this contract since the formation of the department.

Tissue Dosimetry Measurement of a Beta- and Gamma-Emitting Radio-nuclide (Cs-137).

Nold, Max Marcell, submitted as Ph.D. thesis, 1960.

Diagnosis of Thyroid Function in the Dog.

Lombardi, Max Habib, submitted as M.S. thesis, 1961.

Absorption and Transport Rate Studies in the Rat Alimentary Tract Using Liquid and Solid Food Labeled with Calcium-45, Strontium-85, Barium-133, and Yttrium-91.

Marcus, Carol Silber, submitted as M.S. thesis, 1961.

Absorption and Fetal Metabolism of Cesium-137 in the Rat.

Moore, Wellington, Jr., submitted as Ph.D. thesis, 1961.

Calcium and Strontium Secretion by the Lactating Goat.

Twardock, Arthur Robert, submitted as Ph.D. thesis, 1961.

Renal Excretion of Calcium and Strontium.

Georgi, Jay Robert, submitted as Ph.D. thesis, 1962.

The Effects of Lactose on Cellular Metabolism and its Relation to Calcium Absorption from the Ileum of the Rat.

Ingersoll, Ronald John, submitted as M.S. thesis, 1964.

Characteristics of the Rat Gastrointestinal Mucosa during the Absorption of Alkaline Earths.

Marcus, Carol Silber, submitted as Ph.D. thesis, 1964.

The Distribution of Sodium and other Ions in the Mammalian Kidney and its Relationship to the Counter-Current Hypothesis for Urine Concentration as Studied by Autoradiography.

Mercer, Paul Frederick, submitted as Ph.D. thesis, 1964.

Contributions to Multi-Tracer Assay Techniques using Gamma Ray Spectra.

Talisayon, Serafin Demandante, submitted as M.S. thesis, 1966.

Strontium Retention as a Function of Age in the Dog.

Anderson, John Joseph Baxter, submitted as Ph.D. thesis, 1966.

Kinetics of Thyroid and Mammary Gland Iodide Transport in the Guinea Pig.

Corradino, Robert Anthony, submitted as Ph.D. thesis, 1966.

Autoradiographic Studies of DNA Synthesis and Alkaline Earth
Incorporation in Nuclear Material of Human White Blood Cells.
Herzog, Rita Kremer, submitted as Ph.D. thesis, 1966.

Effects of Adrenalectomy on Vitamin D Function and Calcium
Metabolism in Rachitic Rats.
Kallfelz, Francis Anthony, submitted as Ph.D. thesis, 1966.

Induction and Repair of Chromatid Aberrations in the Testes and
Bone Marrow of the Chinese Hamster.
Brooks, Antone Leavitt, submitted as Ph.D. thesis, 1967.

Aspects of Strontium and Calcium Metabolism.
Ebel, Joseph George, submitted as Ph.D. thesis, 1967.

III. EXPERIMENTAL STUDIES

This section emphasizes those phases of our research program that have progressed sufficiently to provide information of interest. None of this work has yet appeared in the formal literature although many of the items are ready for submission for editorial review. Most of the work is presented in sufficient detail to provide an understanding of the experiments as carried out. In a few instances, however, especially where the work represents a graduate thesis it was not possible from a space standpoint to present the details; the interested reader can obtain copies of such theses if he desires. A complete picture of the research program is best gained by consideration of the publication list and the attached reprints as well as the items reported in this section.

A. Phase I - Fission Product Metabolism and Response in Laboratory and Domestic Animals.

The studies under Phase I have been primarily concerned with:

1. Effects of Strontium-90 and Yttrium-90 on Embryological Development.
2. Strontium Retention in Various Parts of the Dog Skeleton.
3. Reduction of Radiostrontium in Cow's Milk with Sodium Alginate.
4. The Calcium-Binding Protein and Alkaline Earth Absorption.
 - I. Vitamin D₃ Induction of Calcium-Binding Protein in the Uterus of the Laying Hen.
 - II. Purification and Some Properties of Vitamin D-Dependent Calcium-Binding Protein.
 - III. The Effect of Certain Physiological and Nutritional Variables on the Concentration of Vitamin D-dependent Calcium-Binding Protein in Chick Duodenal Mucosa.
5. Studied on the Secretion of Iodide Into the Milk of Thyroidectomized Goats.
6. Effect of Age on the Retention of Cesium by Rats.
7. The Retention of Cesium as Affected by Weaning.
8. Estimation of Body Composition and Cs¹³⁷ Body Burdens in College Women Using the Whole Body Counting Technique.
9. Intestinal Absorption of Oleic Acid I^{131} and Triolein I^{131} in the Differential Diagnosis of Malabsorption Syndrome and Pancreatic Dysfunction in the Dog.

10. Comparison of Resin Sponge Uptake of Triiodothyronine - I^{131} from Serum with other Commonly used Thyroid Function Tests as Indication of Thyroid Dysfunction in the Dog.
11. Blood Fe^{59} and Cr^{51} Kinetics in Normal Horses for Comparison with Horses Infected with Equine Infectious Anemia.
12. Comparison of Radiation-Induced Chromatid Aberrations in the Testes and Bone Marrow of Chinese Hamster.
13. Estimation of Erythrocyte Loss in Hookworm Infection by Whole-Body Fe^{59} Counting.

1. Effects of Strontium-90 and Yttrium-90 on Embryological Development.

Alison Casarett and Roger Coomes

Abstract: There are a few reports in the literature suggesting that relatively low doses of ^{90}Sr produce marked effects on reproductive function and embryological development. However, there has not been enough work to permit unequivocal conclusions. This study is an attempt to systematically investigate the effects of Strontium-90 (and daughter yttrium-90) on early embryological development using improved techniques and sufficient numbers to clarify the question. This is a new line of work in the program. So far there has been concentration on the development of some of the needed techniques for incorporation of the radio-nuclides into sperm and for in vitro embryo culture.

I. Introduction

Ionizing radiations have been shown, repeatedly, to produce very marked effects on embryos in terms of lethality and in the production of abnormalities (e.g. Russell & Russell (1954), Rugh (1962)). Most reports have been concerned, however, with changes in litter size or morphological observations on developing embryos or fetuses removed from the mother at particular stages of gestation. There are no reports of repeated observations on individual embryos, using techniques such as in vitro embryo culture. Many of the reproductive studies have utilized x-or gamma radiations, rather than internal emitters. This study will be concerned primarily with the effects of strontium-90 (and yttrium-90) on development of rat embryos.

Strontium-90 is an important constituent of radioactive fallout produced during detonation of thermonuclear devices. It ranks as one of the most important biologically hazardous isotopes due to its long physical half life of 28 years and its relatively long biological half life in mammals. Strontium-90 decays by emission of a spectrum of beta particles, with a maximum energy of 0.545 Mev, to its daughter nuclide, yttrium-90. ^{90}Y has a relatively short physical half-life of 64.2 hours and decays to zirconium-90 primarily by emission of an energetic beta particle with 2.26 Mev maximum energy. When considering the biological activity of ^{90}Sr , therefore, one must also consider the activity of the ^{90}Y .

The metabolism of strontium, a bone-seeking radionuclide, is similar to that of calcium. It is not unlikely, however, that radiostrontium can produce effects on reproductive organs and developing germ cells either by irradiation of the reproductive organs by strontium incorporated in nearby bone or by irradiation from small accumulations of the radionuclide in gonads. Slight transient peripheral testicular accumulation of strontium was reported in mice (Nilsson & Ullberg, 1962) within minutes after intraperitoneal injection of $0.67\mu\text{Ci}$ of ^{90}Sr per gram of body weight. Quantitative histologic examination of testes revealed damage equivalent

to about 25 rads of x-rays. Other authors (Aberg & Giltner, 1966) have injected rams with ^{89}Sr (10 and 31.5 mCi) and have shown that ^{89}Sr in ram blood is taken up by spermatozoa. Maximum uptake was found 3 to 7 days after administration with a second peak at day 20 which could be associated with testicular uptake.

Abnormal chromosomes have been described in mouse embryos sired by strontium injected males (Henricson and Nilsson (1964)) and Luning and co-workers (1963a, 1963b) reported an increase of 3 to 4% in lethality among embryos sired by male mice given $18\mu\text{Ci } ^{90}\text{Sr}$ as compared with saline injected animals. This peak increase occurred in matings made 3 weeks after injection, and was presumably associated with labeling of post-meiotic spermatogenic cells. Matings from the 10th to 15th weeks after injection (associated with spermatogonial uptake of ^{90}Sr) resulted in 1.7% more intrauterine deaths than saline injected controls.

Dosimetry measurements by Brues et al. (1963) have suggested that radiation from ^{90}Sr deposited in bone (and from the ^{90}Y daughter) and from ^{90}Sr leaked into the scrotal sac may contribute a considerable dose to the testes. Although studies have indicated no major translocation of ^{90}Y from bone after its formation from ^{90}Sr , the movement of small amounts might be important in the production of gonadal effects. It is apparent that additional quantitative studies of radiation effects are needed to accurately assess the effects of strontium-90 and yttrium-90 on embryological development.

II. Experimental:

Studies have been initiated on the effects of strontium-90 on reproductive function in rodents. It is intended, first, to study by in vitro culture the early (1 to 4 day) rat embryos produced from sperm containing radionuclides incorporated in vivo or in vitro. These studies will then be compared with others involving a quantitative evaluation of histologic damage of spermatogenic and oogenic cells following exposure to radionuclides. Calculated exposures will be supplemented by quantitative dosimetry - autoradiography and tissue counts (scintillation or G-M) as appropriate.

At a later time, developing embryos will also be studied following irradiation of embryonic stages from 2 cell to blastocyst by external sources or by internally-introduced radionuclides.

Strontium uptake by sperm in vitro

A technique has been developed for studying the incorporation of radionuclides into sperm in vitro. Male rats (300-400 grams) are sacrificed with ether and the reproductive tracts removed. Sperm from both vasa deferentia or from the caudal portion of one epididymis ($1-4 \times 10^7$ sperm) are expressed by manual pressure into a vial containing 10 ml of Locke's solution. To this is added 10 ml of a solution

containing $2\mu\text{Ci}$ per ml of a radionuclide in 0.9% NaCl. Radionuclides used to date are ^{89}Sr , ^{90}Sr - ^{90}Y , ^{59}Fe (ferric) and ^{45}Ca . The system is maintained at 37.5°C .

One ml samples are removed at intervals from 1 minute to 2 hours after introduction of the radionuclide solution and are passed through a Millipore filter (0.45μ pore size) used with a pyrex microanalysis filter holder. The filter paper and sperm are rinsed with Locke's solution in which, for the strontium isotopes, there has been an equimolar replacement of strontium chloride for calcium chloride. The filters are counted with an end-window G-M tube, previously calibrated for the radionuclide in use. Sperm counts are made from the radionuclide-containing solution, on a standard blood hemocytometer.

Peak uptake in the ^{90}Sr series (0.76 dpm per sperm at time of filtration) was reached between 10 and 30 minutes after the start of incubation and remained relatively constant for the 2 hour sampling period. By half-life determinations and Feather analysis it was demonstrated that ^{90}Y accounted for 99.6% of the observed radioactivity.

Activity from incubation with ^{89}Sr was lower than from the ^{90}Sr experiments (.013 dpm per sperm). It could be demonstrated that 68% of this activity was associated with ^{90}Y from the ^{90}Sr contamination (approximately 1%) in the ^{89}Sr solution. Uptake studies with ^{45}Ca resulted in counts only about twice background and ^{59}Fe was not taken up by the sperm to a detectable degree.

A series of experiments measured the removal of activity from sperm. Sperm were incubated with ^{90}Sr - ^{90}Y solutions for 30 minutes, as described above, and passed through a millipore filter. The system was then flushed continuously for times up to 5 minutes with either distilled water, Locke's solution or strontium-Locke's solution. It was determined that less than 10% of the activity was removed by washing with Locke's or strontium-Locke's solution and less than 25% was removed by distilled water.

This technique is considered suitable as an in vitro method for incorporating ^{90}Y into sperm. It is anticipated that the sperm can be used for artificial insemination to determine effects of incorporated radionuclides on embryologic development.

Strontium uptake by sperm in vivo

Preliminary experiments have been started to measure the uptake of intraperitoneally injected equilibrium mixtures of ^{90}Sr - ^{90}Y by rat sperm from the epididymides or ductus deferens or by testicular cells. The uptake by either epididymis or ductus deferens containing maturing sperm is not significantly greater than background at time intervals from 1 to 42 days after I.P. injection of $10\mu\text{Ci}$ of ^{90}Sr into 300 gram rats. Testicular uptake in the same animals is low, but significant at the earliest time periods ($1.2 \times 10^{-2}\%$ of injected dose per testis at 24 hours). Cellular distribution of the radionuclides has not yet been determined.

Tissues have also been assayed 1 to 42 days after intraperitoneal injection of rats with a ^{90}Sr - ^{90}Y equilibrium mixture at a level of $100\mu\text{Ci } ^{90}\text{Sr}$ per animal. Maximum values were obtained at 1 day ($1.0 \times 10^{-3}\%$ injected dose per 1" segment of ductus deferens; $3.1 \times 10^{-3}\%$ injected dose per head or tail of single epididymis; $1.1 \times 10^{-2}\%$ injected dose per testis). By 72 hours after injection the radionuclide concentration was reduced to approximately half of the above values. Further studies will be started soon to confirm these results, to quantify the radionuclide uptake at shorter time periods to determine cellular and intracellular localization and to determine the relative uptake of the strontium and yttrium isotopes. Thermoluminescent dosimetry will be used to estimate testis and sperm doses from the injected radionuclides.

Rat ovaculture

Numerous reports of successful ovaculture have been made for the 2-cell to blastocyst stages of mouse and rabbit embryos (Review: Austin (1961), New (1966)). Very little or limited success in culturing rat ova or pre-implantation embryos has been reported. It is hoped, however, that a technique can be established using a modification of the method described by Brinster (1963). Embryos will then be cultured which have been sired by males injected with ^{90}Sr - ^{90}Y or which have resulted from ova inseminated by sperm containing ^{90}Y . The development of these embryos will be followed and abnormalities, cleavage delay, etc. will be recorded. It is anticipated that such information will further clarify the response of the preimplantation embryo to radiation by supplementing existing reports on preimplantation anomalies, and helping to explain the decreased litter size which has been reported.

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2. Strontium Retention in Various Parts of the Dog Skeleton

P. H. Craig

Abstract: Body burdens of bone seeking radionuclides are associated with the formation of tumors of the skull in both animals and man. Cancers of the mastoid (man) and external ear canal (rabbits) suggest that such nuclides may localize in ear bones preferentially. A pilot study has been started, using dogs, to determine the extent to which ear bones can incorporate radiostrontium rapidly and release it slowly, as compared to other bones. Such behavior has been reported in rabbits, and a critical factor appears to be the age at time of exposure to the nuclide. Techniques have been worked out for whole body counting, autoradiography, and bone specimen analyses in connection with special problems that arise in this type of study.

I. Introduction

It is well known that sarcomas have arisen at a high frequency level in the radium dial-painter population. It is less widely appreciated that epithelial tumors, usually carcinomas, are prone to occur in the skull in this group of people. Similar epithelial lesions have been seen in dogs and rodents given other bone-seeking radionuclides, including ^{90}Sr , ^{32}P , and ^{239}Pu . More recently, Vaughn has observed squamous cell carcinoma of the ear canals of rabbits given 500 μCi or more of ^{90}Sr neonatally.

Such evidence suggests the hypothesis that tumors in the cephalic region are the result of specific anatomic localization of a nuclide at high concentrations. It is further hypothesized that the localization is primarily a function of exposure in relation to age. More specifically, it may be that the petrous temporal bone of the skull forms early during the development and growth of an individual, and remodels only very slowly thereafter. If this is the case, contact with a bone-seeking nuclide during formative stages would result in a very high incorporation rate. Subsequent slow remodeling would allow unusually long persistence of the incorporated material. The following experiments are designed to examine the validity of the hypothesis, and to establish the "critical incorporation time" of the petrous temporal bone.

II. Experimental

Design:

Littermate groups (4 dogs per group) are given injections of ^{85}Sr (NO_3)₂ intramuscularly, 75 μCi per dog, according to the following array

Bone Collection and Autoradiography

At the age of 7 months, all dogs are killed, and the skeletons are collected and dissected free of most soft tissue. The bones are frozen and slab specimens of standardized thickness (1/8") and orientation are removed. The slabs are counted for ^{85}Sr content as a general guide for autoradiographic exposure. Autoradiographs are prepared by the contact method with type AA industrial X-ray film (Eastman Kodak).

1. Head

- a. Posterior ventral midline, including basal synchondroses. (R & L).
- b. Transverse section of frontal region, including nasal turbinates, palatine region, facial bones, and maxillary teeth with supportive structures.
- c. Two transverse sections of each petrous temporal bone including the bony otic capsule, the osseous bulla, and the adjacent squamous portion of the temporal bone (R & L).

2. Humerus

- a. Proximal end, including epiphysis, growth plate, metaphysis and proximal diaphysis (R & L).

3. Tibia

- a & b as in humerus (R & L).

4. Femur

a & b as in humerus & tibia (R & L).

c. Distal end, including epiphysis, growth plate, metaphysis and distal diaphysis. (R & L).

5. Sternum -- longitudinal section of sternbrae.

6. Costochondral junction (10th rib), (R & L).

7. Vertebrae

a. Longitudinal sections (R & L) of C_{III}, T_{III}, L_{III}, and coccygeals.

Thereafter, individual small pieces of bone are sawed from the slab specimens, extracted with alkali, dried, weighed, and counted with standards for ⁸⁵Sr content (ccpm/gm bone).

III. Whole Body Counting

All dogs injected with ⁸⁵Sr are initially counted 3 times weekly to observe the change in their body-burdens of the nuclide.

The animals are restrained in a box, placed in a geometrically reproducible position, and counted for 1 minute in a whole body counter (Los Alamos type). A "phantom," containing approximately half the injected dose, is counted as well. Counting data are corrected for coincidence, background, and are finally expressed as corrected counts per minute per phantom. The latter data are plotted against time on log-log scales. Short portions of the curve permit linear regression of log transforms of the data, and calculation of slope.

Rationale of Method

It is the purpose of the present investigation to establish the outlines of incorporation and removal of a bone-seeking radionuclide (⁸⁵Sr) in the petrous temporal bone, versus age, in comparison with other anatomic sites. It is well known that uptake rates in bone vary greatly from place to place within a given bone. Generally speaking metaphyses and regions of cortical apposition exhibit the greatest immediate incorporation. As growth continues, the "hotspots" are removed by remodeling and the distribution of the nuclide changes radically. It is a further purpose of the investigation to establish quantitative relationships between carefully chosen anatomic structures, as a basis for the further understanding of mineral kinetics in bone.

In order to make appropriate choices of individual bone specimens for analysis, oversight autoradiographs are used to provide a gross picture of nuclide distribution within the sample. On this basis

it is possible to select small segments of bone which are representative of anatomically definable structures and are reasonably homogeneous with respect to nuclide distribution. The final expression of the data will be in terms of the fraction of administered dose adjusted for body weight. The quantity represents the summation of exchange, incorporation, and resorption which occur between injection and death. It permits not only valid comparisons between individuals and groups, but also allows evaluation of rational values for pairs of anatomic sites. The latter, when available in sufficient numbers, should greatly extend quantitative predictive capabilities for nuclide localization.

Results

Groups initiated (12/15/67)

Two dogs, one male and one female, were given 75 μCi ^{85}Sr (NO_3)₂ each at approximately 7 months of age, and killed 4 days later. Specimens therefrom were processed first in order to clarify and establish technical details. The results allow quantitation of acute incorporation rates for this age group.

One group of 5 dogs (IV) was injected at 5 months of age with 75 μCi ^{85}Sr (NO_3)₂ each, and killed at 7 months of age.

Group II animals (4) were injected at two months of age. Three of the dogs died from a variety of causes, including an outbreak of distemper. One animal remains and is scheduled for sacrifice on 10 January 67. Specimens were collected from all dogs which died and provide some of the interim information which follows.

Seven puppies were injected at one month of age (group I) with 75 μCi of ^{85}Sr . Six dogs remain, and are being counted weekly in the Cisco whole-body counter. Three healthy puppies have been very recently injected with 75 μCi at two months of age (Group II).

Autoradiographic Observations

- A. Dogs A & B (injected at 7 months of age with Strontium-85, killed four or five days p.i.). All bones were diffusely labelled, and conspicuous accumulations were present in the areas of enchondral growth. These occurred in the metaphyses of long bones, vertebrae, and rib. Midshaft sections were labelled as well, primarily on the external surfaces. The petrous temporal bones were essentially unlabelled.
- B. Group 4 (injected at 4 months, killed 110 days p.i.). The epiphyses of all long bones were diffusely but definitely labelled. The metaphyses, areas of most recent bone formation, contained a very low level of strontium. Midshafts were heavily labelled; the radionuclide was buried deeply in the cortical bone in most instances. All petrous temporal bones were labelled in a spotty or mottled fashion. Generally speaking, the adjacent segments of the squamous temporal bones were more heavily labelled than the petrous.

- C. Dog L-5 (injected at 2 months, died 51 days p.i.). The long bones and vertebrae presented patterns which were essentially similar to those observed in group 4. However, the petrous temporal bone, especially the otic capsule, were quite heavily labelled.
- D. Dog F-13 (injected at 1 month, died 16 days p.i.). All portions of all bones were labelled, with particular concentrations in regions of enchondral ossification. There was a very striking uptake of the nuclide by the petrous temporal bone.

In summary, the specimen autoradiographs demonstrated expected qualitative patterns of uptake in a variety of well-known anatomic sites. In the petrous temporal bone, acute uptake rates were very high at the age of one month; they were, by contrast, very low at the age of seven months.

Bone Specimen Counts

Final quantitative data for dissected portions of slab specimens of bone are not yet available. As a general indication of progress, crystal scintillation counts of a selected group of bone slabs are arrayed in Table 1. The data have been expressed as a proportion of the original dose at the time the animal was killed, and correlate in a very striking fashion with autoradiographic observations. Very high incorporation rates in bones of the ear are seen in F-13 and L-5, dogs injected at one and two months of age. It is very clear that dissected specimens counts, corrected for bone mass and body size, will provide even more striking differences.

Body Burden Determinations

Whole body counts, related to counting standards and normalized to permit slope comparisons, are presented in Figure 1. Standard deviations (+) are depicted for randomly selected values by vertical brackets.

The relatively large standard deviations associated with the one-month dogs are an indicator of great individual variation in excretion rates in this group.

The more rapid loss of nuclide associated with active growth and turnover in one-month dogs is an expected result. A flatter excretion curve in the Group II than in IV, however, is surprising. It is quite possible that the intercurrent disease (characterized by diarrhea, vomiting, fever and dehydration) that eventually decimated Group II interfered with both growth and remodeling, so as to increase retention of the nuclide. On the other hand, such phenomena should be accompanied by discontinuities of slope, and no such breaks are obvious.

Table 1. Relative fraction of administered dose of ^{86}Sr per slab specimen of bone, for dogs injected at specific ages.

Dog	F-13	L-5	3	4	5	6	7	A	B
Age at injection (months)	1	2	4	4	4	4	4	7	7
Sacrifice (days post-injection)	16	51	110	110	110	110	110	4	5
R. Distal femur	1.0	.63	.46	.64	.58	.50	.51	1.1	.77
R. Prox. Tibia	1.0	.78	.84	.62	.51	.43	.53	1.5	1.2
R. Femoral shaft	.46	.33	.22	.15	.17	.24	.23	---	.13
R. Ear (1)	1.9	1.2	.36	.30	.35	.40	.28	.31	.24
R. Ear (2)	2.2	1.1	.42	.39	.36	.37	.32	.34	.18

Fig. 1

⁸⁵Sr Body Burdens

vs Time

Dogs injected at Age

□ 1 month

△ 2 months

● 4 months

± 1.0 S. D.

Data Normalized to

$$R_{10 \text{ days}} = 1.0$$

3. Reduction of Radiostrontium in Cow's Milk with Sodium Alginate

R. A. Wentworth, C. L. Comar and J. J. Anderson

Abstract: It is known that sodium alginate reduces the gastrointestinal absorption of strontium compared to calcium in human beings and rats. The preliminary studies reported here indicate that about a two-fold reduction in $OR_{\text{milk/diet}}$ can be accomplished by feeding these materials to lactating cows. The physical properties of the alginates are important as far as practical feeding practice is concerned. Other factors to be considered include the acid composition and simultaneous use of other conditions that are known to reduce the $OR_{\text{milk/diet}}$ values.

The feeding of sodium alginate has been shown effective in reducing absorption and retention of radiostrontium in rodents and humans (1-7). In rats the reduction in strontium uptake is accomplished with only minor alterations in calcium metabolism and thus making it a potentially useful product from a radiological health standpoint.

Sodium alginate is a hydrophilic colloidal polysaccharide derived from certain marine algae of the class Phaeopyceae (brown algae). Sodium alginate is commercially used in food products, pharmaceutical and cosmetic preparations, and in industrial processes and products. Its use in food products has been as an emulsifying, thickening, suspending and/or stabilizing agent and is often included in such products as baby foods, syrups and sauces, and in many dessert-type foods.

This study was intended to determine whether sodium alginate might be useful for reducing radiostrontium transfer to milk.

Initial Cow Experiments

Two lactating dairy cows were given gelatine capsules containing ^{45}Ca and ^{85}Sr twice daily for periods of 30 to 37 days. During the first ten days of the experiments the cows were fed a control ration consisting of hay and a commercial daily grain mixture. During the period from day 10 to 20 the cows were fed a supplement of purified sodium alginate (Fisher). The sodium alginate was mixed with the grain at a rate equal to 10% of the total feed intake of the cows during the initial control period of the experiment. Following day 20, the cows were again fed the control ration without supplementation. The cows were milked twice daily and the milk analyzed for radionuclide content.

As the following data indicate, the addition of sodium alginate to the ration tended to reduce the radiostrontium concentration in milk and the $OR_{\text{milk/ration}}$, however the magnitude of the reductions was not

consistent. Also the level of ^{85}Sr observed following the cessation of the feeding of sodium alginate did not return to the level seen in the pre-alginate control period.

	Treatment Period*		
	Control (Pre-alginate)	10% Alginate	Control (Post-alginate)
^{85}Sr (% Daily dose/l. milk)			
Cow 168	0.121	0.076	0.087
Cow 164	0.098	0.088	0.095
$\text{OR}_{\text{milk/ration}}$ **			
Cow 168	0.099	0.065	0.074

* Values are averages during the last 4 days of each period.

$$** \text{OR}_{\text{milk/ration}} = \frac{{}^{85}\text{Sr}/{}^{45}\text{Ca in milk}}{{}^{85}\text{Sr}/{}^{45}\text{Ca in ration (dose)}}$$

The data obtained may in part be explained by properties of the sodium alginate employed. The product fed the cows in this experiment produced a very viscous solution when mixed with water. It is thought that the production of a highly viscous mass in the rumen may have caused the depression in appetite observed in the cows. While palatability of the grain mixed with sodium alginate was fair when first fed to the cows, as the experiments progressed the cows would eat only a fraction of the grain-alginate mixture offered to them.

Low Viscosity Alginates

In light of the difficulties in getting cattle to eat sodium alginate it was decided to investigate products with a lower viscosity. An experiment was carried out with rats to find an acceptable product.

Rat Experiment

Two commercially available sodium alginate products were investigated: "Kelgin LV" (low viscosity) and Kelgin XL" (extra-low viscosity), both obtained from the Kelco Company, Clark, N. J. The manufacturer lists viscosities for a 3% solution in cps. @ 25°C as 3,000 for Kelgin LV and 650 for Kelgin XL. These products were mixed with a ground laboratory ration in amounts equal to 10% by weight.

Twenty-four rats were divided into three groups of eight rats each. One group was fed the control ration while the other groups received one of the sodium alginate supplemented grain mixtures. Following a 2-day preliminary period in which the respective diets were fed, ^{45}Ca and ^{85}Sr were added to the drinking water of each rat. After receiving the radioactivity for 9 days the rats were killed and femurs removed for radio-nuclide assay.

The following data were obtained:

Treatment	<u>H₂O Consumed</u> (ml./day)	<u>Feed Consumed</u> (grams/day)	<u>⁴⁵Ca</u> (% dose/ bone)	<u>⁸⁵Sr</u> (% dose/ bone)	<u>OR_{bone}/dose</u>
Control	16.4	10.8	1.67	0.42	0.25
Kelgin LV	22.0	10.8	1.77	0.25	0.14
Kelgin XL	19.2	10.1	1.71	0.23	0.13
Standard Error	0.65	0.29	0.05	0.017	0.006

Statistical analysis revealed no significant differences among feed intakes but water consumption was significantly greater ($P < .01$) in the rats receiving the low viscosity sodium alginate than in rats in either of the other groups, and significantly greater ($P < .01$) in rats receiving the extra low viscosity sodium alginate than in the rats on the control ration. The ⁴⁵Ca content of femurs of the rats did not differ significantly among treatment groups. The ⁸⁵Sr content of femurs and the OR_{bone}/diet were significantly lower ($P < .01$) in the animals receiving either form of sodium alginate than those observed in the control group. Although the animals fed the extra low viscosity sodium alginate retained slightly less ⁸⁵Sr and had a lower OR_{bone}/diet than the rats which received the low viscosity product, the difference was not statistically significant.

Current Bovine Experiments

To date one experiment has been completed in which a cow was fed "Kelgin XL" (extra low viscosity sodium alginate). Calcium-45 and ⁸⁵Sr were fed twice daily for 32 days. During the first 10 days a control ration of alfalfa-grass hay was fed plus a commercial grain mixture. From day 11 through day 18 the ration of the cow was supplemented with 8% Kelgin XL and from day 19 through day 22 the level of sodium alginate was increased to 12% of the feed intake. Following this period the cow was again fed the control ration.

Feed consumption and milk production was readily maintained even during the feeding of 12% alginate. The cow showed no aversion to the product at the levels fed.

<u>Period</u>	<u>% Daily Dose ⁸⁵Sr/l.milk</u>	<u>% Daily Dose ⁴⁵Ca/l.milk</u>	<u>OR_{milk}/dose</u>
Control (Pre-Alginate)	.072	.79	.091
8% Alginate	.046	.80	.057
12% Alginate	.037	.75	.050
Control (Post Alginate)	.094	1.03	.092

The differences in ⁸⁵Sr levels and ⁴⁵Ca levels between the initial control period and the post-alginate control period indicate that most likely the radionuclide levels in milk had not reached a plateau level during the initial 10 day control period. This is supported by the fact that

the levels of ^{45}Ca and ^{85}Sr in the milk in the post-alginate control period leveled off at a constant value for several days before the experiment was terminated. The reductions in $\text{OR}_{\text{milk}}/\text{dose}$ compared favorably with those seen in the rat experiment previously discussed. Although further experimentation along these lines are in progress, it does appear that the low viscosity sodium alginate produces could be useful in reducing radiostrontium contamination of milk.

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4. The Calcium-Binding Protein and Alkaline Earth Absorption
R. H. Wasserman

Abstract: Vitamin D was shown to induce the formation of a calcium-binding protein in the small intestine and kidney of rachitic animals. This protein, abbreviated CaBP, may be involved in some fashion with the translocation of Ca across epithelial tissues. Considerable attention was given to several aspects of the protein, and detailed studies over this report period have led to the following: (a) demonstration of the presence of a vitamin D-dependent CaBP in the dog. (Previously this protein had been observed in intestinal mucosa of the chick, rat and monkey); (b) demonstration of the presence of a vitamin D-dependent CaBP in the uterus of the laying hen, a tissue across which large amounts of Ca moves during egg-shell formation; (c) the improvement of the mathematical treatment of CaBP assay data such that a straight line relationship now can be obtained when CaBP concentration is plotted against the degree of Ca binding; (d) the purification of CaBP from chick intestinal mucosa by a combination of procedures; including ammonium sulfate precipitation, gel filtration on Sephadex G-100 and preparative discontinuous electrophoresis; (e) the determination of some of the physical and chemical properties of CaBP, prominent among these being the determination of molecular weight by two procedures which gave values ranging from 24,000 to 28,000, and the measurement of a formation constant which yielded values of 2.6×10^5 , 3.9×10^4 and $5.9 \times 10^3 \text{ M}^{-1}$ for Ca, Sr and Ba, respectively; (f) the correlation of the concentration of CaBP in intestinal mucosa with several physiological and nutritional variables that alter the ability of chick intestine to absorb Ca.

The following represent excerpts from manuscripts that either have been submitted for publication or are in preparation.

4. I. Vitamin D₃ Induction of Calcium-Binding Protein in the Uterus of the Laying Hen.

R. A. Corradino and R. H. Wasserman in collaboration with
M. H. Pubols and S. I. Chang of the Department of Animal
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It was shown that small intestine and kidney elaborate on calcium-binding protein (CaBP) in response to vitamin D₃ but CaBP was not detected in colon, muscle and liver (1, 2). Small intestine, kidney and bone, the latter of which has not yet been examined for CaBP, are known target organs of vitamin D₃, i.e., they respond to vitamin D₃ treatment by enhanced calcium and/or phosphorus translocation. It seemed plausible, therefore, that the uterus (shell gland) of the laying hen, which is the site of copious calcium transfer in egg-shell formation (3), might also elaborate CaBP and represent an additional site of vitamin D₃ action. We have found that, indeed, hen uterus does produce CaBP in response to vitamin D₃, while hen oviduct does not, and that the uterine protein is identical to that produced in chick duodenal mucosa.

White Leghorn pullets (4) were housed in individual cages at 18 weeks of age and fed laying mash for the next 26 weeks after which experimental treatment was begun. Four hens were fed a vitamin D₃ deficient (basal) diet (5). Two hens were fed the basal diet supplemented with vitamin D₃ (1200 I.U./kg diet). During the 4 week experimental period, the hens on the basal diet laid 6 eggs/hen whereas the hens fed supplementary vitamin D₃ laid 22 eggs/hen. After the 4 week period, 2 of the hens fed the basal diet were given a capsule containing 5000 I.U. vitamin D₃. After 72 hours, all six hens were sacrificed by cervical dislocation. The duodena and reproductive tracts were excised and immediately frozen and shipped in dry ice to Ithaca. The organs were thawed, the duodena slit and the mucosa scraped from the underlying muscle layer. All tissues were homogenized in a Virtis blender and the homogenates centrifuged at 3800 g for 20 minutes. The supernatants were heated at 60°C for 10 minutes in a constant temperature water bath; this process had been shown to reduce total protein concentration without appreciably influencing Ca⁺⁺-binding activity (1). The heat treated supernatants were then recentrifuged and the resulting supernatants reserved for analyses. The supernatants were analyzed for protein by the Lowry method (6), for calcium-binding activity by a previously described procedure (1) and subjected to acrylamide slab gel electrophoresis for visualization of CaBP (2).

Table 1 illustrates the effect of vitamin D₃, either dietary or single dose administration, on calcium binding activity of supernatants prepared from hen duodena and reproductive tracts. It is apparent that vitamin D₃ administration increased the calcium binding activity of both duodenum and uterus, but not oviduct. Acrylamide gel electrophoretic patterns of the tissue supernatants support these conclusions. It was observed that there was a protein in duodenum and uterus, but not in oviduct, which migrated identically with chick duodenal mucosal CaBP, indicating that they may be the same protein. A second protein (P₂) was present in supernatants of uteri from vitamin D₃-treated hens that was not present in the D₃-deficient hen uteri. It migrated just behind the presumed CaBP.

To determine which protein(s) possessed calcium binding activity, the uterine supernatants from hens No. 5 and 6 were concentrated on a Diaflo membrane filter and then subjected to gel filtration on Sephadex G-100 (2). Each Sephadex fraction was analyzed for protein and calcium binding activity. Fig. 1 illustrates that calcium binding activity was associated only with fractions 44 to 55. When selected fractions were electrophoresed on acrylamide gel slabs, only fractions from the range 44 to 55 contained a protein which migrated identically with chick duodenal mucosal CaBP. The second protein (P₂), which was eluted from the Sephadex column in fractions 41 to 48, did not exhibit calcium binding activity. Although CaBP was found in supernatants prepared from uteri of vitamin D₃-replete laying hens obtained in Ithaca (7), the second protein (P₂) was not. It is possible that P₂ may be a CaBP breakdown product (8).

The discovery that vitamin D₃ induces CaBP formation in the laying hen uterus is strong circumstantial evidence for a special calcium

translocation process in this organ. The observations that the efferent blood supply from the uterus contains less calcium than the blood vessels leading to the uterus during shell calcification (3) and that the uterus itself contains very little calcium in contrast to the isthmus of the oviduct (9, 10) supply additional evidence for an efficient calcium transport mechanism in the uterus. Indeed, evidence has been obtained indicating that the transport of calcium across the shell gland is a metabolic dependent mechanism (11) and proceeds against an electrochemical gradient (12).

The present observations may help clarify the results of previous experiments on the role of vitamin D₃ in egg production. It was found that vitamin D₃ deficiency resulted in a marked decrease in the number of eggs layed but there was no pronounced effect on ovary or oviduct weight (5). When vitamin D was administered to other vitamin D deficient hens, a sustained increase in number of eggs layed began within 3-4 days (13). In another experiment, a single 5000 I.U. dose of vitamin D₃ to severely vitamin D₃ deficient hens (10 months on a vitamin D₃ deficient diet) restored egg production to within 50% normal level over a five week period. Again, there was no substantial effect on oviduct weight (14). A daily dose of Estradiol Cypionate (1 mg/hen/day) for five weeks to another group of vitamin D₃ deficient hens, however, neither restored egg production nor increased oviduct weight (14). These observations suggest that the vitamin D₃ effect on egg production is not mediated through estrogen-sensitive processes. Alternatively, the present finding that vitamin D₃ induces the formation of a calcium-binding protein in the shell gland constitutes evidence (a) that vitamin D₃ has a direct action on the shell gland and (b) that the shell gland may have a special calcium-translocation mechanism upon which shell formation is dependent.

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Table 1

Effect of Vitamin D₃ on Calcium Binding Activity in Duodenum
and Reproductive Tract of Laying Hen

<u>Chicken #</u>	<u>Treatment</u>	Calcium Binding Activity ¹ (% dose ⁴⁵ Ca/mg protein)		
		<u>Duodenum</u>	<u>Oviduct</u> ²	<u>Uterus</u> ³
1	Vitamin D ₃ deficient	1.35	0.41	1.38
2	" " "	1.60	0.89	1.06
3	Vitamin D ₃ in diet	3.07	0.35	1.88
4	" " "	2.66	0.32	1.72
5	Vitamin D ₃ injection	2.83	0.34	1.82
6	" " "	2.84	0.34	2.27

¹ A tracer dose of ⁴⁵Ca is added to a tube containing supernatant and Chelex 100, a cation exchange resin. After mixing and centrifuging, the amount of ⁴⁵Ca remaining in the supernatant is a function of CaBP concentration (1).

² Oviduct refers to the entire oviduct from ovary to shell gland (uterus) including magnum and isthmus.

³ Uterus refers only to the shell gland.

Fig. 1. Sephadex G-100 fractionation of uterine supernatants prepared from hens No. 5 and 6. Solid line = protein concentration; broken line = calcium binding activity in % dose ⁴⁵Ca per ml of fraction.

Purification and Some Properties of Vitamin D-Dependent
Calcium-Binding Protein

R. H. Wasserman, R. A. Corradino and A. N. Taylor

The vitamin D-induced CaBP has been obtained in high purity, using ammonium sulfate precipitation, gel filtration and preparative disc-electrophoresis, from supernatants of homogenates from vitamin D-treated rachitic chicks. The purified material gave single protein bands on acrylamide gel at pH 8.3 (slab gel) and at pH 7.9 (analytical disc) but, at pH 8.9 (disc anal.), two bands (one dense, the other diffuses) appeared. Comparative electrophoresis, however, indicated that under all conditions the purified material was detectable only in vitamin D supernatant and not in rachitic supernatant. The separation seen at pH 8.9 could be the result of destructive effects during some phase of purification or to some change due to the electrophoresis procedure per se.

The protein concentration and Ca-binding activity of fractions from the various purification steps are shown in Table 1. The binding activity, assessed by the Chelex 100 assay method (1), is expressed both as % ^{45}Ca remaining in the supernatant and as the ratio, $\text{Ca*Pr}/\text{Ca*R}$. $\text{Ca*Pr}/\text{Ca*R}$ is the ratio of distribution of radiocalcium between binding protein and resin in the assay system. On theoretical grounds, it can be shown that the value of this ratio is a direct, linear function of protein concentration and is given by the equation:

$$\text{Pr} = \left[\frac{k_{\text{Pr}}}{k_{\text{R}}} \right] \text{R} \frac{\text{Ca*Pr}}{\text{Ca*R}} \quad (1)$$

where: Pr = unbound protein concentration, k_{Pr} and k_{R} are the formation constants of protein and resin with Ca, respectively, and $\text{Ca*Pr}/\text{Ca*R}$ = ratio of distribution of Ca* between protein and resin in the assay system. Under the conditions of the assay, the total concentration of resin(R) is constant. Therefore, all terms that are bracketed on the right side of equation 1 are constants and equation (1) can be re-written simply as:

$$\text{Pr} = (\text{K}) \frac{\text{Ca*Pr}}{\text{Ca*R}} \quad (2)$$

Several assumptions and conditions are required for equation (2) to hold under the conditions of the assay as performed. However the existence of a linear relationship between total protein concentration and $\text{Ca*Pr}/\text{Ca*R}$ was tested experimentally and, as shown in Fig. 1, the expression of binding data as $\text{Ca*Pr}/\text{Ca*R}$ yielded a straight line when plotted against relative CaBP concentration. The observed linear relationship indicated that the required assumptions and conditions were reasonably valid and that the present procedure is suitable for the quantitative assay of calcium-binding activity. When the data was expressed as "% ^{45}Ca remaining in the supernatant," the plot was not linear. Referring again to Table 1, the relative specific activity values indicate that CaBP constitutes about (1/36)th or about 2.8% of the protein originally present in the crude mucosal supernatant. Other

purification runs gave values of 2.5% and 2.6%.

The molecular weight was determined by the use of a Sephadex G-100 column calibrated with proteins of known size (bovine serum albumen, cytochrome C and α -chymotrypsin). The volume required to elute a given protein is indirectly related to the molecular weight of that protein. The elution volume of CaBP, when compared to a standard curve, indicated that it has a molecular weight of about 28,000 (Fig. 2). An estimate of the molecular weight of CaBP was also derived from high-speed sedimentation equilibrium studies (through the courtesy of Dr. N. Norcross, N.Y. State Veterinary College). Assuming a partial specific volume of 0.72, the value obtained ranged from 24,000 to 26,000 which agrees reasonably well with the values obtained by gel filtration.

The formation constant for the CaCaBP complex was determined using the ion exchange method of Schubert *et al.* (2). The method consists essentially of mixing a cation exchange resin and a solution of CaBP in the presence of a tracer dose of $^{45}\text{Ca}^{++}$ until an equilibrium distribution is achieved. At this time, aliquots of the liquid phase are taken for radio-assay in a liquid scintillation system and for protein analysis by the Lowry method. The formation constant was calculated using the following expression, after Schubert:

$$K_f = \frac{1}{K_c} = \frac{(K_d/K_d^0) - 1}{(A^b)^n} \quad (12)$$

where: K_f = formation constant (M^{-1});

K_c = dissociation constant (M);

K_d = distribution coefficients;

$$= \frac{\% \text{ dose } ^{45}\text{Ca} \text{ in resin}}{\% \text{ dose } ^{45}\text{Ca} \text{ in solution}} \times \frac{\text{volume of sol. (ml)}}{\text{mass of resin (mg)}}$$

K_d^0 = distribution coefficient in absence of CaBP;

A^b = concentration of CaBP of charge b; and

n = molar ratio of Ca^{++} : CaBP in the Ca-CaBP complex which was shown to be one in this laboratory. A similar evaluation of the formation constants of the Sr-CaBP and Ba-CaBP complexes was achieved utilizing ^{85}Sr and ^{133}Ba . The values obtained are presented in Table 2. The Ca^{++} -citrate complex formation constant is included for comparative purposes.

Amino acid analysis of two purified CaBP preparations was performed on 6 N HCl hydrolysates (110°C, 22 hrs.) with the Technicon automatic amino acid analyzer by Dr. M. Morrison, N.Y. State College of Home Economics, Ithaca. Recovery of amino acid-nitrogen in both samples was about 80-85%. The protein showed a high content of dicarboxylic amino

acids (asparatic, glutamic), which may be partially in the amide form in the native molecule, and a relatively high lysine content.

Purified CaBP was submitted to analysis for various prosthetic groups. These results are summarized in Table 3. It should be noted that the degree of confidence regarding the possible presence of non-protein groups was limited by the amount of protein available for analysis.

Table 1

Purification of Vitamin D-Induced Ca-Binding Protein

Fraction	Protein concentration	Chelex-100 assay	Ca ⁴⁵ binding activity	Specific activity	Relative specific activity
	(mg/ml)	(% super- natant Ca ⁴⁵)	$\left(\frac{\text{Ca*Pr}}{\text{Ca*R}}\right)$	$\left(\frac{\text{Ca*Pr}}{\text{Ca*R}} / \text{mg. protein}\right)$	
Crude	11.00	42.1	0.701	.064	1.00
(NH ₄) ₂ SO ₄	0.555	13.4	0.129	.232	3.63
Gel filtration	0.105	11.6	0.106	1.010	15.78
Prep disc electrophoresis	0.060	14.1	0.139	2.317	36.20
Tris buffer	—	2.5	—	—	—

Figure 1. Relationship of relation CaBP concentration to binding activity by the Chelex assay. The binding data are expressed in two ways, as the ratio of Ca^* associated with protein to that associated with resin (Ca^*Pr/Ca^*R) and as the % Ca^{45} remaining in the supernatant (see text).

Fig. 2. Molecular weight of CaBP as determined by gel filtration (Sephadex G-100). The column was calibrated with known proteins (bovine serum albumin, cytochrome C, α -chymotrypsin and the ratio of elution volume (V) to void volume (V_0) was plotted against the log of the molecular weight. The molecular weight of CaBP was determined by comparing its measured V/V_0 ratio against this curve.

Table 2

Formation Constants, K_f , for CaBP Complexations
of Calcium, Strontium, and Barium ¹

<u>Cation</u>	<u>K_f</u> (M^{-1})	<u>log K_f</u>
Ca^{++}	2.6×10^5	5.4
Sr^{++}	3.9×10^4	4.6
Ba^{++}	5.8×10^3	3.8
Ca citrate	1.66×10^3	3.22 ²

¹. Determined by procedure of Schubert et al (2) at
25° C, pH 7.2 and $\mu = 0.16$

². Ca citrate values from Hastings, McLean et al (3).

Table 3

Prosthetic group analysis*

Lipid - none detectable by direct analysis;
sediments in KBr at sp.gr. 1.21.

Carbohydrate - less than 1% by anthrone procedure.

Phosphate - less than 0.2%.

Sialic acid - less than 1%.

*Limited by amount of pure CaBP available.

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The Effect of Certain Physiological and Nutritional
Variables on the Concentration of Vitamin D-dependent
Calcium-Binding Protein in Chick Duodenal Mucosa.

R. H. Wasserman and A. N. Taylor

General Methods: The experimental animals, variously treated as described below, were killed by decapitation. The duodenum was removed, cooled, everted and the mucosal layer separated from the underlying muscle layers by the use of a glass slide. The handling of the tissue and the homogenization procedure were as previously described (1). In all of these studies the supernatant fluid was heated at 60°C for 10 min., a procedure which previously was shown to remove about two-thirds of the inactive protein without appreciably influencing the calcium-binding activity of the supernatant. The calcium-binding activity was assessed by the Chelex-100 assay procedure (1) except for one study on the alteration of supernatant binding capacity with time. This was done by an earlier employed method (2) in which the homogenate debris served as the phase competing with the supernatant binding factor for added radiocalcium. In several of the studies, the effect of the variables on the protein pattern of the supernatant fluid determined by slab-gel acrylamide electrophoresis that is described in detail elsewhere (3).

The cockerels, pullets and hens were of the White Leghorn breed. The cockerels were obtained from Babcock Hatchery, Ithaca, and the older birds from the Department of Poultry Science, Cornell University.

The calcium-binding data are expressed as the % ^{45}Ca retained in the supernatant fluid or as the ratio, $\text{Ca}^*\text{P}/\text{Ca}^*\text{R}$, described above.

Normal diet: Day old cockerels were raised on a commercially available starter mash to the age of 4 weeks. At 72 hours before experiment, four chicks were given 500 I.U. vitamin D_3 per os in vegetable oil and the same number, vegetable oil only. For comparative purposes the Ca-binding activity and gel patterns of supernatants from rachitic and vitamin D_3 -treated chicks of the same age and same treatment were determined.

The acrylamide gel patterns showed that the duodenal supernatant from chicks raised on a vitamin D adequate diet contained the CaBP protein band. This indicated that the formation of the protein is not an artifactual consequence of the rachitic state subsequently modified by treatment with vitamin D. A comparison of the intensities of the stained bands on the gel further showed that the concentration of CaBP in the normal animal appeared to be less than that in the vitamin D_3 -repleted chick. The Ca^{45} binding activities of these two groups indicated the same trend (Table 1) and, further, that vitamin D_3 administration to the normal animal did not enhance radiocalcium binding. A possible explanation for this "over-shoot" phenomenon could be that another factor is involved in CaBP synthesis in addition to vitamin D, a factor which may originate endogenously and determine the quantity of CaBP formed.

Time pattern of disappearance of calcium-binding activity: Previously it was reported that the appearance of CaBP after vitamin D administration to rachitic chicks closely correlated with the remission of the absorptive defect (1). In the present study, 4 week-old rachitic chicks were given a single oral dose of vitamin D₃ (500 I.U.) and replaced on the rachitogenic diet. The calcium-binding activity of the mucosal supernatants and the duodenal absorption of ⁴⁷Ca of separate groups were followed over the period from 5 to 10 days after vitamin D administration. The data, depicted in Fig.1, showed that the calcium-binding activity of the supernatant fluid decreased at an exponential rate similar to the decrease in the ability of the duodenum to absorb ⁴⁷Ca as vitamin D deficiency redeveloped. The half-time of the absorption curve was 3.0 days and that of the binding curve was 2.7 days. Further, if the respective responses are compared at the 5 and 10 hr. periods, it seems apparent that the ability of the chick to absorb calcium is directly related, qualitatively, to the amount of CaBP present in the duodenum at those times.

Dietary calcium intake: Day-old chicks were raised on a diet containing 1.2% Ca and 0.7% P for eleven days. The chicks were then divided into two groups, one maintained on the normal Ca diet and the other on the same diet less the CaCO₃ supplement. The Ca content of the latter diet was about 0.1%. At 4 and 6 days, the absorption of ⁴⁷Ca by an isolated duodenal loop in vivo was determined and the Ca-binding activity of the heat-treated supernatant fluids assessed by the Chelex-100 procedure. In a similarly designed but separate experiment, the electrophoretic patterns of 30 µl aliquots of the supernatants of individual chicks were determined.

At both time periods, the duodenal absorption of Ca⁴⁷ by the low calcium groups was significantly greater than that of the high calcium groups (Table 2). Concomitant with this was the observation that the calcium-binding capacity of the duodenal supernatant fluid was similarly increased. When the binding data was expressed as the ratio, (Ca*P/Ca*R) per mg. protein, it was calculated that there was a similar factorial increase in absorptive capacity and Ca-binding activity; at 4 days, for example, ⁴⁷Ca absorption and Ca-binding were enhanced by factors of 1.4 and 1.5, respectively.

The electrophoretic patterns of the mucosal supernatants showed clearly that the intensity of the CaBP band, was considerably greater in those chicks receiving a low calcium diet in contrast to those maintained on the normal calcium intake.

Age: Comparison of three-week old pullets with mature non-laying hens showed that duodenal supernatant of the former had about one-half the concentration of Ca-binding activity of the older animals (Table 3). The rapidly growing immature fowl requires considerably more dietary calcium than the mature individual and this coincides with their respective duodenal calcium-binding capacity.

Sex: No significant differences were noted in the calcium-binding activity of the supernatants of homogenates of duodena from 3-week old pullets and cockerels (Table 3).

Egg-laying versus non-laying hens: During the egg-laying cycle, the requirement for ingested calcium increases in order to provide sufficient mineral for the shell (4). As shown in Table 3, the binding activity of the duodenal supernatant of the layer was about three times greater than in the non-layers of about the same age. There was thus an increase in intestinal CaBP in response to the physiological need for calcium. The direct stimulus to the protein-synthesizing mechanism of the duodenal cell could be due to hormones associated with egg-laying, prominent of which is estrogen. The results of the experiments described below indicate that this hormone is not involved in CaBP formation.

Estrogen effects: Since the administration of estrogen to cockerels was shown by others to increase plasma calcium and protein levels (5), the question as to whether the greater binding capacity of supernatants from egg-laying hens in contrast to non-layers was due to this hormone could be approached. In this study, rachitic chicks were given either diethylstilbesterol or vitamin D₃ or both simultaneously (cf. footnote of Table 4 for dosages and protocol). At 48 hours, the absorption of Ca⁴⁷ by the ligated duodenum in vivo and the calcium-binding activity by the supernatant fluids of duodenal homogenates were measured.

As expected from the paper cited above, the injection of diethylstilbesterol alone into cockerels was shown to increase both plasma Ca and plasma protein concentrations (Table 4). The simultaneous administration of vitamin D and diethylstilbesterol increased plasma calcium to a greater extent than when estrogen was given alone, whereas plasma protein concentration were not significantly influenced. Vitamin D, in the absence or presence of estrogen, enhances radiocalcium absorption from the ligated duodenum of the rachitic chicks; estrogen alone had no significant effect. As may be also seen in Table 4, Vitamin D₃ greatly increased the calcium-binding capacity of the duodenal supernatant; estrogen only slightly influenced Ca-binding activity and this may be due to the contamination of Group 4 mucosa with plasma having a higher binding affinity for calcium than that of Grp. 1. The acrylamide gel pattern readily revealed the presence of the CaBP band in those groups given vitamin D; however, this band was undetectable in the groups not given vitamin D, including Grp. 4 in which only estrogen was administered.

From these studies with the cockerels, it is suggested that differences in estrogen output by laying and non-laying hens cannot account for the difference in CaBP content of the duodenal mucosa of these birds. It is suggested that another factor is elaborated that influences the degree of CaBP formation when vitamin D is not limiting.

Comparison of effect of vitamin D₂ and vitamin D₃: Rachitic chicks aged three weeks were given a single dose of either vitamin D (5000 I.U.) or vitamin D₃ (500 I.U.) or vitamin-free carrier alone (Cotton seed oil). About ten times as much vitamin D₂ than vitamin D₃ was administered because, in the chick, the D₂ form has considerably less biological activity. It was observed that the binding activity of the supernatants from the duodenum of the vitamin D₃-treated chicks was 1.53 times greater than that of the vitamin D₂ chicks. This difference was reflected in the acrylamide gel pattern. ²Using CaBP formation as

a criteria of biological effectiveness, it appears that under the conditions of the present experiment, vitamin D₃ has more than ten times the potency of vitamin D₂ in the chick. Bosman and Chen (6) clearly point out that the potency ratio of vitamin D₃: vitamin D₂ in this species is highly dependent upon the absolute quantities of ² vitamin administered and the parameter observed.

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Table 1

Calcium-binding Activity of Mucosal Supernatants of Chicks Raised on Either a Normal or Rachitogenic Diet With or Without Vitamin D₃ Administration.

<u>Diet¹</u>	<u>Vitamin D₃²</u>	⁴⁵ Ca in Supernatant Fluid (%/ml)	Protein Concentration (mg/ml)	Specific Activity (%Ca ⁴⁵ / mg protein)
Normal	—	29.3 ± 1.8	6.2 ± 0.3	4.7
Normal	+	29.0 ± 0.6	6.8 ± 0.8	4.3
Rachitogenic	—	11.2 ± 0.4	5.6 ± 0.3	2.0
Rachitogenic	+	47.4 ± 1.1	5.9 ± 0.1	8.0

Normal diet = chick starter mash. 500 I.U. vitamin D₃ given orally to those groups so designated. Values represent the mean of four determinations. (±) = standard error of the mean.

Figure 1. Effect of redevelopment of vitamin D-deficiency on the binding capacity of supernatants of mucosal homogenates and the absorption of ^{47}Ca by duodenum chicks given a single dose of vitamin D₃. The binding activity was determined by the earlier ³method of Wasserman and Taylor (2) in which the homogenate debris served as the competitive accumulator of ^{45}Ca instead of resin. The absorptive capacity of the ligated duodenum was as previously published (7). One ml of a solution containing 1 mg Ca (as the chloride), 0.19 M NaCl and tracer ^{47}Ca was injected into a tied-off loop of duodenum. At 30 min., the duodenum was excised and counted for ^{47}Ca radioactivity with a gamma scintillation detector and gamma spectrometer set to eliminate the contribution from the ^{47}Sc daughter. The dose was taken as 100%, and the % absorption of ^{47}Ca calculated by difference.

Table 2

Effect of Calcium Intake on Duodenal Absorption of Ca^{47} and Ca-Binding Activity of the Duodenal Supernatant

Ca Intake (%Ca)	Duodenal Absorption of Ca^{47} %	Supernatant Ca-Binding Activity ¹			
		% Ca^{45} in supernatant	$\frac{\text{Ca*P}^2}{\text{Ca*R}}$	Protein Concentration (mg/ml)	Specific Activity $\left(\frac{\text{Ca*P}}{\text{Ca*R}}\right)$ / mg protein
Low (0.1%)	56.9 $\pm$ 3.9 (6)	27.4 $\pm$ 1.4 (4)	0.329 $\pm$ 0.037	4.49 $\pm$ 0.38	0.075 $\pm$ 0.011
Normal (1.2%)	77.7 $\pm$ 3.3 (6)	41.0 $\pm$ 1.4 (3)	0.646 $\pm$ 0.047	5.63 $\pm$ 0.30	0.115 $\pm$ 0.008
Low (0.1%)	60.5 $\pm$ 3.8 (6)	34.3 $\pm$ 1.6 (4)	0.474 $\pm$.037	5.73 $\pm$ 0.13	0.083 $\pm$ 0.007
Normal (1.2%)	86.9 $\pm$ 2.6 (6)	41.3 $\pm$ 1.7 (4)	0.652 $\pm$.044	5.57 $\pm$ 0.08	0.117 $\pm$ 0.008

¹ Values represent mean $\pm$ standard error of the mean. Animals per group given in parenthesis.

² (Ca*P/Ca*R) = ratio of distribution of radiocalcium between protein and resin.

Table 3

Effect of Age, Sex and Egg-laying on Calcium-Binding Activity of Supernatant Fluids of Duodenal Homogenates¹

<u>Age</u>	<u>Sex</u>	<u>Egg-laying status</u>	Ca ⁴⁵ in supernatant fluid ² (% Ca ⁴⁵ /mg protein)
3 weeks	Male	-	5.36 $\pm$ 0.23 (4)
3 weeks	Female	-	4.86 $\pm$ 0.45 (5)
Mature	Female	-	2.50 $\pm$ 0.34 (5)
Mature	Female	+	7.35 $\pm$ 1.19 (4)

¹ These birds were obtained from the Dept. of Poultry Science, Cornell University, immediately prior to use and were raised on commercial poultry rations adequate in vitamin D₃.

² Values represent mean $\pm$ standard error of the mean. Animal per group given in paranthesis.

Table 4

Effect of Diethylstilbesterol and Vitamin D₃ on Plasma Ca and Protein Levels, Duodenal Absorption of ⁴⁷Ca, and on the Ca-Binding Activity of Supernatants of Duodenal Homogenates¹

Grp.	Treatment Vit. D ₃	Estrogen	Plasma		Duodenal ⁴⁷ Ca Absorption (%)	Supernatant Ca-Binding Activity		
			Ca (mg/100 ml)	Protein (g/100 gm)		⁴⁵ Ca in Supernatant Fluid (%/ml)	Supernatant Protein (mg/ml)	Specific Activity (% ⁴⁵ Ca/mg protein)
1	-	-	7.1	3.2	10.3 ± 1.1	4.8 ± 0.5	2.9	1.66
2	+	-	11.2	3.1	71.4 ± 3.3	20.4 ± 0.9	2.7	7.56
3	+	+	26.1	3.8	72.4 ± 3.8	18.0 ± 1.5	2.6	6.92
4	-	+	18.4	4.3	12.9 ± 2.5	7.0 ± 0.2	3.2	2.19

¹ Rachitic chicks at 3 weeks of age were divided into 4 groups, 6 chicks per group. Group 1 given single doses of 0.2 ml propylene glycol intramuscularly and subcutaneously; Grp. 2, 500 I.U. vitamin D₃ in propylene glycol; Grp. 3, 500 I.U. vitamin D₃ in propylene glycol i.m., 9 mg. diethylstilbesterol in propylene glycol; Grp. 4, 9 mg diethylstilbesterol in propylene glycol. Experiment performed at 48 hours after dosing. Values represent mean ± standard error of the mean.

5. Study^s on the Secretion of Iodide Into the Milk of
Thyroidectomized Goats
 F. W. Lengemann

Abstract: L-thyroxine administered to normal lactating cows decreases milk I-131 concentration by decreasing the milk-plasma iodide ratio. To determine if the effect of the thyroxine is directly on mammary tissue or indirectly through its effect upon thyroid stimulating hormone output by the pituitary, a study was carried out using thyroidectomized lactating goats given various combinations of L-thyroxine and/or thyroid stimulating hormone. The results indicated that the withdrawal of thyroid stimulating hormone could lower the milk-plasma iodide ratio.

I. Introduction

In the past an observation was made in this laboratory that administration of L-thyroxine to normal cows decreased the milk I-131 concentration by decreasing the milk-plasma iodide ratio. This effect on the concentration mechanism of the mammary gland could have been due to the direct action of high levels of thyroxine or to the decreased release of thyroid stimulating hormone (TSH) from the pituitary. The data did not permit a decision.

II. Methods

In order to test whether the effect was due to thyroxine or TSH, goats were thyroidectomized using high levels of I-131. The success of the ablation was determined by thyroid uptake tests using tracer doses of I-131 and by observing milk yields after the ablation procedure. When ablation was successful milk yields would hold steady for a period and then decline rapidly. This rapid decline could be reversed by the daily I.V. administration of 1 to 2 mg of L-thyroxine. In the first study the two goats were given 2 mg of L-thyroxine and 2 units of T.S.H. (Nutritional Biochemicals, Inc.) daily for about 10 days before the experiment began and also received twice daily oral doses of I-125.

III. Results and Discussion

Table 1

Effect of L-Thyroxine and Thyrotropic Stimulating Hormone on the Secretion of Iodide into Milk of Thyroidectomized Goats.

Period	Thyroxine (mg/day)	TSH (units/day)	Goat 1			Goat 6		
			Milk I-125 % dose/1	Milk 1	M/P	Milk I-125 % dose/1	Milk 1	M/P
1	2	2	40	0.89	6.1	134	0.28	14.9
2	2	0	20	0.89	3.8	47	0.31	7.0
3	2	5	38	0.75	3.7	141	0.12	19
4	0	5	14.6	0.34	3.1	23	0.17	4.6

From Table I it can be seen that there was a rather different response of the two animals to thyroxine. The milk production of Goat 6 did not respond markedly to thyroxine administration while that of Goat 1 did. We have no explanation for this. The general pattern of response for the other criteria was quite similar for both goats. Cessation of TSH injection reduced the milk I-125 concentration and lowered the milk/plasma ratio. When TSH injection was resumed the milk I-125 concentration returned to the previous levels. Cessation of thyroxine injection produced the same reactions as the cessation of TSH administration.

The results of the experiments are interesting since they suggest a direct effect upon an extra-thyroidal iodide concentrating mechanism. This is somewhat unexpected from the literature in this field although the latter is sparse. For this reason it seemed of value to repeat the experiment and to use a more reliable source of the thyroid stimulating hormone.

For the next experiment, Goat 1 was used again along with 2 other goats that had also been subjected to thyroid ablation using I-131. The source of the T.S.H. was the National Institutes of Health (NIH-TSH-B-3 Bovine). The experimental details are essentially the same as for Goats 1 and 6; the results are shown in Table II and each period is related on a percentage basis to the initial period when the animals were receiving only supplemental thyroxine.

Table II

The Effect of L-Thyroxine and the N.I.H. Thyrotropic Stimulating Hormone on the Secretion of Iodide in Milk of Three Thyroidectomized Goats.

<u>Period</u>	<u>Thyroxine</u> <u>mg/daily</u>	<u>T.S.H.</u> <u>units daily</u>	<u>Milk</u>		<u>Plasma</u>	
			<u>Liters</u>	<u>% I-125 dose/liter</u>	<u>%I-125 dose/liter</u>	<u>M/P</u>
1	2	0	100	100	100	100
2	2	2	93	119	116	101
3	2	0	94	70	80	89
4	2	2	64	112	95	128
5	0	2	53	87	80	109
6	2	0	61	87	96	90

When comparing the results of these experiments with that of Table I the following accord was noted:

- a. Milk production dropped when thyroxine was withdrawn and increased when thyroxine was supplied (Periods 4, 5, and 6).
- b. Cessation of T.S.H. injection reduced the milk I-125 concentration and lowered the milk/plasma ratio (Periods 2 and 3). When T.S.H. injection was resumed the milk I-125 concentration again increased.

These data support the idea that T.S.H. can affect iodide concentrating mechanisms external to the thyroid. On the other hand these results do little to help decide if high levels of thyroxine given to normal lactating animals reduce milk radioiodide by a direct effect of thyroxine or by the thyroxine acting to reduce TSH secretion by the pituitary.

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6. Effect of Age on the Retention of Cesium by Rats. F. W. Lengemann

Abstract: The retentions of repeated single doses of radiocesium were followed over the lifetimes of 30 male albino rats. These studies showed that the turnover time of Cs-134 was shortest in the youngest animals and then increased markedly with age until the animals were about 4 months old. After this time increases were still observed but were of much less magnitude. Resolution of the retention curves into 3 compartments showed that the last compartment increased markedly in size up to about 4 months of age; small increases were noted thereafter.

Introduction

A survey of the literature has shown that the retention half time of radiocesium in the human is quite variable with values ranging from 30 to more than 140 days. The shortest retention times were observed in infants and the longest in adults and suggest that age might be an important contributor to the differences in cesium retention half times seen between individuals. Since it is difficult in human beings to determine with certainty the variables that interact to produce a given result it is necessary to use experimental animals to look at specific factors separately.

Methods

In one study, we used an experimental group of 30 male albino rats (Carworth Farms, Inc.) for repeated determinations of cesium retention over their lifetime. At each selected age interval 8 rats were chosen at random from this group and given a single I.P. injection of Cs-134. The injected dose contained 0.5 μ c of Cs-134. The retention of the radiocesium was determined by counting the animals individually in a small animal counter (Tobor, Nuclear Chicago) about twice a week. The counting period extended for a minimum of 51 days with the animals then being returned to the experimental reserve group. The data were corrected for radioisotope decay and for variations in body size; and the data normalized so that all the counts at day 0 were identical.

Results and Discussion

The equation for the retention curve for the average of the rats of each time period was expressed in the exponential form:

$$\text{Retention (\% of initial dose)} = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} + a_3 e^{-k_3 t}$$

The constants are found in Table 1.

Table 1. The Retention of Cesium by Rats as a Function of Age

Age at Dosing (Mo.)	Approx. Wt. (g)	<u>1st Comp.</u>		<u>Middle Comp.</u>		<u>Last Comp.</u>	
		a_1 %	k_1 —	a_2 %	k_2 —	a_3 %	k_3 —
1	50	15.4	1.04	72.5	0.25	12.1	0.08
1.5	100	30.7	1.55	59.6	0.18	9.7	0.05
2	200	19.7	2.24	33.8	0.23	46.6	0.07
3	300	22.6	2.05	22.6	0.20	54.9	0.07
4	400	10.5	3.36	22.7	0.56	66.8	0.06
6.5	500	11.5	1.87	18.2	0.41	71.3	0.06
13	600	17.8	0.99	9.8	0.18	72.5	0.05
16	570	18.8	0.78	----	----	81.2	0.06
21	570	15.0	0.81	----	----	85.0	0.05
25	475	7.6	0.75	18.8	0.19	73.5	0.06

The initial group of 30 rats was large enough to leave sufficient survivors so that at 25 months we had sufficient rats to make an experimental group. It should be noted that for all groups except the 16 and 21 month old groups, the retention curve could be described by 3 components. In a preliminary analysis of the data there appears to be little regularity evident in the first component that can be described as a function of age. The middle component does show some tendency for the size of the compartment to decrease with time with no discernible trend in the transfer rate out of the compartment. Unfortunately, the lack of a middle component for the 16 and 21 month groups reduces the significance of these observations. The last component shows a marked trend towards an increase in the size of the compartment with age. This compartment expanded by a factor of 8 between the age of 2 months and 16 months. The biggest change between consecutive groups was observed between the 1.5 and 2 month old animals. While there is some suggestion that the rate constant became smaller with age (the residence time is longer) this is not clear cut.

The net result of the increase in size of the last compartment is to decrease the rate of turnover in the animals as they age. When the curves for the various ages are plotted on a single graph the changes with age are quite pronounced. From the equations for the curves it is apparent that the last component is the factor which is producing the change. The reason for the expansion of the last compartment is unknown as is the identity of the compartment.

7. The Retention of Cesium as Affected by Weaning
F. W. Lengemann

Abstract: In these experiments rat pups were given I.P. injections of Cs-134 at 4 days of age and the retention of the radiocesium followed. It was noted that up to the time of weaning, the pups lost half of the radiocesium every 10.9 days. After weaning the rate of loss increased markedly and became a multiexponential rate of loss rather than the single exponential loss rate during nursing. Experimental procedures definitely ruled out recycling of radiocesium through the dams and the physical nature of the diet after weaning as being contributory factors to the observed changes in cesium retention.

I. Introduction

In a preliminary study of the effect of age upon the retention of radiocesium by rats, it was noted that suckling rats exhibited a much more marked retention of cesium than was the case for rats weighing 50 or 100 grams. It was also noted that at weaning the retention of cesium decreased sharply and was in accord with data obtained using 50 grams rats. It was also observed that at the time of weaning, the dam contained as much radioactivity as did the average suckling young. Since it is characteristic of the mother to induce the nursing pup to urinate by licking its anal region, the results of the experiment suggested that the rather long retention of cesium in the suckling could be due to recycling of radiocesium from dam to the suckling.

To look at the problem with more preciseness it was decided to repeat the experiments and attempt to keep the dam from having oral contact with the pups.

II. Methods

Pregnant albino female rats (Sprague-Dawley strain) weighing between 250-300 grams were purchased from Blue Spruce Farms. For about four days after the birth of the pups the female was allowed to nurse the young undisturbed. On the fourth day, in most cases, each pup in the litter was injected I.P. with 0.05 ml of a solution of radiocesium that contained 95 μ c of Cs-134 in 50 ml of distilled water. From this time on till weaning the dam was separated from the pups during the period 8 a.m. to 5 p.m. At 5 p.m. the dam was fitted with a wire screen head mask and returned to the cage containing the pups. The dams had access to food and water only during the period away from the pups. In this manner, the intake of foods other than maternal milk by the pups could be controlled. The pups were counted individually in a small animal counter, Tobor (Nuclear Chicago). To test the effect of weaning upon the retention of radiocesium the pups were weaned at either 16 days, 3 weeks, or 4 weeks. In most cases the pups were provided access to the normal pelleted rat diet (Agway, Inc.), during the absence of the dam; however, in some situations the pups were provided with fresh cows milk instead of the solid diet. Table 1 describes the variables during these experiments.

Table 1. Protocol for management of rats during the series of experiments looking at the effect of weaning upon cesium retention

Supplemental Diet Provided Pups	Age at Weaning	Number of Litters	Number of Pups in Litter	Age of Pups at Dosing
Grain	16 days	2	6,6	4 days
Grain	3 weeks	1	10	7 days
Grain	3 weeks	1	3	4 days
Grain	4 weeks	2	7,5	5 days
Grain	4 weeks	1	6	4 days
Fresh Cows Milk	4 weeks	3	5,5,6	4 days

III. Results and Discussion

Figure 1 is a graphic presentation of the results of the series of trials in which the weaning time was varied to determine its relation to the retention of radiocesium. In this figure the group average has been obtained by averaging litter values rather than averaging by weighting for the numbers of animals in a litter. From the figure it is evident that during the period the pups were nursing the retention of cesium was greatly different than the period after weaning. Moreover the sharp break in the curve coincided relatively closely with the time of weaning. All groups showed a single exponential rate of loss of cesium from the bodies of the pups during the suckling period that had a $T_{1/2}$ of 10.9 days. After weaning the falloff was multi-exponential, and somewhat faster than that observed previously for rats dosed at 50 grams of weight. At the time of weaning the dams were sacrificed and the count of skin, visera, and empty carcass were determined. In all cases the dam contained less than 10 percent of the count of the individual pups of the litter; most of the count being associated with the pelt.

Figure 2 presents the results of a trial in which the pups were given either the normal pelleted rats diet or fresh cows milk as the sole food after weaning. It is to be noted that both groups reacted in the same manner with the sharp break in cesium retention occurring at weaning. This experiment definitely rules out the possibility that the physical form of the diet (particulate vs. liquid) could be responsible for the observed results.

The experiment presented in Figure 2 was intended also to subject the pups to weaning but yet to continue them on a diet that was quite similar in composition to the maternal milk. This would determine if the composition of the diet provided after weaning was responsible for the results. Table 2 presents a comparison of rat and cow's milk to show how well this was accomplished. As can be seen from this table, the actual composition of the two milks differ markedly with rat milk being far more concentrated than cow's milk. If it is assumed, however, that the weanling rats will attempt to consume more of the cows' milk than of the rat milk in order to meet their caloric needs, then the contrast is less striking. On an isocaloric basis (last column of Table 2) if 234 ml of cows' milk is consumed for every 100 ml of rats milk that might have been consumed then there are few differences in the intakes of the various constituents. It would then appear that feeding of cows milk continued the character of diet supplied during the suckling period. Since the commercial rat diet is composed to a large part of plant materials, it would tend to exclude the chemical composition of the diet as being the cause of the change in the retention of radiocesium.

Table 2. Comparison of Rat and Cows Milk

	Rat	Cow (Actual)	Cow (Isocaloric)
Calories/100 ml	ca 170	ca 72	
% H ₂ O	70-78	85-88	
% Protein	12.0	3.5	8.2
% Lactose	3.0	4.8	7.0
% Fat	9-15	4.3	10.0
% Ash	1.5-2.0	0.7	1.7
Ca(% of Ash)	26.1	17.1	
P (% of Ash)	23.2	14.3	
Fe (μg/100 ml)	700	30	70
Bifidus Factor Activity/unit Vol (man=100)	60	1	--
Riboflavin (μg/100 ml)	400	150	350
Pantothenic Acid (mg/100 ml)	900	350	820
B ₁₂ (μg/100 ml)	1.2	0.5	1.2
Ascorbic Acid (mg/100 ml)	0.4	2	4.7

Figure 1. The Age of Rat Pups at Weaning and the Retention of a Single I.P. Dose of Radiocesium.

Figure 2. The Retention of Radiocesium by Rat Pups Weaned at 4 weeks of Age to a Diet of Liquid Cows Milk or a Stock Laboratory Diet

8. Estimation of Body Composition and Cs^{137} Body Burdens in
College Women Using the Whole Body Counting Technique
F. A. Kallfelz

Abstract: A cooperative study with Professor C. Young of the Graduate School of Nutrition is underway in which the determination of lean body mass and fat content of college age women is being determined by several different methods including the K^{40} method using the Cisco counter. The results from whole-body counting will then be compared with the results obtained from skin-fold measurements and densitometric techniques. In addition, an estimation of Cs^{137} content has been made on all subjects counted to-date.

The values obtained from women have been compared with results obtained on men by us and other investigators.

Generally, the results obtained by K^{40} counting have given estimates of body fat that are higher than those usually obtained by other workers. The reasons for this are discussed. Cs^{137} body burdens are generally in the range expected in this geographical region and at this time.

Introduction

The K^{40} counting method has been used quite extensively in the estimation of body composition in human beings. What is needed is the validation of the K^{40} method with other indirect methods of measuring LBM and/or fat, e.g. anthropometric techniques and densitometric and hydrometric techniques.

For this purpose, the Cornell University Campus is possibly uniquely equipped since facilities are available for determinations of lean body mass and fat content using all of these techniques. The whole body counter (Cisco) is being used to estimate body composition by the K^{40} method and these data will then be compared with results from the other techniques which are being run on the same individuals at other sites on the campus. In addition, an estimate is being made of the Cs^{137} body burden of these individuals.

Methods

Subjects have been and are being recruited from the University population through public appeal. Thus they all fall within the same age group (18-25 yrs.) To date approximately 35 girls have been counted, and approximately 10 men.

Natural potassium contains 0.012% of the isotope K^{40} which is naturally radioactive and has a half life of about 10^9 years. K^{40} decays by beta emission (89%) and electron capture (11%). Associated with the E.C. is an α -ray emission of 1.46 Mev. Natural potassium has a gamma activity of 2.96 photons/sec/gram K. The natural potassium in the body can be counted with a whole body counter, and with suitable corrections, the values obtained can be converted into a value for lean body mass (assuming that all body lean has a constant concentration of potassium).

By subtraction, then, the fat mass can be determined.

Cesium can be determined at the same time by using a double channel analyzer connected to the whole body counter and correcting for cross channel counting.

Efficiency for K^{40} and Cs^{137} counting was determined by preparing 130 lb. phantom standards made of plastic carboys filled with water. Suitable amounts of K^{40} and Cs^{137} were then dissolved in the water. The Phantom standard has roughly the same geometry as a man. The settings for Cs^{137} and K^{40} were selected by the E^2/B method and the efficiency for K^{40} counting was approximately 8.7% while that for Cs^{137} was about 6.7%. Background depression was corrected for by using a phantom man* filled with H_2O to weigh 120 pounds.

The counter was calibrated daily and the figures for K^{40} and Cs^{137} efficiency, as well as background depression and cross-channel correction data from each daily calibration were used only for the subjects counted on that day.

In addition, one man has been counted repeatedly at daily intervals during the time of this study in order that an error estimate on an individual count might be obtained.

Results

Most authors have published body composition as a function of age (3, 5, 6, 7). However, since all of the women were in the same age group (18-25) and most of the men were in the same age group (23-40), the data here have been plotted as fat content related to total body weight. To-date, the % fat in individuals appears to be directly proportional to body weight. This trend is apparent in the data obtained from females (Fig. 1) and also in the small amount of data obtained from males (Fig. 2). For women, the data followed roughly the equation

$$\% \text{ Fat} = (\text{Body wt. (kgs)} - 27.5) \times 1.25 \quad (1)$$

For men, the equation is

$$\% \text{ Fat} = (\text{Body wt (kgs)} - 32) \times 0.649$$

Thus a 70 kg female would be expected to contain about 53% fat while a 70 kg man would contain only about 25% fat. Of course, this analysis does not take into account variations in height of the individuals, i.e. difference in physique, which would most likely influence the results. Also it must be remembered that all individuals counted are adults which explains the observation that % fat reaches zero at a considerable total body weight.

* Remcal, Picker Nuclear Corp.

Data for the individual who was counted repeatedly at daily intervals for a period of twenty days showed that his average fat content was 28.5% with a standard deviation of 4.1%. Thus the 95% confidence limits for this individual were 20.3-36.7%. Thus for any given individual count, the 95% confidence limits might be taken as plus or minus 8%.

Cesium levels for the thirty-six females studied to date show an average body burden of 2.6 nanocuries of Cs^{137} for the group. The standard error is ± 0.2 nanocuries. In general, the cesium level varied directly with the K^{40} count as would be expected. Cesium levels for males averaged higher than those for females ($5.13 \pm .61$ nanocuries) undoubtedly the result of the increased muscle mass in men.

Discussion

Although the data generally follow expected trends, the K^{40} estimates of lean body mass were lower than might be anticipated from other work in this area. The most probable cause for this is a decreased estimate of K^{40} content occurring through an artificially high estimate of K^{40} counting efficiency. At present, the efficiency of the K^{40} standard is taken as the efficiency of human counting. It may be that the efficiency of K^{40} photon detection by the counter is less for human beings, due to absorption by bone etc., than it is for the standard.

This possibility will be investigated by determining K^{42} efficiency in a number of individuals. K^{42} is a very short lived ($12.5 \text{ hr } T_{1/2}^1$) isotope of potassium which has a gamma photon very similar to that of K^{40} (1.51 Mev K^{42} , 1.46 Mev K^{40}). Thus the efficiency of counting K^{42} should be almost identical to that of K^{40} . By injecting a number of individuals and the K^{40} standard with a small amount of K^{42} (approx. $0.25 \mu\text{c}$) the efficiency of counting of the isotope from the human and from the standard will be determined. The K^{40} counting efficiency will then be assumed to be the same as that of K^{42} . If a difference is found in counting efficiency, the data collected previously will be corrected.

To-date, none of the data have been compared with results from other methods of determining body composition but such comparisons will be made upon completion of this study.

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Fig. 1. Body fat content as a function of body weight in women aged 18-25 years.

WOMEN

Fig. 2. Body fat content as a function of body weight in a group of men aged 24-40 years.

9. Intestinal Absorption of Oleic Acid I^{131} and Triolein I^{131} in the Differential Diagnosis of Malabsorption Syndrome and Pancreatic Dysfunction in the Dog.

Francis A. Kallfelz

Abstract: The use of oleic acid I^{131} and triolein I^{131} appears to be a useful diagnostic tool in the diagnosis of pancreatic dysfunction and malabsorption syndrome in the dog. After oral administration of either drug in capsular form, the amount of absorption can be determined by counting the feces or counting periodic blood samples and correcting for the amount present in the entire blood stream. In normal dogs an average maximum of 13.0% of a dose of oleic acid I^{131} and 11.0% of triolein I^{131} was absorbed. In dogs affected with pancreatic insufficiency, the absorption of oleic acid I^{131} was normal while the absorption of triolein was below 2%. In one dog showing both pancreatic and intestinal pathology, the absorption of both substances was below the average.

Introduction:

Measuring the intestinal absorption of triolein I^{131} and oleic acid I^{131} has proved to be a valuable diagnostic technique in the differential diagnosis of malabsorption syndrome and pancreatic insufficiency in man (1). In addition, there is at least one report in the literature citing the use of labeled triolein and oleic acid in the diagnosis of malabsorption syndrome in the dog (2). The following report describes the use of labeled triolein and oleic acid in the diagnosis of both pancreatic insufficiency and malabsorption syndrome in the dog.

Methods:

The clinical cases described were animals which had been referred to the Small Animal Clinic during the past year with a general complaint of progressive emaciation. Each was given on the first day of study, a capsule containing 25 uc of triolein I^{131} . Immediately before administration of the test drug, the dog was given a capsule containing approximately 0.25 ml Lugol's solution. This was done to block thyroidal uptake of absorbed I^{131} . Blood samples (4 ml) were taken at 1-2 hour intervals for the next six to ten hours and counted in a well type scintillation counter using a NaI (TL) crystal and a single channel pulse height analyzer. The blood volume of each animal was estimated according to Dukes (3) and the count was then expressed as the total percent dose absorbed.

A two day lapse was allowed between the administration of the triolein I^{131} and the subsequent administration of the oleic acid I^{131} . The oleic acid was then administered and samples were taken and calculated as with the labeled triolein.

* Labeled triolein and oleic acid was obtained from either Abbott Radiopharmaceuticals or Malinckrodt Nuclear Corporation.

Results:

The findings in five studies (3 normal, 2 affected) are reported in table I. The range of maximum absorption for triolein I^{131} in normal dogs was about 8-15% with an average of 11%. The time of maximum absorption was 4-6 hours after administration which probably reflects the time necessary for digestion of the neutral fat by pancreatic lipase and subsequent absorption. Oleic acid absorption in the normal dog appears to reach a maximum in a shorter period of time (2 hours) and, in general, the oleic acid I^{131} is more completely absorbed (13%).

In both affected animals the absorption of triolein I^{131} was severely depressed with maximum values of less than 1% absorbed in both cases. In addition, the absorption of oleic acid I^{131} may have been slightly reduced in affected case 1 while it was significantly reduced in case 2.

Figure 1 shows the pattern of absorption in affected case number 1 and in a control animal. The severe reduction in triolein absorption is readily apparent in the affected animal while oleic acid absorption appears to be about the same in the control and the affected animal.

Discussion:

The normal values reported here agree well with those of Kaneko (2) in normal animals. Both affected animals studied show a severe reduction in absorption of the labeled triolein. This reduction in absorption is evidently due to a lack of pancreatic lipase which is necessary for the breakdown of neutral fats into fatty acids and monoglycerides.

Affected case number 2 was subsequently euthanized and post-mortem examination was performed. The pancreas consisted of a lacy network of tissue macroscopically with most of the tissue seemingly absent. Microscopically the tissue consisted of small clumps of large inactive pancreatic acinar cells and functional islet cells. The final diagnosis was hypoplasia or atrophy of the pancreas. In addition there was a chronic catarrhal enteritis present in this dog with heavy mononuclear infiltration of the mucosa. This type of an enteritis is not uncommon in man occurring concomitantly with a chronic pancreatitis. The enteritis seen here, might have been caused by the aberrant environment of the mucosal cells due to the lack of pancreatic enzymes, and could possibly result in a "malabsorption syndrome." Such a situation may explain the reduced oleic acid I^{131} absorption seen in case number 2 which, although higher than that of the triolein, was less than normal.

Affected case number 1 was diagnosed as chronic pancreatitis and returned to the owner; the dog was placed on pancreatic enzyme replacement and a bland, high nutrient diet was prescribed. No biopsies were taken on this dog.

The use of this test has been well accepted by the clinicians in the New York State Veterinary College and the number of cases presented for this and other diagnostic tests involving the use of radioisotopes has increased greatly during the past year.

Table 1

Results of Oleic Acid I¹³¹ and Triolein I¹³¹ Absorption
Studies in Five Dogs

<u>Condition</u>	Triolein Absorption (%)	Time After Admin. (hours)	Oleic Acid Absorption (%)	Time After Admin. (hours)
Normal 1	15.5	6	13.4	2
Normal 2	8.8	4	12.4	2
Normal 3	8.5	6	13.1	2
Affected 1	0.9	8	10.8	8
Affected 2	0.3	6	4.6	7

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Fig. 1. Results of Oleic acid I^{131} and triolein I^{131} absorption study in a control dog and in a dog suspected of pancreatic dysfunction. Note the severely reduced absorption of triolein I^{131} in the affected dog.

TRIOLEIN 131 AND OLEIC ACID 131 ABSORPTION STUDY
IN CONTROL DOG AND A DOG SUSPECTED OF
PANCREATIC INSUFFICIENCY 11-13-67—11-16-67

10. Comparison of Resin Sponge Uptake of Triiodothyronine I^{131} from Serum with other Commonly used Thyroid Function Tests as Indication of Thyroid Dysfunction in the Dog.

Abstract: The resin sponge uptake of triiodothyronine I^{131} ($T-3^{131}$) from dog serum has been compared with cholesterol level, thyroid uptake of I^{131} and the protein-bound iodine I^{131} conversion ratio ($PBI^{131}CR$) in a group of beagle dogs. The normal $T-3^{131}$ uptake is approximately $50.0 \pm 8\%$ (95% confidence limits). Results of the other tests compare favorably with previously reported values. The advantages of the $T-3$ test as a practical clinical diagnostic test are discussed.

Introduction

For many years, the diagnosis of thyroid gland dysfunction in the dog was rather difficult with plasma cholesterol levels being the only indicator that had wide use. With the availability of artificial radioisotopes, the radioiodine thyroid uptake test and the protein-bound iodine conversion ratio ($PBI^{131}CR$) test were developed for use in the dog (1, 2, 3). These tests greatly helped the clinician to diagnose when dealing with thyroid problems. These tests, however, suffer from the disadvantage that the animal must be brought to a university or research institute for the performance of the test since practitioners in the field are not usually prepared to handle radioisotopes.

In 1960 Mitchell et al (4) reported the development of the resin sponge uptake of $T-3^{131}$ from serum as a diagnostic test for thyroid dysfunction and pregnancy in humans. This test was a great improvement over the red cell uptake of $T-3^{131}$ since it reduced the technical difficulties of the test and also reduced the variability.

This report deals with the adaptation of the $T-3^{131}$ resin sponge uptake test for use in the dog.

Methods

Twenty-five adult beagle dogs were maintained on a normal dog diet containing normal amounts of iodine. At zero time a 10 ml blood sample was taken using a vacutainer syringe. Immediately thereafter the dogs were given a dose of I^{131} (carrier free) by intravenous injection ($50-60 \mu c$). The thyroid uptake and $PBI^{131}CR$ was determined daily for 5-6 days using standard procedures (2). The blood taken at time zero was allowed to clot and the serum was collected and then used for determination of $T-3^{131}$ * binding and cholesterol levels. At the end of this experiment, five dogs were placed on propylthiouracil (PTU) at a level of 200 mg/day for two weeks and the above tests were repeated. A second group of five dogs was given 20 I.U. of thyroid

* Triosorb, Abbott Radiopharmaceuticals.

stimulating hormone and the tests were again repeated. In addition a number of clinical cases from the small animal clinic have been tested with one or more of the above tests and the results are reported here.

Results

Table I shows the results of the four tests used on normal animals. It can be seen that the thyroid uptake of I^{131} rises to a peak of about 17% at about 2-4 days and then decreases gradually with time. The $PBI^{131}CR$ gradually increases with time as would be expected. $T-3^{131}$ values showed a mean of about 50% with 95% confidence limits of approximately 8% (i.e. approximately 42-58%); most of the observed readings fell between 45% and 58%. Values for cholesterol level were within the normal range for dogs.

In Table II are shown the results obtained in dogs injected with TSH before the tests were run. Although the $T-3$ values tended to rise and the cholesterol levels tended to fall, as would be expected, none of these results were significantly different from the normal.

Results in propylthiouracil treated dogs were also not significantly different from normal (Table III). However the $T-3^{131}$ values did tend to fall and the cholesterol levels tended to rise as would be expected.

In the human being, results of the $T-3$ test vary according to the time of incubation and the number of times the resin sponge is washed with dionized water after incubation. In the current experiments all $T-3^{131}$ tests were incubated for one hour at room temperature. Results were determined after 3 and 5 washings of the sponge. It can be seen from Table IV that there is no significant change in the data with 3 or 5 washings. Thus it can be assumed that 3 washings removes essentially all of the unbound $T-3^{131}$ from the resin sponge.

A number of cases presented at the small animal clinic of the New York State Veterinary College and suspected of having thyroid dysfunction were also submitted to one or more of these tests. Results are shown in Table V. All four cases had a low normal or subnormal $T-3^{131}$ value while results on the other tests seemed to vary.

Discussion

Results of the radioiodine thyroid uptake test, $PBI^{131}CR$ determinations, and cholesterol levels, in normal dogs agreed with previous results from this and other laboratories (1, 2, 3). The $T-3^{131}$ test results in these normal dogs showed a mean of about 50% with 95% confidence limits of about $\pm 8\%$ (50.0 ± 8.0). Although TSH pretreatment tended to raise and PTU pretreatment tended to reduce the $T-3^{131}$, neither treatment produced values significantly different from the normal. In the clinical cases reported, however, a low normal or subnormal value was found in a number of animals. It may be that the experimental treatments employed to produce hyper or hypo-thyroidism were not sufficient to produce clinical manifestations of dysfunction.

In the future, it is planned to produce hypothyroidism by surgical removal or radiolysis of the thyroid gland and to then recheck results in these animals.

The development of the T-3¹³¹ test for use in veterinary medicine represents, possibly, the first nuclear medical technique with field applicability in veterinary medicine. Since only a serum sample is needed to run the test, and there is no radioactivity injected into the animal, it is not necessary that the animal be referred to a veterinary college or research institution for the test to be run. The practitioner need only withdraw a blood sample, collect the serum and send it in a refrigerated container to a central laboratory where the test can be run. Our results indicated that refrigeration for two to three days or frozen storage for up to two weeks does not influence the results of the test. The laboratory can then perform the T-3¹³¹ test and report the results to the practitioner.

It is hoped that the development of the T-3 test for use in veterinary medicine will be of value to practitioners and plans are now in progress to extend the scope of our findings to cover cattle, swine and horses.

Table I. Thyroid Function Tests in Normal Dogs

<u>Day</u>	<u>Thyroid Uptake</u> (%)	<u>PBI¹³¹CR</u> (%)	<u>T-3¹³¹</u> (%)	<u>Cholesterol</u> (mg %)
1	15.36 $\pm$ 1.07	2.61 $\pm$ 0.40		
2	15.58 $\pm$ 0.92	7.70 $\pm$ 1.10		
3	16.95 $\pm$ 1.08	15.89 $\pm$ 1.83	49.63 $\pm$ 0.72	146.0 $\pm$ 5.13
4	15.07 $\pm$ 0.61	26.43 $\pm$ 2.87		
5	14.09 $\pm$ 0.88	32.31 $\pm$ 2.75		
6	11.94 $\pm$ 0.70	30.70 $\pm$ 2.60		

Values represent the mean $\pm$ the S.E. of the mean of 20-25 observations.

Table II. Thyroid Function Tests in TSH Treated Dogs

<u>Day</u>	<u>Thyroid Uptake</u> (%)	<u>PBI¹³¹CR</u> (%)	<u>T-3¹³¹</u> (%)	<u>Cholesterol</u> (mg %)
1	20.96 $\pm$ 1.97	2.28 $\pm$.14		
2	15.13 $\pm$ 1.20	10.55 $\pm$ 2.65		
3	15.34 $\pm$ 1.87	14.04 $\pm$ 2.16	55.55 $\pm$ 1.56	134.76 $\pm$ 6.25
4	12.97 $\pm$ 2.04	27.78 $\pm$ 2.88		
5	11.22 $\pm$ 2.05	32.44 $\pm$ 2.41		
6	9.03 $\pm$ 1.61	34.42 $\pm$ 3.15		

Values represent the mean $\pm$ S.E. of the mean of 4 observations. TSH given 10 iU I.M. 48 hours before injection of radioiodine, 5 iU I.M. 24 hours before injection of radioiodine and 5 iU I.M. at time of injection of I¹³¹ at 24 hours after injection of I¹³¹. Blood for T-3 test drawn just before injection of radioiodine.

Table III. Thyroid Function Tests in PTU Treated Dogs

<u>Day</u>	<u>Thyroid Uptake</u> (%)	<u>PBI¹³¹CR</u> (%)	<u>T-3¹³¹</u> (%)	<u>Cholesterol</u> (mg %)
1	7.31 $\pm$ 1.03	2.32 $\pm$ 0.53		
2	4.38 $\pm$ 0.90	4.35 $\pm$ 0.86		
3	4.16 $\pm$ 0.73	5.24 $\pm$ 1.38	46.30 $\pm$ 0.56	167.7 $\pm$ 22.5
4	2.91 $\pm$ 0.69	15.46 $\pm$ 3.53		
5	1.92 $\pm$ 0.54	38.85 $\pm$ 8.09		

Values represent the mean $\pm$ of the mean of 6 observations. PTU given orally 200 mg bid for 10 days before exp.

Table IV. Comparison of T-3¹³¹ values in serum of normal dogs following 1 hour incubation with 3 or 5 washings of the resin sponge.

<u>Subjects</u>	<u>Inc. Time</u>	<u>Number of Washings</u>	<u>T-3¹³¹ (%)</u>
Normal Dogs	1 hr.	3	55.30 $\pm$ 3.24
Normal Dogs	1 hr.	5	54.76 $\pm$ 3.33

Results indicate mean $\pm$ standard error of the mean of 20 observations.

Table V. Thyroid Function Tests in Selected Clinical Cases

Dog No.	Presumptive Diagnosis	Maximum Thyroid Uptake of I ¹³¹ (48-72) hours (%)	PBI ¹³¹ CR 72 hrs. (%)	T-3 ¹³¹ (%)	Cholesterol (mg %)
66-6154	Hypothyroidism	41.10	4.02	42.4	297
67-1000	Hypothyroidism	5.97	10.50	32.7	---
67-2944	Hypothyroidism	-	-	39.5	---
67-5969	Hypothyroidism	-	-	42.4	---

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11. Blood Fe^{59} and Cr^{51} Kinetics in Normal Horses for Comparison with Horses Infected with Equine Infectious Anemia.

F. A. Kallfelz and P. H. Craig

Abstract: Several parameters of hematopoietic function in normal horses have been studied as a prelude to similar studies in horses infected with equine infectious anemia. These include red blood cell and plasma volumes, $T_{1/2}$ of removal of Fe^{59} from plasma, $T_{1/2}$ of Cr^{51} labeled red blood cells, incorporation time of Fe^{59} in red blood cells. Results appear to correspond favorably with other published values for these parameters in normal horses.

Introduction

At present, the only definitive diagnostic test for equine infectious anemia is the horse inoculation test; i.e. blood or other biological material from the suspected animal is injected into an E.I.A. free animal to see if the free animal becomes infected with the disease. This, of course, is a lengthy and expensive process and is generally used only for extremely valuable animals. It is not practical as a screening test for E.I.A. infection.

In addition, the pathogenesis of the disease is not entirely clear. It is felt that an allergic response may be involved as well as the viral attack itself. That is, the breakdown products of cells resulting from the viral attack may act as foreign protein and elicit a severe allergic reaction. This response is accompanied by a large increase in the gamma globulin fraction of the blood but these antibodies appear to be directed against the breakdown products formed from infected cells and not against the virus itself.

To the authors' knowledge, no work has been reported on observations of blood kinetics during the disease. However, such studies would be relatively easy to perform. The techniques for determining erythrocyte survival times, plasma iron transport rates plasma clearance of Iron-59, appearance rates of Fe^{59} in erythrocytes and blood volume have been well worked out for the human, and for sheep, and could easily be adapted for use in the horse.

Since it is known that changes occur in the blood cells and blood protein during the course of the disease of E.I.A., it was felt that blood kinetic studies would be of interest from a pathogenetic point of view and also, might prove to be of some diagnostic value.

Initially, the normal parameters of blood kinetics in ponies have been studied. This has required the injection of approximately eight ponies with Cr^{51} and Fe^{59} . After a normal pattern is established, infected ponies will be studied at various times during the course of the disease e.g. incubation period, acute phase, recovery phase, relapse, etc.

Materials and Methods:

Each horse used in this experiment was injected with approximately 200 μCi Cr^{51} and 35 μCi Fe^{59} according to the procedure outlined below. Ten milliliter blood samples were removed periodically for three to four weeks and analyzed.

Tagging Procedure:

Thirty ml. of blood was withdrawn with a sterile heparin wetted syringe and placed in a 125 ml sterile flask. To this blood was added 8 ml. of ACD solution, approximately 200 μCi of Cr^{51} in the hexavalent form and 35 μCi of Fe^{59} . The blood sample was then incubated at room temperature for 30 minutes with intermittent swirling. During this period, the hexavalent Cr^{51} was bound to the erythrocytes and the Fe^{59} became bound to the plasma apoferrin. At the end of the thirty minute incubation time 50 mg of ascorbic acid was added to the blood. The ascorbic acid reduces any unbound Cr^{51} to the trivalent form making it unavailable for any further erythrocyte binding.

An accurately measured volume (approx. 20 ml.) of the labeled solution was then reinjected intravenously into the test animal with repeated flushing of the syringe to insure complete delivery of the tagged solution.

Samples of blood (approx. 10 ml) were withdrawn in a heparin wetted syringe at 10, 20, 30 and sixty minutes; 2, 4, and 24 hours; and daily from 2 to 28 days after the injection of the labeled blood.

Sample Preparations:

A. Standards

1. From the 10 ml of suspension remaining after injection, 0.5 ml was taken and added to a 100 ml volumetric flask containing 50 ml. of a 0.1% saponin solution to which had been added 1 ml of a 1% Na Chromate solution and 1 ml of a 1% Ferrous Citrate solution. This was diluted to 100 ml and three 1 ml samples were counted for chromium and iron in an automatic gamma counter equipped with two single channel analyzers which had been previously calibrated for cross counting with a pure Cr^{51} and a pure Fe^{59} standard. The procedure was run in duplicate.
2. Five ml of suspension was pipetted into a 15 ml centrifuge tube and spun down. The plasma was collected and 0.5 ml was placed in a 50 ml volumetric flask to which had been added 25 ml of distilled H_2O containing 1 ml of a 1% Ferrous Citrate solution. The flask was then filled to the mark with distilled H_2O and three 1 ml samples were counted for Fe^{59} and Cr^{51} .
3. Samples
 1. The hematocrit of all samples was determined in triplicate.
 2. From each sample drawn, 2.0 ml of blood was counted in duplicate for Fe^{59} and Cr^{51} content.

3. The plasma from 5 ml of blood was collected and 1.0 of plasma was mixed with 1 ml of distilled H₂O and counted in duplicate for Fe⁵⁹ and Cr⁵¹ content.

Calculations:

1. The erythrocyte volume was calculated by the general isotope dilution equation:

$$V_t = V_i \frac{C_i}{C_r} - 1 \quad (1)$$

where

V_t = total volume of the compartment

V_i = volume of injected tag

C_i = concentration (cpm/ml) of injected tag

C_r = concentration (cpm/ml) of recovered tag

If V_t is much greater than V_i , as it is in this case, then equation (1) can be modified to

$$V_t = V_i \frac{C_i}{C_r} \quad (2)$$

The erythrocyte volume was calculated by using the data of Cr⁵¹ determinations in the red cells in the early time periods (10-30 minutes) and extrapolating to zero time.

The plasma volume was calculated in similar fashion but in this case the data used was the Fe⁵⁹ values from the plasma.

Plasma clearance of Fe⁵⁹ was determined by counting equal volumes of plasma at early time periods after injection (10, 20, 30, 60, 120 min.) and plotting the counts recorded on semi-logarithmic paper. After drawing the straight line of best fit through these points, the time required for radioactivity to decrease to one half of the initial injected value was designated as the "half-clearance time" for Fe⁵⁹.

An appearance curve of Fe⁵⁹ in erythrocytes was obtained by collecting periodic blood samples for seven to ten days after the initial injection, counting the activity of Fe⁵⁹ in the red cells, and calculating the daily plot by the following formula.

$$\% \text{ dose in RBC} = \frac{V_c \times \text{cpm/ml packed cells} \times 100}{\text{cpm Fe}^{59} \text{ injected}}$$

where

V_c = red cell volume

In determining red blood cell survival times, the Cr⁵¹ content of erythrocytes was determined for samples drawn at 24 hours and thereafter. These samples were plotted as % dose/ml of erythrocytes on semi-log paper and the $T_{1/2}$ of Cr⁵¹ labeled erythrocytes was determined.

For the first eight animals, the data were calculated manually. As this was found to involve an exorbitant amount of time, a computer program was developed to analyze the data. For all future runs, the data will be punched directly from the counter tape onto cards and fed into the departmental computer (IBM-1800) for analysis.

Results:

Of the eight normal horses run, only six provided useable data. The data for these animals are presented in table I. Red blood cell volumes and plasma volumes cannot be directly compared with other observations since the weights of the animals were unknown. The average half-time of clearance of Fe^{59} from the plasma was 1.96 hrs. (118 min.) which compares favorably with the work of Obara et al. (1) in Japan. Compared with removal of Fe^{59} from the plasma, the first appearance of Fe^{59} in the red cells occurred at 1 to 2 days and reached a maximum at an average of about 9 days after injection when the % dose of Fe^{59} in the erythrocytes was about 55% (56.4 ± 5.4). The shape of the incorporation curve of Fe^{59} into red blood cells (Fig. 1) is also essentially the same as that found by Obara et al. (1).

The half-time of survival for Cr^{51} labeled erythrocytes was found to be an average of about 10 days in the six horses. Although this appears to be quite low, it compares reasonably with the results of Obara & Nakajima who found a Cr^{51} rbc survival half-time in normal horses of about 12-16 days (2).

Discussion:

The studies performed were undertaken in an attempt to characterize some of the normal parameters of Fe^{59} and Cr^{51} labeled rbc kinetics in normal horses. In general, the results obtained are comparable to those reported elsewhere in the literature (1, 2). However, more normal horse data will probably be advantageous before proceeding with work on infected horses.

Survival half-times for Cr^{51} labeled erythrocytes appeared to be quite low which may be partially the result of the techniques employed. The ACD solution used in these studies was designed for use with human blood, and it is planned to formulate a solution which may be more physiological for equine erythrocytes and result in less damage to them.

The use of DFP^{32} as an erythrocyte label has been tried; however, the preparation of such samples for counting is extremely time consuming, and the results are not significantly different than those obtained with Cr^{51} labeled horse erythrocytes (3).

Computer analysis will no doubt reduce the time necessary for calculation of the results and facilitate the running of more horses in a given period of time. Three infected horses have been sampled at this time and the data are now being processed.

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4, 229 (1962).

Table I

Results of Blood Cr⁵¹ and Fe⁵⁹ Kinetics in Blood of Normal Horses

Horse No.	RBC vol. (ml)	Plasma vol. (ml)	T _{1/2} Clearance Fe ⁵⁹ Plasma (min.)	T _{1/2} RBC(Cr ⁵¹) (days)	Fe ⁵⁹ Incorp. in RBC max. time (days)	Max. Incorp. of Fe ⁵⁹ in RBC (%)
58	3226	7143	100	10.5	7.0	64.5
61	2005	6135	64	14.0	8.0	54.1
63	2632	7813	104	9.6	10.0	50.0
66	3345	8333	168	7.0	9.0	53.5
70	1949	7194	140	11.4	10.0	39.0
72	4831	9009	132	7.0	9.0	77.3
<hr/>						
Mean	2998	7605	118	9.9	8.8	56.4
<u>±</u> S.E.	<u>±</u> 438	<u>±</u> 412	<u>±</u> 14.8	<u>±</u> 1.1	<u>±</u> .5	<u>±</u> 5.4

Fig. 1 Results of Cr^{51} and Fe^{59} kinetic studies in a typical normal horse.
All other horses studies provided essentially similar data.

-94-
MINUTES

20 40 60 80 100 120 140 160 180 200 220 240 260 280

horse #58

%DOS - /ml.

.1

.01

.001

Fe^{59} in R.B.C. (days)

Cr^{51} in R.B.C. (days)

Fe^{59} IN PLASMA (min.)

1 .2 3 4 5 6 7 8 9 10 11 12 13 14

DAYS

12. Comparison of Radiation-Induced Chromatid Aberrations in the
Testes and Bone Marrow of Chinese Hamster
A. L. Brooks and F. W. Lengemann

Abstract: In research upon radiation-induced mutations, chromosome aberrations present in somatic tissues are used as an index of the genetic damage to the animal organism. Little is known about the relation of damage produced in a somatic tissue to that produced in a reproductive tissue. The purpose of this work was to compare the chromatid breaks of testicular tissue to that of bone marrow in the Chinese Hamster. The results indicated that at 6 hours after irradiation the testicular cells showed twice as many chromatid breaks as did the bone marrow. A time study indicated that differences were maximum at early time intervals and approached unity at 24 hours. The data suggest that the damage observed in one tissue can not be extrapolated to another and that characterizing the sensitivity of chromosomes requires a collection over several different time periods.

I. Introduction

It is well accepted that ionizing radiation can cause chromosome aberrations which result in genetic alterations. This has led to extensive research on the genetics of radiation-induced mutations in mammals and on the induction of chromosome aberrations in mammalian somatic tissues. Yet, very little research has been directed toward the measurement of radiation-induced aberrations in the chromosomes of reproductive tissues.

The experiments reported here were undertaken to compare the sensitivity of the testes and bone marrow of the Chinese hamster to the induction of chromatid aberrations by gamma irradiation.

Materials and Methods

The Chinese hamsters used in this study were all irradiated at a dose rate of 16.6 R/min at a distance of 10.5 inches from the cobalt-60 source. The total dose delivered was varied, from 50 to 125 R by changing the exposure time. In the first experiment the cells were sampled 6 hours after exposure. The animals in the second experiment were exposed to 100 R of cobalt-60 gamma irradiation, killed, and the cells sampled at 3, 6, 9, 12, and 24 hours postexposure.

Two hours before killing, the animals were injected intraperitoneally with 0.3 ml of 1% colchicine solution in normal saline. They were killed with chloroform; the femurs and testes were dissected out and placed in prewarmed normal saline solution.

The bone marrow cells were prepared according to the Feulgen's staining procedure as described in Darlington and LaCour (1) with minor modifications. The staining step was eliminated, and the cells were suspended directly in the 45% acetic acid for 3 minutes. After the cells were reconcentrated by centrifugation, they were placed on a slide and squashed.

The testes were removed from the saline, the tunica was cut, and the seminiferous tubules were placed in a prewarmed 2.2% sodium citrate solution. The tubules were washed, placed in fresh sodium citrate solution, and then placed on a cover slip and lightly squashed with a second cover slip. This increased the proportion of spermatogonia and therefore the number of mitotic figures present, since these tend to associate with the tubule wall. The tubules were then placed in a hypotonic 1.1% sodium citrate solution and incubated for 5 minutes at 37°C. To the hypotonic solution an equal volume of ethyl alcohol and acetic acid (1:1) was added, and the tissue was allowed to fix for 18 to 24 hours at 4°C. They were placed in a 45% acetic acid solution, then squashed, and scorable metaphase figures were located.

The scoring of the cells was done at a magnification of 1500X with a Nikon inverted microscope with Zeiss phase contrast objective and condenser. The number of chromosomes was counted and then they were classified into four major groups, according to their length and centromere location. After this karyotyping, the chromosome number and the type of abnormalities present were recorded. Where there was a doubt as to whether a chromosome contained an aberration, it was scored as normal; only chromatid-type aberrations were scored. Breaks were scored only when there was a complete separation in the chromatid. Gaps were noted and recorded, but owing to difficulty in scoring them accurately they were not included in the totals. For scoring, 50 cells were taken from each of two animals per experimental point. There were 300 control cells scored for each tissue during the course of the study. Standard errors are plotted for each experimental point.

Results

Bender and Gooch (2) demonstrated that the number of chromatid-type aberrations changes as a function of time after irradiation. If a comparison is to be made between the breakage frequency of the chromosomes of two tissues, the cells must be sampled at the same time interval postirradiation. Since most workers use 6 hours postirradiation as the sampling time, this time interval was used in the first experiment. From this experiment the breakage frequency in terms of breaks per cell per roentgen can be derived. This is done by plotting the breaks per cell against the dose in roentgens, then finding the slope and intercept of the derived lines by using a least-squares analysis. By comparing the slopes of the lines, a reasonable estimate of the sensitivity of the chromosomes in the two tissues can be made. A plot of the single-hit breaks (chromatid plus isochromatid deletions) plotted against dose showed that these increased linearly with increased dose as described by the following regression equations:

Testes:

$$\text{Breaks/cell} = 0.032 + 0.0041 (\text{dose in roentgens})$$

Bone marrow:

$$\text{Breaks/cell} = 0.015 + 0.0015 (\text{dose in roentgens})$$

This suggests that, at 6 hours postirradiation, the testicular chromosomes are more than twice as sensitive to radiation-induced breakage as are the chromosomes of the bone marrow. This increase in sensitivity to breakage is also reflected by an increase in the number of chromatid exchanges observed in the testes.

To make a valid comparison of the radiation sensitivity of the chromosomes in two tissues, using this type of experimental design, it is essential that several factors be the same for each tissue. These factors include the following: the breaks in the chromosomes of both tissues have the same repair rate; the tissues suffer about the same radiation-induced mitotic delay; and both tissues have about the same proportion of cells in each stage of the cell cycle at the time of irradiation. If there is a change in any of these factors induced by the radiation treatment, the change must be of the same magnitude for each of the tissues before a valid comparison of the radiation sensitivity can be made. A change in any of these variables can result in changes in the number of breaks observed in the chromosomes at any fixed time after irradiation.

As a check on the influence of the above variable, a time-course experiment similar to the one reported by Bender and Gooch(2) was run. Table I summarizes the data from this experiment. The table shows that the single-hit breaks seem to be decreasing as an exponential function with time postirradiation. The testes values were significantly higher than the bone marrow values. Another difference between the two tissues in their response to radiation can be seen in the number of exchanges present as a function of time postirradiation. For the testes the exchanges start from a low value, peak at 6 hours postirradiation, and then decline rapidly thereafter. The number of exchanges in the bone marrow, on the other hand, was at their highest observed value at 3 hours and then continually decreased with time. This peaking behavior in the exchange frequency, as seen with the testicular tissue, has also been noted by Brewen (3) in plant cells.

The total number of breaks per cell is plotted against time post-irradiation in Fig. 1. The breakage frequency in the chromosomes of the testes was higher than in those of the bone marrow at all times sampled after irradiation, but the difference decreased markedly with time. Because of the techniques used (2-hour time delay between injection of colchicine and sampling of the cells), it was not possible to sample immediately after exposure. If such sampling had been possible, the data may well have indicated that the initial breakage value would have been the same for each tissue, perhaps about one break per cell. By 24 hours after irradiation the number of chromatid aberrations observed in each tissue was no longer significantly different. If, at each period, the number of breaks in the testes is divided by the number of breaks in the bone marrow, a ratio can be established which reflects the radiation sensitivity of the chromosomes. This apparent sensitivity ratio has values ranging from 1.34 to 3.19. The range of ratios indicates that conclusions drawn concerning the radiation sensitivity of the chromosomes are highly dependent on the time interval selected between irradiation and sampling of the cells. It is then important to use more than one standard sampling time postirradiation for comparing different types of cells.

Discussion

The experiments reported in this paper indicate that the testes chromosomes have more than twice as many radiation-induced chromatid breaks as do the chromosomes of the bone marrow when scored at 6 hours postexposure. This difference in the breakage frequency was also noted in the second experiment. Thus the chromosomal damage in a somatic tissue measured at a constant time postexposure may not reflect the number of chromatid aberrations produced by the same dose of radiation in a reproductive tissue. The estimates of the radiation-induced genetic damage, arrived at by using bone marrow, leukocyte cultures, corneal epithelium, etc., may not accurately reflect damage in gonadal tissue.

In explaining the differences observed in the breakage frequencies, several factors may enter into consideration. First, it has been noted that the length of the G-2 phase in the testes (6 hours) is longer than the G-2 phase in the bone marrow (2 hours). Moreover, the cells in the G-2 stage of the cell cycle may have a higher frequency of chromatid breakage than cells in any other stage. Second, the repair rate of the slow-healing type of chromatid breaks might be faster in the bone marrow than in the testes, leaving fewer scorable aberrations. Third, differences in the amount of mitotic delay induced in the two tissues could cause different proportions of the sensitive cells to be scored in the two tissues.

The effect of the length of the G-2 stage of the cell cycle can be deduced by considering the changes of the apparent sensitivity ratios with time. Because testicular cells have a longer G-2 stage, a higher proportion of the cells were in this sensitive phase at the time of exposure than for the bone marrow. Thus, at early time intervals the testicular cells could be expected to show more aberrations per cell, creating a high apparent sensitivity ratio. At later sampling times fewer of the cells samples would have been in the sensitive G-2 phase, thus decreasing the number of aberrations per cell. The effect, however, should be much more marked for the testicular cells than for the bone marrow cells, causing the apparent sensitivity ratio to decline. By 24 hours, when few of the dividing cells scored were in the G-2 phase at the time of irradiation, the difference between the two tissues should be minimal. The data of the second experiment support this by showing high apparent sensitivity ratios at early time intervals and a ratio that approaches unity at 24 hours.

The 3 hour time interval, however, does not quite fit this case. If these are significant, a possible explanation would be that there was a greater effect of mitotic delay on the testes cells than was observed for the marrow cells. This is a tentative suggestion, however, and needs further investigation.

In the testes and bone marrow of the Chinese hamster, if the sampling time was 6 hours postexposure, one would conclude that the testes chromosomes were three times as sensitive as those of the bone marrow. If the 24-hour period was used, the conclusion would be that the chromosomes of the testes are no more sensitive to radiation-induced damage than the chromosomes of the bone marrow.

These data then emphasize that several different time intervals of sampling must be used in characterizing the sensitivity of the chromosomes in any previously unmeasured tissue.

Table 1. Chromatid Aberrations Present in the Testes and Bone Marrow of the Chinese Hamster after Varied Doses of Cobalt-60 Gamma-Irradiation^a

Tissue	Doses(R)	CTD ^b	ICTD ^c	CTD + ICTD	Exchanges	Total breaks/cell ^d
Testes	0	3	1	4	0	0.013
	50	21	6	27	4	0.35
	63	25	5	30	8	0.46
	100	35	4	39	14	0.67
	100	41	5	46	12	0.70
	125	50	5	55	32	1.19
Bone Marrow	0	2	0	2	0	0.006
	50	6	2	8	1	0.10
	63	12	1	13	2	0.17
	100	11	4	15	3	0.21
	100	15	5	20	3	0.26
	125	15	3	18	8	0.34

a) All cells were exposed at a dose rate of 16.6 Rs/min with the total time of exposure being varied. There were 100 cells sampled per experimental point for the irradiated tissues, and 300 cells for the nonirradiated controls.

b) CTD = chromatid deletions

c) ICTD - isochromatid deletions

d) Total breaks/cell - CTD + ICTD + 2 (exchanges)

Table 2. Number of Chromatid Aberrations Remaining in the Bone Marrow and Testes of the Chinese Hamster as a Function of Time after 100 Roentgens of Cobalt-60 Gamma Irradiation^a

Tissue	Time after irradiation	CTD ^b	ICTD ^c	Exchanges	Total breaks/cells ^d
Testes	3 hours	62	6	3	0.74
	3	71	7	3	0.84
	6	35	4	14	0.67
	9	21	9	9	0.48
	12	15	2	3	0.23
	24	4	1	2	0.09
Bone Marrow	3	45	4	5	0.58
	6	11	4	3	0.21
	9	9	4	2	0.18
	12	10	2	1	0.14
	24	1	1	1	0.04

a) All cells were exposed at 16.5 R/min to a total of 100 R. There were 100 cells per experimental point.

b) CTD = chromatid deletions

c) ICTD - isochromatid deletions

d) Breaks - CTD - ICTD + 2 (exchanges)

Breaks - Chromatid + Isochromatid

Deletions + (2) (Exchanges)

Figure 1

13. Estimation of Erythrocyte Loss in Hookworm Infection by Whole-Body Fe⁵⁹ Counting
J. R. Georgi

Abstract: The volume rate of erythrocyte loss suffered by A. caninum infected dogs was estimated by periodic measurement of whole-body Fe⁵⁹ retention and erythrocyte Fe⁵⁹ concentration. Potential sources of systematic bias ("normal" Fe⁵⁹ excretion, intestinal Fe⁵⁹ reabsorption, and variations in whole-body counting efficiency caused by changes in bodily Fe⁵⁹ distribution) were studied experimentally. The first two sources tended to balance each other, the third was ordinarily insignificant and always amenable to correction. Objection is raised to the custom of expressing observations on the movement of erythrocyte labels as "blood loss" and the appropriateness of various commonly applied blank corrections is challenged. In harmony with the observations of others, the peak erythrocyte loss was found to precede the onset of egg production.

The advantages of Fe⁵⁹ over Cr⁵¹ as an erythrocyte label and of whole-body counting over fecal assay as a measurement technique are discussed.

I. Introduction

Hahn and Offutt (1949) proposed a technique for estimating blood loss in hookworm infection that utilized the decrease in erythrocyte Fe⁵⁹ concentration observed in compensated cases. Their data analysis was based on a simple exponential decay model that required steady state conditions with total incorporation of the tracer dose in the circulating erythrocytes and a relatively constant erythrocyte volume. They met these rather stringent requirements by bleeding 2 dogs for many months to deplete their iron stores and by restricting their analysis to data obtained during periods of negligible hematocrit change.

Similar assumptions were basic to my original analysis of whole-body Fe⁵⁹ retention in Haemonchus contortus infected sheep (Georgi, 1964a). The restrictions imposed by this set of assumptions precluded practical field application so means were sought to adapt the technique to dynamic situations.

A solution was found in the periodic measurement of both erythrocyte Fe⁵⁹ concentration and whole-body Fe⁵⁹ retention. Evaluation of the data in terms of volume rate of erythrocyte loss now rested on a set of testable assumptions:

1. The circulating labeled erythrocytes are homogeneously distributed. (In view of the constant mixing of the circulating blood, this might be viewed as a trivial assumption; no evidence has appeared to discredit it).

2. Fe⁵⁹ excretion is usually negligible so that any decrease in whole-body Fe⁵⁹ retention can be attributed to a loss of erythrocytes from the body.
3. There is negligible re-entry of Fe⁵⁹ into the blood or iron stores from labeled erythrocytes lost into the lumen of the alimentary tract.
4. Variations in the distribution of Fe⁵⁹ within the body have no appreciable influence on the Fe⁵⁹ counting efficiency.

These assumptions were investigated for sheep (Georgi and Whitlock, 1965) and found to hold reasonably well in sheep under hemorrhagic stress. Application of the technique to dogs demanded their reinvestigation in that species as well as development of a suitable restraint technique. This report is therefore concerned principally with the measurement technique itself although some preliminary data relating erythrocyte loss and parasite burden are presented.

Materials and Methods

Readers interested in the details of the basic mathematical model and alternative procedures for analysis Fe⁵⁹ retention and concentration data in terms of erythrocyte volume flow should consult Georgi and Whitlock, 1965 and 1967. In the experiments reported here, volume loss of erythrocytes was calculated by dividing the observed reduction in whole body tracer retention by the geometric mean of the corresponding tracer values i.e.,

$$\sqrt{\frac{\left(\begin{array}{c} \% \text{ dose Fe}^{59} \\ \text{retained at} \\ \text{time } t \end{array} \right)}{\left(\begin{array}{c} \% \text{ dose Fe}^{59} \text{ per} \\ \text{ml. erythrocytes} \\ \text{at time } t \end{array} \right)}} \div \sqrt{\frac{\left(\begin{array}{c} \% \text{ dose Fe}^{59} \\ \text{retained at} \\ \text{time } t + \Delta t \end{array} \right)}{\left(\begin{array}{c} \% \text{ dose Fe}^{59} \text{ per} \\ \text{ml. erythrocytes} \\ \text{at time } (t + \Delta t) \end{array} \right)}} = \text{volume (ml) of} \\ \text{erythrocytes lost during} \\ \text{the interval } \Delta t.$$

Similar calculations were carried out with the Cr⁵¹ data although, as pointed out later, a rather large proportion of the calculated volumes in this case represented elution and excretion of Cr⁵¹ rather than actual loss of erythrocytes.

The characteristics of the dogs used in these experiments are summarized in table 1. Dry dog meal was fed ad libitum to all; mean and milk supplemented the diets of younger pups.

Whole-body radioactivity measurements were made with "Cisco" (Metrix, Inc.) a 2-pi liquid scintillation whole-body counter (Twardock et al., 1964). This very dependable and sensitive instrument has remained in continuous operation (except during power failures and other extrinsic interferences) since the fall of 1962 with only minor maintenance. All counts were corrected for radioactive decay by reference to the observed decrease in radioactivity of plastic bottle phantoms containing aqueous solutions of the appropriate radionuclide.

Blood samples were drawn with heparinized syringes, duplicate microhematocrit tubes filled, and either 1 or 2 ml aliquots pipetted for radioactivity measurements. A Nuclear-Chicago well-scintillation counter with automatic sample changer made 30 minute radioactivity measurements on each sample. These protracted measurements were desirable in view of the small amounts of total body Fe^{59} involved (usually a fraction of a microcurie). When Cr^{51} and Fe^{59} were present concurrently, their individual contributions to the sample count rate were resolved by pulse height analysis. All counts were corrected for radioactive decay and fluctuations in efficiency by reference to appropriate standards. Microhematocrit tubes were centrifuged in an International microcapillary centrifuge, model MB and read on an International microcapillary reader, model CR. The sample count rate per ml. of blood divided by the hematocrit reading yielded the sample count rate per lm. of erythrocytes.

A slight modification of the Cornell technique (Whitlock, 1941) was used for fecal egg counts. Specimens were collected from the pens or cages of individually confined dogs. Subsamples of 10 grams were suspended in 150 ml. of water with an electric mixer, the suspension passed through a tea strainer to remove the larger particles of inert material and duplicate 0.30 ml. aliquots drawn with a large bore pipette. Each aliquot was added to a 0.30 ml pool of saturated sucrose solution in the counting chamber and the two materials thoroughly mixed with a dissecting needle. All eggs in each aliquot were counted and the average of these two counts expressed as eggs per 0.02 gm feces.

Erythrocytes were labeled with Cr^{51} in vitro by incubating 50 microcuries of sodium chromate 51 (Abbott) with 5-10 ml. of heparinized whole blood. These erythrocytes were then washed three times with 0.85% sodium chloride solution to remove unbound Cr^{51} . Erythrocytes were labeled with Fe^{59} in vivo by injecting 500 microcuries of Fe^{59} citrate (Abbott) intravenously into donor dogs. Erythrocyte labeling occurred in the course of normal hemoglobin synthesis and blood containing adequate concentration of labeled erythrocytes could be obtained by venipuncture at any time from 1 to about 6 weeks after administration of the Fe^{59} .

Restraint of dogs for whole-body counting.

The restraint box used in these experiments for whole-body counting of fully conscious dogs is diagrammed in figure 1. The length and depth of the animal chamber were varied to accommodate dogs of different sizes by substituting appropriate end and bottom supports. The center of the animal chamber was used as a reference point to position the restraint box in the whole-body counter. This helped minimize shifts in detection efficiency that might otherwise have attended enlargement of the animal chamber necessitated by the growth of pups. The high precision obtained in normal Fe^{59} retention measurements (v.i.) attests to the effectiveness of this restraint technique.

Fe^{59} retention by hookworm-free dogs.

Normal Fe^{59} retention was measured 25 times over a period of 69 days in 5 collie-shepherd pups (group 3a) and 120 times over a period of

169 days in 2 adult female beagles (group 1).

Reabsorption of Fe^{59} from the lumen of alimentary tract.

Surgical preparation of dogs for intraduodenal injection of Fe^{59} : Five mongrel dogs (group 4) were fasted overnight, anesthetized with sodium pentobarbital and prepared for right flank laparotomy. A 2 inch incision was carried through all layers of the abdominal wall about 1 inch below and parallel to the lumbar muscles. The peritoneum and transverse fascia were sutured to a 2 x 3 cm. elliptical area of descending duodenum and then in turn to the subcutis thus bringing the edges of the skin incision into apposition with the borders of the exposed portion of duodenal wall. The incisions healed uneventfully and the exposed area of the duodenal wall became covered with epidermis. This preparation permitted insertion of a 12 gauge needle into the duodenal lumen without observable discomfort to the dogs.

Each dog was infected with A. caninum by depositing 300 larvae in the buccal cavity.

Two trials were conducted in tandem with these five dogs. In the first, Fe^{59} labeled erythrocytes were injected into the duodenum of two dogs chosen at random and administered by stomach tube to the other three. The second trial was identical to the first except that Fe^{59} was now administered by stomach tube to the two dogs previously dosed by duodenal injection and by duodenal injection to the three dogs previously dosed by stomach tube.

In order to obtain an unbiased estimate of the proportional Fe^{59} absorption, it was necessary to account for loss of absorbed Fe^{59} resulting from the blood sucking activities of the hookworms during the period of observation, and in the case of the second trial, to account for the residual label remaining from the first dose. The following analysis was found to satisfy both requirements:

The linear regression of log (decay corrected, whole-body Fe^{59} counting rate) vs. time was calculated from the third day after Fe^{59} administration until the second dose or the end of the experiment. The time-zero value predicted by this equation represented the total Fe^{59} retention with the effects of excretion and blood loss removed. In the first trial, this figure represented the amount absorbed. The residual Fe^{59} predicted by the first trial regression equation was subtracted from the time zero value of the second trial regression equation to obtain the amount absorbed in the second trial.

Comparative absorption of Fe^{59} and Cr^{51} from labeled erythrocytes.

Homologous Fe^{59} erythrocytes labeled in vivo were also labeled in vitro with Cr^{51} and administered by stomach tube to the group 6 pups. This experiment preceded all of the other involving this group. Whole-body retention of Cr^{51} and Fe^{59} was measured over a period of 11 days.

Whole-body Fe^{59} counting efficiency as a function of in vivo distribution of the tracer.

Iron-59 ferrous citrate was injected intravenously into 5 pups (group 5). Whole-body counts were taken on each pup immediately following the injection, and blood samples were drawn for assay of radioactivity at 3 to 4 minutes.

Comparison of Fe^{59} and Cr^{51} estimates of erythrocyte loss.

Homologous, in vivo Fe^{59} labeled erythrocytes and autologous, in vitro Cr^{51} labeled erythrocytes were simultaneously injected intravenously into 7 beagle pups (group 6). Four pups selected at random were given 150 A. caninum larvae by stomach tube and 3 were held as controls. Whole-body retention and erythrocytes concentration of each tracer were measured twice weekly for five weeks. Whole-body retention values were corrected, in each case, for losses due to blood sampling.

The volume of erythrocytes lost between observations was calculated by dividing the observed reduction in whole-body tracer retention by the geometric mean of the corresponding erythrocyte tracer concentration values.

Relationship of fecal egg counts to erythrocyte loss estimates.

Erythrocyte loss and worm egg production were measured in 5 collie-shepherd (group 3a) and 2 terrier cross (group 3b) pups over a period of 14 weeks after the oral administration of 200 A. caninum larvae per individual.

At the sixth week post-infection, group 3a was removed to gravel surfaced outdoor runs and group 3b retained indoors on concrete. This step was necessitated by space limitations and was not part of the experimental design. However, the increased opportunity for reinfection in the outdoor runs is relevant to the interpretation of the results.

Results

Fe^{59} retention by hookworm-free dogs.

Regressions of $\log_e$ whole-body Fe^{59} on time did not differ significantly among pups (group 3a) ($F(4, 115) = 1.45$). Their average slope, -0.00346 ± 0.000125 , was equivalent to a daily loss of 0.346% of the total body Fe^{59} or, roughly, 1.7 ml/day of erythrocytes. Two adult beagles (group 1) displayed a lower Fe^{59} removal rate of 0.094% per day (average slope -0.000937 ± 0.0000261). It should be pointed out that erythrocyte loss estimates calculated by dividing the observed reduction in whole-body tracer retention by the geometric mean of the corresponding erythrocyte concentration values would display greater variation than is implied by the rather modest error of slope.

Reabsorption of Fe⁵⁹ from the lumen of the alimentary tract.

Ideally, all of the tracer extracted by hookworms would pass out with the feces because reabsorption of a given proportion would lead to equivalent underestimation of erythrocyte loss.

We found minimal absorption of Fe⁵⁹ by sheep after oral and abomasal administration of labeled erythrocytes and labeled hemoglobin (Georgi, 1964b; Djurdjevic and Georgi, 1967) and therefore did not correct for tracer reabsorption in our haemonchosis work. However, the significant Fe⁵⁹ absorption by humans that has been reported (Roche and Perez-Gimenez, 1959; Turnbull, Cleton and Finch, 1962) prompted the re-investigation of this potential source of error in the experiments with dogs reported here.

A priori, stimulation of the conditions of interest demanded the deposition of Fe⁵⁹ labeled erythrocytes in the duodenal lumen of A. caninum infected dogs. To facilitate luminal injection and to minimize the risk of accidental intraperitoneal injection, the duodenum was surgically fixed in the right flank as described under Materials and Methods.

No significant effect attributable to route of administration was observed in either trial. The mean Fe⁵⁹ absorption values were: gastric, 5.8% duodenal, 8.5%; standard error of the difference 3.6%, for the first trial and gastric, 3.2%; duodenal, 6.4%; standard error of the difference 3.0% for the second trial.

The absorption of Fe⁵⁹ from labeled erythrocytes was studied further in 4 A. caninum infected pups and 3 uninfected controls (group 6) just in case the duodenal adhesion preparation of the previous experiment might have altered the normal absorption process. Labeled erythrocytes were administered by stomach tube and whole-body Fe⁵⁹ retention was measured over a period of two weeks. The percentage of Fe⁵⁹ absorption was calculated according to the analysis outlined above.

No significant difference in Fe⁵⁹ absorption between infected and noninfected pups was observed. The mean Fe⁵⁹ absorption values were: Infected, 2.1%; controls 5.6%; standard error of the difference 2.3%.

Comparative absorption of Fe⁵⁹ and Cr⁵¹ from labeled erythrocytes.

No measurable Cr⁵¹ remained in any pup after the third day but a mean of 5.5 ± 1.1% of the Fe⁵⁹ was absorbed and retained, without any detectable Fe⁵⁹ excretion occurring during the period of observation.

Whole-body Fe⁵⁹ counting efficiency as a function of in vivo distribution of the tracer.

With constant erythrocyte volumes and negligible Fe⁵⁹ loss during the examination period, the percentage of initial blood Fe⁵⁹ concentration would represent the proportional distribution of tracer between the

circulating blood and other tissues, and the percentage of initial whole-body Fe^{59} count rate would represent the counting efficiency relative to that observed when all of the tracer was in the circulation. At the time of this experiment, the dogs were under no significant hemorrhagic stress and the observed exponential rate of Fe^{59} loss from day 6 to 23 was only 0.65% per day.

The regression equation of $\log_e$ % initial whole-body Fe^{59} counting rate on $\log_e$ % initial blood Fe^{59} concentration, calculated from the pooled group 5 data for days 1, 2 and 3 and corrected for initial tracer loss* was: $Y = 4.796 - 0.0415X$. This equation is almost identical to the equation reported for sheep (Georgi and Whitlock, 1965). Although statistically significant ("t" for 13 d.f. H:b = 0 was 3.61), it predicts less than 2% variation in efficiency attributable to the range of tracer distributions (60-100% erythrocyte incorporation) usually encountered 1 to 2 weeks after dosing and thereafter. Therefore, counting efficiency fluctuations brought about by shifts in tracer distribution may, for practical purposes, be ignored in Fe^{59} whole-body counting provided at least 60% of the total Fe^{59} remains in the circulating erythrocytes. At less than 60% incorporation, significant systematic error exists.

Comparison of Fe^{59} and Cr^{51} estimates of erythrocyte loss.

The controls suffered a mean cumulative 5 week loss of 19 ± 8 ml as measured by Fe^{59} and a mean cumulative 5 week loss of 112 ± 20 ml as measured by Cr^{51} . The difference in estimates is probably attributable to elution and excretion of Cr^{51} . The difference in estimates is probably attributable to elution and excretion of Cr^{51} and is not to be confused with the so-called "normal" fecal blood loss as measured by techniques based on fecal Cr^{51} assay.

In order to isolate the effect of hookworms and render the Fe^{59} and Cr^{51} estimates comparable, the control values were subtracted from the cumulative erythrocyte loss of each A. caninum infected pup. Thus, Fe^{59} estimates of cumulative erythrocyte loss were reduced by 19 ml and Cr^{51} estimates were reduced by 112 ml. The resulting "control-corrected" estimates differed on the average by a nonsignificant 13 ± 9 ml of erythrocytes.

* The day zero values were excluded from the calculations because our experience with sheep has shown that a small portion of an intravenous dose of Fe^{59} citrate is usually excreted during the period between injection of the tracer and its initial removal to the bone marrow and other tissues. This loss modifies the intercept but has no effect on the slope of the regression equation. The corrected intercept was calculated using an arbitrary (100% 100% coordinate in place of the mean values of the variables.

Relationship of fecal egg counts to erythrocyte loss estimates.

Erythrocyte loss estimates and egg counts of each group 3 pup are plotted against time in Figure 2. Radioactive decay and cumulative loss of labeled erythrocytes resulted in considerable fluctuations in the erythrocyte Fe^{59} concentration measurements due to low counting rate of samples toward the end of the 100 day observation period. Although the whole-body Fe^{59} retention measurements remained stable, both sets of data were subjectively fitted with regression curves and values taken from these for the calculation of erythrocyte loss. I doubt that this procedure introduced bias although, as comparison with the group 6 data of Figure 3 suggests, detail was probably lost as a result of the consequent averaging effect.

The maximum rate of erythrocyte loss was observed during the period before the onset of egg production in 6 out of 7 cases. All dogs transferred to gravel runs and one of the two kept indoors displayed an initial maximum followed by a minimum and then by another maximum. The underlying cause of the second maximum cannot be established with certainty although reinfection cannot be ruled out.

After initial oscillations, the egg counts tended to vary in sense and in degree with the erythrocyte loss estimates although, as variations in the proximity of the curves reveal, the ratio of one to the other varied with time and among pups.

The time of onset of erythrocyte loss was determined more precisely by comparing the erythrocyte loss estimates of 3 control and 4 A. caninum infected pups (group 6). A significant difference was first observed during the period from 7 to 10 days post infection ($0.01 < P(4.5 \text{ df.} = 2.69) < 0.025$). The erythrocyte loss rose rapidly to a regional maximum that peaked between the twelfth and sixteenth days. The peak, occurring during the late prepatent period, was in each case the highest value observed during the course of the experiment. The daily rate of erythrocyte loss of the four A. caninum infected pups (group 6) is plotted against time in Figure 3.

Discussion

Normal Fe^{59} retention

Normal adult animals are exemplified by the group 1 beagles, suffer only very slow reduction in total body Fe^{59} retention. The more rapid removal rates exhibited by the pups of groups 3a and 6 may be attributed variously or collectively to: 1) bona fide erythrocyte loss of unidentified cause, 2) excretion of Fe^{59} unrelated to erythrocyte loss or 3) a reduction in detection efficiency such as might be brought about by increased gamma self-absorption in the growing pups. Miller (1966) designated an analogous Cr^{51} loss observed during the prepatent period (patency referring to onset of blood loss, not egg production) as "normal" fecal blood loss. This loss, equivalent to 0.05 to 2.1 ml of blood/day, was comparable to the control erythrocyte loss estimates reported above. From the standpoint of erythrocyte loss estimation, the identities of the processes

involved are less important than objective assay of their net effect. For exact estimation of erythrocyte loss in absolute terms, a relevant control set could conceivably be carried for each treatment set. This is discussed in greater detail below.

Fe⁵⁹ reabsorption

The unusually high Fe⁵⁹ reabsorption values (range 13.1) to 75.4%) reported for human subjects by Roche and Perez-Gimenez (1959) were derived by subtracting the endogenous fecal iron as measured by fecal Fe⁵⁹ from an estimate of intestinal iron loss based jointly on fecal blood loss (Cr⁵¹ estimate) and the blood hemoglobin concentration. Possibly this novel technique contains an unidentified systematic bias. Roche and Layrisse (1966) have reported that: "Studies in progress in our laboratory have shown that, when Fe⁵⁹ and Cr⁵¹ tagged rabbit hemoglobin is administered to humans, the value for iron absorbed is much less when determined from the resulting circulating Fe⁵⁹ than when calculated from the fecal difference between Cr⁵¹ and Fe⁵⁹ excretion, although they sought other explanations than the one I have suggested for the discrepancy.

If, in any experimental situation, uncertainty exists relative to the extent of Fe⁵⁹ reabsorption it can be dispelled empirically by conducting absorption trials in tandem with the principal observation period. These trials may include every experimental animal and not interfere materially with the erythrocyte loss estimates except during the first 3 or 4 days after alimentary administration of labeled erythrocytes.

Correction of erythrocyte loss estimates for reabsorption, extraneous losses and variation in detection efficiency of Fe⁵⁹ .

Inherent in any deliberate effort to control systematic error by additive or multiplicative correction terms, is the tacit but frequently ignored assumption that controls are similar to treated units in all respects but one. Such efforts, though pious, are often misdirected.

Suppose, for the sake of argument, that we wish to correct our data for extraenous "normal" blood loss. We may, as in the group 6 data of this report, subtract the values obtained from control animals from those obtained from hookworms infected ones and almost no one will object. However, what assurance have we that our dogs will continue to suffer "normal" losses after they have become infected with hookworms? In the experiment comparing Fe⁵⁹ and Cr⁵¹ erythrocyte loss estimates (group 6), the correspondence of estimates lends credibility to the application of this correction. But this is, after all, a circular argument because we have set out to compare estimates and wind up judging the validity of the analysis by the results of the test.

We have consciously neglected to correct for "normal" erythcoryte loss in our haemonchosis work (Whitlock et al., 1966; Georgi & Whitlock, 1967) because 1) we were principally interested in comparisons rather than absolute values, 2) the "normal" loss estimates tended either to be negligible or so variable as to cause us to doubt their relevance to the

physiopathological status of the infected sheep.

Thus, erythrocyte loss estimates may be corrected for "normal" Fe^{59} losses and for Fe^{59} reabsorption by conducting tandem and parallel control experiments provided the controls are biologically relevant. However, these two sources of systematic error tend to balance each other so the benefits to be derived from applying modest corrections with immodest error terms appears limited indeed. The controls are absolutely essential for comparison and to avoid major pitfalls in interpretation but their data needn't necessarily generate correction factors.

Fluctuations in whole-body counting efficiency related to variations in the distribution of Fe^{59} within the body are generally trivial except during large scale shifts as may occur, for example, during the period of initial Fe^{59} incorporation and mobilization of the newly labeled erythrocytes. Such fluctuations are amenable to correction, when necessary, through an empirical equation like the one reported above.

One point relative to the interpretation of tracer data deserves careful consideration and this applies to whole-body as well as fecal assay of either Fe^{59} or Cr^{51} . In each case, the tracer is associated with the erythrocytes, only minor concentrations being found in the plasma. Therefore, any observed reduction in whole-body retention or accumulation of tracer in feces explicitly represents (ignoring elution, excretion, etc.) a corresponding transfer of erythrocytes. The interpretation of such changes as "blood loss" carries with it an implicit assumption that, so far as I can determine, has yet to receive the critical examination it deserves. This assumption is that the concentration of erythrocyte in the blood passing through the gut of the worm is the same as that drawn from a subcutaneous vein. At least two subsidiary assumptions immediately present themselves: 1) equal erythrocyte concentrations must exist in the blood of mesenteric and peripheral veins and 2) the worms must not discriminate between erythrocytes and plasma. These assumptions could indeed prove valid but we can't be sure until a suitable plasma label is tested simultaneously with an erythrocyte label. In the meanwhile, the expression of estimates based on erythrocyte labels as "blood loss" must be viewed as imprecise at best.

Relationship of erythrocyte loss to egg production.

The occurrence of peak erythrocyte loss during the period preceding the appearance of eggs in the feces has been observed before (Miller, 1966; Clark *et al.*, 1961). This behavior is in sharp contrast to the sheep - *Haemonchus contortus* *cayugensis* system (Whitlock *et al.*, 1966) and is therefore of considerable comparative physiological interest.

The close relationship observed between erythrocyte loss and egg counts indicates that a major portion of egg count fluctuations represents real changes and not mere random error.

Comparison of labels and techniques.

Iron-59 and Cr^{51} have certain contrasting properties as erythrocyte

labels that bear on their suitability for various experimental applications. As a label for erythrocyte loss estimation, Fe^{59} presents at least two significant advantages over Cr^{51} .

1. Iron-59 labels new erythrocytes with a normal life expectancy of months as an integral part of the heme nucleus; a permanent label for the life of the erythrocyte. Chromium-51, on the other hand, labels erythrocytes of random ages and is lost from the system by elution of label and senescence of labeled cells. Thus, Fe^{59} affords a longer period of observation than does Cr^{51} . Usually a proportion of the Fe^{59} dose is retained initially in body iron reserves and subsequently mobilized in response to hemorrhagic stress. This tends to prolong the period of efficient observation still further.

2. Iron-59 has a high energy gamma emission (1.1 and 1.3 Mev) suitable for whole-body counting or large-sample counting whereas internal absorption of the lower energy Cr^{51} gammas (0.33 Mev) in large masses of tissue or excrement hinders efficient measurements of Cr^{51} . Although this disadvantage can be overcome by using more Cr^{51} , the benefits of working with low levels of radioactivity are thereby lost.

A most conspicuous disadvantage of whole-body counting as opposed to fecal sampling techniques is the initial cost of the instrument. Our whole-body counter which is large enough to accommodate people, sheep or large pigs (we have yet to devise a restraint technique for pigs that renders the work enjoyable), cost \$70,000. However satisfactory systems for whole-body counting of rats, rabbits, cats or small dogs are only slightly more expensive than ordinary sample counting equipment. If fecal assay must be resorted to for larger animals, these smaller systems permit radioactive assay of total fecal collections thus overcoming the necessity and uncertainty of subsampling.

The advantages of whole-body counting over fecal assay for the estimation of erythrocyte loss are otherwise overwhelming:

1. Separation of urine and quantitative collection of feces is unnecessary with whole-body counting. This affords great freedom of experimental design requiring only that the animals (or people) be rounded up periodically for whole-body counting and collection of blood samples. The fecal assay method, on the other hand, requires bending the experimental design around the metabolism cage or the bedpan, as the case may be, and enduring the restrictions imposed by these devices.

2. The high precision inherent in measurement of substantial quantities of radioactivity (e.g., the animal body) permits use of much smaller individual doses in the case of whole-body counting than when subsamples representing small fractions of the total radioactivity are assayed. We have found 1 to 2 microcuries of Fe^{59} sufficient for whole-body counting experiments lasting months as compared with reported use of 80 microcuries (Roche, *et al.*, 1957) to 1 millicurie (Miller, 1966) of Cr^{51} than of Fe^{59} for whole-body counting but for a different reason; our liquid scintillation counter is less efficient than a crystal scintillation counter in detecting low energy gammas.

Low doses of radioactivity made possible by the whole-body counting

technique reduce the hazards of individual exposure and environmental contamination to the point where safe and practical field application may be realized.

Surely any large scale investigation of human hookworm disease would benefit from the advantages offered by the whole-body counter.

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Table I. Characteristics and Functions of the Experimental Dogs.

Group	Breed	Number	Function
1	beagle	2 females	normal Fe ⁵⁹ retention
2	basset cross	1 female	{ donors of Fe ⁵⁹ labeled erythrocytes.
	beagle cross	1 male	
3	A) collie-shepherd cross litter mates	2 females 3 males	normal Fe ⁵⁹ retention. experimental <u>A. caninum</u> infection
	B) terrier cross litter mates	2 females	Experimental <u>A. caninum</u> infection
4	mongrel litter mates	5 females	Fe ⁵⁹ reabsorption
5	mongrel litter mates	5 females	Whole-body counting efficiency vs. Fe ⁵⁹ distribution study.
6	A) beagle litter mates	1 female 1 male	{ Fe ⁵⁹ reabsorption; Experimental <u>A. caninum</u> infection
	B) beagle litter mates	3 females 2 males

Figure 1. Restraint box for whole-body counting of conscious dogs.

Figure 2. Weekly erythrocyte loss (solid line) and egg counts per 20 mg feces (dashed line) as a function of time after the administration of 200 infective *A. caninum* larvae to group 3a (5 lower pairs of curves) and group 3b (2 upper pairs of curves) pups. The ordinal scale is logarithmic and is repeated for individual pups.

Figure 3. Daily erythrocyte loss as a function of time after administration of 150 infective A. caninum larvae to group 6 pups. Control data are recorded in Table II.

Phase II -- Planning Study for Evaluation of Radioactive
Contamination of the Food Chain

Studies under Phase II during this period have been primarily concerned with:

1. Urine/Diet Studies Among Teenagers -- Continuation of
Alternative Methods Development for Estimating
Dietary Sr^{90}
2. Pooled Urine Samples for Estimating Radionuclide Levels.
3. Non-Milk Contribution of I^{131} to Diet
 - a) Comparison between milk and non-milk foods
 - b) Experimental I^{131} studies in pasture and food plants.
4. Decontamination Processes and Dietary Sr^{90} Intake as
Influenced by Developmental Status
5. Radionuclide Plant and Soil Relationships Under Normal
Cultivation Practices
6. Climatological Aspects of Sr^{90} Levels in Milk.

1. Urine/Diet Studies Among Teenagers -- Continuation of
Alternative Methods Development for Estimating Dietary Sr^{90}
J. C. Thompson, Jr.

Abstract: Studies conducted with two groups of teenagers (13 and 16 years) further quantify the age dependent relationship of the $OR_{urine/diet}$ which was observed in earlier studies among adults and pre-school children. Incorporation of the teenage data into prediction equations gives a more reliable base for the use of urinary samples to estimate dietary Sr^{90} .

Introduction

Since urinary calcium excretions are considerably age dependent, specific studies are needed to relate this dependency to the $OR_{urine/diet}$. Under normal dietary calcium intakes (.7 to 1.0 grams per day) urinary excretions have been reported as follows (1):

<u>Age</u>	<u>Calcium Excretion (mg/day)</u>
1-4	47
5-9	79
10-14	94
15-19	178
20 and over	167

Previous studies in this department have shown that in the young age group (4-5 years) and the adult group (21-33 years) there is a definite age dependent association between urinary calcium and the $OR_{urine/diet}$. On the basis of normal urinary calcium excretions it seemed necessary to examine other age groups in order to fully characterize the $OR_{urine/diet}$ relationship. Specifically, some observations seemed necessary in the teenage category. The indicated change in urinary excretions between the ages of 10-14 and 15-19 were of the greatest magnitude and were chosen for further study. The 15-19 age group was similar to adults in excretion patterns, whereas the 10-14 age group excreted about half the adult level. In addition, the 10-14 age group could provide some insight for the 5-9 age group because of the similarity in calcium excretions.

Methods

Two groups of children were selected for study - one from junior high school, averaging 13 years, and the other at high school level averaging 16 years. Fullst cooperation was obtained from the school and parents. The study was considered to be an excellent complement to biology studies being conducted in the school during this period. School officials incorporated the ideas and aims of the study into their classwork as examples of biological research and an awareness of metabolic pathways.

After detailed consultations with teachers and parents, ten male subjects were selected for each group. The subjects were required to retain duplicates of all foods eaten and to make total urinary collections. Emphasis was placed on the critical nature of the experiment insofar as completeness was concerned. Omissions were to be noted on daily record sheets and would be excluded from the compositing process. Special eating facilities were reserved in the school cafeteria and food service personnel exercised the strictest control over portion servings. Urinary collections were supervised by the school nurse and study personnel.

The study was conducted for four consecutive days. Food and urine samples representing morning and evening collections were under the supervision of parents. Collections during the school day were supervised by study personnel and all subjects completed the study. Payment for food costs incurred during the study amounted to \$5.00 per person per day. Follow-up reports of the results were forwarded to each participant.

Results

Average intake data, ages and weights of the junior high school group are shown in Table 1. Sr^{90} intakes averaged about 31 picocuries per day, ranging from 14 to 52 picocuries. Calcium intake averaged 1.16 grams per day with only one subject having less than .9 gram calcium. The $\text{Sr}^{90}/\text{gCa}$ ratios ranged from 15 to 35.

Food intake levels for the high school group were somewhat higher and more variable than junior high levels with calcium intake being noticeably greater. Table 2 shows that high school calcium intakes were about 1.6 grams per day and Sr^{90} intakes averaged 40 picocuries per day. However, the dietary $\text{Sr}^{90}/\text{gCa}$ ratio was quite similar in both groups, averaging 24.9 for high school and 26.6 for junior high school. Comparable values for diet samples taken in the Northeast during this same period averaged 22.8 $\text{pCiSr}^{90}/\text{gCa}$ (2,3).

Daily urinary excretions for the junior high group are given in Table 3. Urinary calcium levels were lower than expected, averaging 52 mg per day. This was also true for Sr^{90} levels with less than 1 picocurie being excreted per day. The resulting average $\text{Sr}^{90}/\text{gCa}$ ratio was 13.3.

Subjects in the high school group had considerably higher calcium and Sr^{90} excretions on a daily basis, as shown in Table 4. Average calcium excretions were 115 mg per day and average Sr^{90} excretions were 1.6 picocuries per day. Considerable variation in calcium excretions was evident among the subjects (ranging from 18 to 262 mg per day). However, the $\text{Sr}^{90}/\text{gCa}$ average of 17.9 compares favorably with expected values.

In comparing $\text{OR}_{\text{urine/diet}}$ data, the high school group had much higher OR's than the junior high group (0.82 versus 0.52). Table 5 shows these results by individual subjects. The low OR's for the junior high group are consistent among all subjects except one.

Discussion

Previous studies have shown the feasibility of using urinary samples for monitoring dietary Sr^{90} levels (4,5,6,7,8). The basis for this approach depends on whether variations in the $\text{OR}_{\text{urine/diet}}$ are compatible with the accuracy necessary for purposes of food monitoring from a health physics standpoint. In general, experiments have established the feasibility of this approach provided that the samples represent normal, healthy persons consuming diets that are indicative of the general population intake. During periods of rapidly increasing dietary $\text{Sr}^{90}/\text{gCa}$ levels the use of the $\text{OR}_{\text{urine/diet}}$ concept would tend to underestimate the dietary value for the few days immediately following the sudden increase. However, it is unlikely that such sudden increases could occur without the observance of numerous other indicators of sudden releases or depositions. Thus, a forewarned knowledge of such increases would permit an awareness of possible underestimations for $\text{OR}_{\text{urine/diet}}$ calculations for the short time period following the release. This would not seriously reduce the value of urinary observations for purposes of routine monitoring; particularly when the long term hazards of Sr^{90} are to be considered.

In addition to the use of the $\text{OR}_{\text{urine/diet}}$ concept for estimating dietary Sr^{90} levels, there is a need to refine the relationship between urinary calcium and the $\text{OR}_{\text{urine/diet}}$. Such a relationship has been discussed and good correspondence between theoretical values and observed data have been shown (8). The equation has been described as follows:

$$\text{O.R.} = 26.2 (\text{U}_{\text{Ca}})^{-0.7} \quad (1)$$

This equation was shown to be applicable for data from the adult and preschool children studies. When these data were combined with the junior high school and high school observations, the equation was modified as follows:

$$\text{O.R.} = 5.21 (\text{U}_{\text{Ca}})^{-0.38} \quad (2)$$

The modified equation now incorporates data for the four age groups and has a smaller constant and exponent. Figure 1 shows a log-log plot of the individual observations for all four groups (adults, high school, junior high and preschool children). The line of best fit as described by equation 2 is also shown.

In general, the observations follow expected patterns as based on known urinary excretions and expected Sr^{90} and calcium interrelationships. However, data for the junior high group appear to be lower than expected with an average $\text{O.R.} = .52$. Values in this group were consistently low except for one subject with an $\text{O.R.} = .86$. All remaining O.R. 's were less than 0.7. The lower O.R. 's might be the result of changed metabolic patterns during this stage of growth or counting errors associated with the low levels present in urinary samples. Some comparisons can be made with the other study groups on the basis of relative urinary excretion rates, as shown in Table 6. It is evident that the junior high group had a much lower calcium excretion rate and an even greater reduction in Sr^{90} urinary excretions. Further studies of this group seem necessary in order to resolve the lower than expected urinary Sr^{90} excretion rate and smaller O.R. values.

Data relevant to the Cs¹³⁷ determinations on these studies (Diet, Urine and Whole Body Levels) have been reported previously (9).

Table 1. Calcium and Sr^{90} Intakes of Junior High Students
Lansing Central School - 1966

Subject	Age	Weight lbs.	Total daily intake(kg)	Daily Ca intake (g)	Daily Sr^{90} intake(pCi)	Sr^{90}/Ca (pCi/g)
1	13	132	1.12	0.55	15	26.8
2	13	122	1.55	1.06	37	35.2
3	13	88	1.38	1.15	24	21.0
4	13	96	1.20	0.91	14	15.5
5	13	93	2.10	1.89	52	27.8
6	13	109	1.40	0.82	24	29.9
7	13	75	1.55	1.11	38	34.5
8	12	105	1.90	1.45	46	31.5
9	13	86	1.89	1.51	26	17.4
Average			1.566	1.16	31	26.6

Table 2. Calcium and Sr^{90} Intakes of High School Students
Lansing Central School - 1966

Subject	Age	Weight lbs.	Total Daily Intake(kg)	Daily Ca Intake(g)	Daily Sr^{90} Intake(pCi)	Sr^{90}/Ca (pCi/g)
1	16	127	2.00	1.65	60	36.2
2	16	148	2.38	1.93	25	12.8
3	16	144	1.95	1.10	35	31.7
4	17	143	1.83	1.51	23	15.2
5	16	136	2.07	1.40	51	36.3
6	16	110	1.98	2.08	83	39.9
7	16	159	3.12	1.91	39	20.4
8	16	145	1.64	0.84	15	17.6
9	16	131	2.06	1.50	22	14.9
10	16	136	2.45	1.99	44	21.9
Average			2.15	1.59	40	24.7

Table 3. Urinary Excretions of Ca and Sr^{90} by Junior High Students
Lansing Central School - 1966

Subject	Urine Vol. (ml/day)	Ca in Urine (mg/day)	Sr^{90} in Urine (pCi/day)	Sr^{90}/Ca in Urine (pCi/day)
1	782	70	0.57	8.2
2	921	30	0.46	15.3
3	800	51	0.60	11.7
4	630	72	0.75	10.4
5	994	32	0.35	10.9
6	899	33	0.85	25.6
7	740	49	0.67	13.6
8	944	101	1.27	12.6
9	759	33	0.39	11.8
Average	830	52	0.66	13.3

Table 4. Urinary Excretions of Ca and Sr^{90} by High School Students
Lansing Central School - 1966

Subject	Urine Vol. (ml/day)	Ca in Urine (mg/day)	Sr^{90} in Urine (pCi/day)	Sr^{90}/Ca in Urine (pCi/g)
1	1067	58	1.81	31.5
2	917	133	2.44	18.3
3	768	57	1.12	19.7
4	1173	77	1.83	23.6
5	1400	262	1.82	7.0
6	1036	18	0.49	27.8
7	1653	172	2.13	12.4
8	1055	115	2.04	17.7
9	832	160	1.89	11.8
10	1108	99	0.91	9.2
Average	1101	115	1.65	17.9

Table 5. Comparison of $OR_{\text{urine/diet}}$ Between Junior High and High School Groups, Lansing Central School - 1966

Subject	Junior High Group OR	High School Group OR
1	0.31	0.87
2	0.44	1.43
3	0.56	0.62
4	0.67	1.55
5	0.39	0.19
6	0.86	0.70
7	0.39	0.61
8	0.40	1.01
9	0.68	0.79
10	*	0.42
Average	0.52	0.82

* Subject collected combined samples (Urine and Feces).

Table 6. Relative Urinary Excretion Rates of Sr^{90} and Ca
Among Various Age Groups under Controlled Studies

	Sr^{90}	Urinary Excretions Calcium
Preschool (a)	(% of intake)	
Period I	10.0	5.9
Period II	11.9	6.2
Junior High (b)	2.1	4.5
High School (b)	4.2	7.2
Adult (c)		
Male	12.0	15.1
Female	10.3	10.5

a) Studies conducted in 1965

b) Studies conducted in 1966

c) Studies conducted in 1964

Figure 1. Relationship between $OR_{urine/diet}$ and urinary calcium (x = children, • = adults, □ = high school, ◊ = junior high).

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2. Pooled Urine Samples for Estimating Radionuclide Levels J. C. Thompson, Jr.

Abstract: Sampling procedures and collection patterns have been developed for urinary sampling of defined populations. Analytical problems have prevented accurated estimation of dietary Sr^{90} levels from urinary samples. Poor Sr^{90} and calcium determinations by commercial laboratories as well as losses incurred in sample shipments have delayed the verification of this method of estimation. Further attempts at resolving these problems include laboratory intercomparisons and repeat of the experiment in which samples were lost in transit.

Cs^{137} determinations on urinary samples from this project have been used to estimate body burdens with good accuracy.

Strontium - 90

Introduction

Techniques developed from controlled dietary and urinary studies indicate the feasibility of using urinary analyses to estimate dietary Sr^{90} levels. The concept of an $\text{OR}_{\text{urine/diet}}$ relationship can be used directly in making such estimates. This method requires the analysis of urine samples for $\text{Sr}^{90}/\text{gCa}$ and a projection of dietary levels based on an understanding of the $\text{OR}_{\text{urine/diet}}$ for the respective group or population represented in the sample. However, since it has been established that the $\text{OR}_{\text{urine/diet}}$ varies with age, it is desirable to determine more precise estimates of this value. A better understanding of this relationship has been described and estimates of the specific $\text{OR}_{\text{urine/diet}}$ can now be made from average daily urinary calcium excretions. Once the proper $\text{OR}_{\text{urine/diet}}$ is determined it can be used with values obtained from urinary Sr^{90} analyses to estimate dietary levels. The use of one item (urine) for such estimates contrasts with the problems of determining representative diets from the many food items normally consumed.

Methods

The simplest and most precise method for collecting urinary samples must involve the medical community. Since one of the commonest tests undertaken during routine physical examinations involves a urine sample, it is logical to design the sampling plan around this practice. All members of the medical profession in the city of Ithaca were contacted and asked to cooperate in a project involving urinary collections. Seven pediatricians (total in the city) and five general practitioners were selected to participate in the study. The general plan was to collect unused urinary samples and pool them into specific age categories for Sr^{90} and calcium analyses.

The scope and intent of the study was described to each doctor and staff. Measuring devices, collection bottles and daily report forms were provided. Urine samples were to be collected from each person considered to be in normal health. Each sample consisted of 25 ml which was to be pooled into collection bottles on the basis of the following age categories:

- a) 1 year to 4 years and 6 months
- b) 4 years and 7 months to 9 years and 6 months
- c) 9 years and 7 months to 14 years and 6 months
- d) 14 years and 7 months to 19 years and 6 months
- e) 19 years and 7 months to 24 years and 6 months
- f) 24 years and 7 months and over

Samples were taken for individuals living in the immediate Ithaca area. Sampling was continued for a 90-day period during July-August-September, 1966. Analyses were performed on the pooled samples for Sr^{90} , Cs^{137} , K^{40} and calcium.

Results

The successful completion of the study was due to the full cooperation of the medical practitioners in the Ithaca area. Samples were obtained for all age groups except the 19-24 year group. Samples for this group could have been obtained if the collection period had been extended but it was felt that a 90-day period was adequate for testing.

Laboratory analyses of urinary samples for Sr^{90} have been extremely variable. Results from one commercial laboratory are not comparable with expected values for samples taken during this period. Table 1 shows the nature of the variations encountered in Sr^{90} determinations. Samples from the same age group had determinations varying by factors of 2 to 5. Repeat analyses of some of these samples did not improve the range in variability. Calcium determinations were also questionable in some instances because of the high values reported for samples from the younger age groups. For example, if normal excretion rates were applied to calcium determinations for the three youngest age groups in Table 1, the calcium excretions would be nearly twice the average expected levels. In view of the counting difficulties, no conclusions will be drawn for this experiment until the variability in results can be resolved.

Duplicate analyses of samples from the same groups will be attempted as a means of resolving these problems. It is hoped that we will be able to perform some of these analyses in our own laboratory. Estimates of dietary Sr^{90} levels will not be accomplished until further analyses are complete.

Discussion

A portion of this study was included in the special summer research program for college biology teachers (AEC supported). The participant in this instance wished to duplicate the study in his home area. Since it was a unique opportunity to compare two communities of similar sizes and yet geographically remote (New York and Nebraska) a duplicate study was planned. The study was conducted during October and November 1966 with the complete cooperation of local Nebraska medical authorities. All techniques, collecting and sampling methods were duplicates of the Ithaca study. Upon completion of the study, most of the samples were shipped air express and have been declared "lost enroute" by the Air Express Agency. Efforts to trace the shipment have been unsuccessful. Duplicate samples were available for one age group (24 years and older). This study is being repeated during the current year.

Cesium - 137

Introduction

Despite the problems of obtaining Sr^{90} analyses for the urinary samples, it has been possible to determine Cs^{137} levels for samples collected from the medical community. These data are useful for monitoring the level of Cs^{137} contamination in an area. General relationships have been established between urinary levels of Cs^{137} and body burdens (or dietary levels) which give a good approximation to observed levels. More precise measurements may be needed to specify the nature of these relationships among various population groups where age has an important bearing on this method of estimation.

Results

Data on Cs^{137} levels in urine are presented in Table 2 along with the potassium levels as determined by K^{40} counting. The data are in good agreement with expected levels. They can be compared with data from the teenage studies conducted three months earlier:

Age Group	Teenage Study	Pooled Urine Study
	(pCiCs ¹³⁷ /gK)	
13 years	27 ⁶	20
16 years	20	23

The slightly lower levels of the pooled study are expected as Cs^{137} deposition continued to decline during 1966. Estimates of Cs^{137} body burdens can be made from urinary Cs^{137} /gK measurements by use of appropriate factors. For example, the normal procedure for estimating body burdens involves multiplying urinary Cs^{137} /gK by factors ranging from 2 to 5 (2,3,4,5,6,7). Assuming a factor of three for population groups would estimate the body burdens in Ithaca at the 70 pCi Cs^{137} /gK level.

during late 1966. This is in close agreement with the levels observed in the teenage study conducted three months earlier (49 and 76 pCi Cs¹³⁷/gK). These levels can then be translated into total Cs¹³⁷ body content of approximately 8 to 10 nanocuries.

Table 1. Sr^{90} and Calcium Determinations for Pooled Urinary Samples, Ithaca, N. Y. July-August-September, 1966.

Age Group	Sr^{90} pCi/liter	Ca g/liter	$\text{Sr}^{90}/\text{gCa}$
1 year to 4 years 6 months	3.94 0.72	.242 .216	16.3 3.3
4 years 7 months to 9 years 6 months	1.36 0.53	.187 .177	7.3 3.0
9 years 7 months to 14 years 6 months	0.92 0.78	.187 .233	4.9 3.3
14 years 7 months to 19 years 6 months	0.53 0.28	.088 .047	3.1 11.2
19 years 7 months to 24 years 6 months	---	---	---
24 years 7 months and over	1.44 1.92	.247 .207	5.8 9.3

Table 2. Cs^{137} and Potassium Levels in Urinary Samples,
Ithaca, N. Y. 1966

Age	Cs^{137} (pCi/liter)	K (g/liter)	$\text{Cs}^{137}/\text{gK}$ (pCi)
1 year to 4 years 6 months	48.5	2.64	18.4
4 years 7 months to 9 years 6 months	66.0	2.90	22.7
9 years 7 months to 14 years 6 months	51.7	2.58	20.0
14 years 7 months to 19 years 6 months	60.1	2.61	23.0
19 years 7 months to 24 years 6 months	---	---	---
24 years 7 months and over	69.0	2.02	34.2
Average	59.1	2.55	23.7

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3. Non-Milk Contribution of I^{131} to Diet J. C. Thompson, Jr.

a) Comparison between milk and non-milk foods.

Abstract: Non-milk I^{131} could make a significant contribution to total I^{131} intake among certain population groups. Estimations have been made for some of these groups during the June-September 1962 period. The possibility of a 20 to 40 per cent non-milk I^{131} contribution was indicated for farm families, rural families with gardens and other non-urban population categories. Since there are nearly 50 million persons in the United States included in these groupings, the nature and magnitude of this contribution should be more carefully examined. Appropriate remedial measures may simply require converting to processed foods when the occasion demands action, but no procedures or plans have been established to meet this need.

Introduction

The problems of I^{131} intake from fallout have been well documented in many reports (1,2,3). Questions of yield, half-life, deposition processes, pathways and dose estimations have also been considered from an individual and multiple incident nature. Events such as the Windscale reactor accident (4), the Troy Rainout (5) and the Utah experience with localized fallout (6,7) have indicated the possibility of high I^{131} intake levels among population groups. Although there are several potential avenues of entrance into the body, only one has been considered vitally important in all fallout situations. Fluid milk occupies this position and forms the basis for nearly all I^{131} uptake and dose estimations. It has been the consensus that water, other foods and inhalation are minor contributors of I^{131} (8,9,10).

Despite the minimal I^{131} contribution assigned to pathways other than milk there is a need to clarify and quantify, if possible, the specific role of other pathways. This is particularly true for the plant based contribution to special population categories such as farm families, home gardeners and vegetarians. The unique features of such food chains often preclude the normal reductions in I^{131} intake that occur with other consumer groups (delayed marketing, extensive processing, etc.). The result of these processes is a greater potential I^{131} intake during localized or high release conditions. Thus, thyroid doses to such select population groups may be beyond our present estimates. The nature of these parameters must be determined so measures can be initiated in time of need. Much of this type of information can then be translated into action programs around any potential area of high I^{131} levels. Adaptations of these techniques can be especially helpful where consumption patterns are higher than average or are concentrated on plant based foods.

The location of nuclear reactors in populated areas and the possibility of accidental releases will be the subject of increasing concern in future years. More than 50 nuclear power reactors were in use throughout

the world in 1966 with well over 100 units expected to be in operation by 1970 (11). In addition, there were 270 research reactors operating around the world in 1966 (11). Other uses of nuclear energy involving releases of radioactive materials such as canal, harbor and mine excavations are equally important in future intake and dose considerations. The agricultural systems surrounding these sites and the persons inhabiting them require a high degree of classification if minimal dose levels are not exceeded. Continued above-ground nuclear testing by China and France plus planned or accidental ventings by other nuclear powers will probably continue for many years. The uncertainty of these releases make it necessary to maintain an even greater action capability than normally required.

In the light of data collected on plant ^{131}I contamination levels during the 1961-1962 nuclear tests it seems that sources of food other than milk could make a material contribution to total ^{131}I intake. The magnitude of this contribution should be determined and appropriate comparisons made to establish the milk and non-milk intake fractions for the more susceptible population groups.

Methods

Limited observations on ^{131}I in food plants were reported during the intensive nuclear testing period of 1961-62 (12,13,14,15,16). Although previous analyses had minimized the importance of this route of entry, the data taken during this period can be used to estimate the general nature of plant based ^{131}I contamination. For example, the Japanese collected 19 samples of spinach during the October-December 1961 period and ^{131}I levels ranged from 100 to 16,000 pCi/kg (12,13,14). Data on leaf lettuce from 6 farms in the United States (Utah) during July-August 1962 ranged from 3 to 2812 pCi ^{131}I /kg over four sampling dates (15). These correspond with 6 lettuce samples from Germany during September-December 1962 having ^{131}I levels from 0 to 1067 pCi/kg (16). Finally, data from Japan on 27 spinach samples ranged from 0 to 1900 pCi/kg during September-December 1962 (12,13,14). Although the data are limited it is obvious that a general pattern of ^{131}I contamination was evident in food plants.

In order to translate these data into estimates of ^{131}I intake it is necessary to account for reductions in ^{131}I levels resulting from preparation-processing practices and the radioactive decay occurring between harvest and consumption. The adjusted ^{131}I levels can then be used with per capita consumption data to estimate average intakes from the specific food items analyzed during this period. Calculations based on this procedure indicate the possibility of lettuce contributing from 7 to 21 picocuries per day (July-August 1962) to average Salt Lake City area diets whereas the estimated milk ^{131}I intake would have been 75 to 322 picocuries (17). Estimates based on a group of ten "most likely contaminated fruits and vegetables" having similar contamination levels indicate a possible contribution from 13 to 54 per cent of total ^{131}I intake for average diets during the same period (17). Extending this procedure to special consumer groups could reveal a most significant contribution from fruits and vegetables if data on ^{131}I levels were available. Special population categories such as farm families, home gardeners and suburbanites purchasing from fruit and vegetable stands would be examples as well as the "high" intake segment of average consumer groups. Population-wise these categories range from

13 million persons on farms to the 50 or 60 million people considered to be in a rural category. Thus, nearly one-fourth of our population could be included in these groups.

The lack of data on ^{131}I levels in plant foods does not permit a direct determination of the non-milk contribution. However, it is possible to describe an indirect process which can establish some basis for the nature of the non-milk contribution. Basically this process involves use of the ^{131}I levels observed in milk and the application of experimentally derived secretion rates to determine pasture ^{131}I levels. Once pasture levels are estimated, direct or indirect inferences can be made for food plant ^{131}I levels growing in the same vicinity. Although the exact nature of the relationship between pasture and food plants has not been established some estimates can be made on the basis of yield per acre, proportion of ground cover, etc.

Studies have shown that the average ^{131}I secretion rate ranges from one-half to one percent per liter of milk under continuous intake conditions (18, 19). Using this information with estimates of pasture intake and production per cow permits the determination of pasture ^{131}I contamination levels. Table 1 shows an example of this practice using two milk secretion rates for dairy cattle. The levels of 133 to 800 pCi $^{131}\text{I}/\text{kg}$ conform to expected values from observed and experimental data (20,21,22, 23,24).

Various ratios of milk/grass or its inverse have been used and found to be valid for high or low ^{131}I concentrations. As shown in Table 1 the milk/grass ratio might possibly vary from 0.05 to 0.3 depending on milk production and pasture intake rates. In the Windscale accident the milk/grass ratio was 0.126 (20,21). Several subsequent experiments have produced results within the same general range and indicate the possibility of using this procedure for basing estimates of pasture contamination levels on observed milk determinations (22,23,24). Thus, data from milk sampling networks could be used to estimate general pasture or, in this case, plant ^{131}I contamination levels. These estimates could then be combined with consumption data for milk and non-milk food groups in order to compare ^{131}I intakes. An example of this procedure will be described for the ten market areas with highest ^{131}I levels during June-September 1962. The following steps were taken:

- 1) Average milk ^{131}I levels were converted to estimated pasture contamination levels by using two milk secretion rates (one-half and one per cent per liter). A pasture consumption rate of 20 kilograms per day and a daily milk production rate of 10 liters per cow was assumed.
- 2) Derived pasture contamination levels were then used as estimates of food plant contamination levels. Reduction factors of 65 per cent were used for preparation and processing practice removal rates and a 12-hour delay between harvest and consumption was considered normal. A minimum of ten fruits and vegetables considered most likely to be contaminated with ^{131}I were used.

- 3) Average milk ^{131}I levels for the period were combined with actual milk consumption data to obtain estimates of the milk ^{131}I contribution. Delay factors of 8 hours were used between collection and consumption; this contrasts with urban delay times of 50 to 75 hours.

Results

Table 2 illustrates the use of milk ^{131}I secretion rates for estimating pasture ^{131}I contamination levels. This approach compares with the use of multiplying factors as developed by the Subcommittee on Livestock Damage of the National Academy of Sciences Advisory Committee on Civil Defense (25). Such multiplying factors permit an estimation of pasture contamination from milk levels or vice versa. In Table 2 a secretion rate of one per cent per liter resulted in a multiplier of 5 whereas the one-half per cent secretion rate gave a factor of 10. These compare favorably with multiples of 6 and 12 as derived from the subcommittee report under similar feeding conditions (25). Average ^{131}I pasture contamination for the ten highest ^{131}I contaminated areas range from 250 to 2400 pCi ^{131}I per kilogram when calculated in this manner. Little data are available on the high individual values in each area but studies have indicated that levels of 50,000 to 100,000 pCi ^{131}I per liter of milk were not uncommon in the Salt Lake City area (26). Pasture estimates derived from these data would range from 0.25 to 1.0 μCi ^{131}I per kilogram.

The determination of pasture ^{131}I contamination levels provides an indicator of the relative plant based contamination level in the milk sampling areas. Although correlations between pasture and food plant ^{131}I contamination levels are unavailable, it seems obvious that such relationships do exist. The exact nature of this relationship would depend on the location and type of food plant, the amount of ground cover or plant surface area exposed, etc. Using a direct relationship between pasture and food plants can provide some guide to the contamination potential. For example, the pasture levels estimated in Table 2 for Salt Lake City (1200 to 2400 pCi/kg) do correspond to the observations on ^{131}I levels in lettuce during July as cited previously (3 to 2812 pCi ^{131}I /kg).

Table 3 shows the results of using derived pasture ^{131}I levels as estimators of food plant ^{131}I contamination. Milk and non-milk consumption estimates were based on observed data for the general population¹ during June-September 1962 (27). Appropriate delay times for readily available fresh produce were used in all instances. The resulting calculations show a potential non-milk ^{131}I contribution ranging from 24 to 49 per cent among population categories for the time period under study. Although changes in the pasture/food plant relationship might cause some reductions in the relative contribution levels, the overall inference of a significant non-milk ^{131}I contribution seems established. More information on pasture/food plant relationships along with improve food intake estimates for special groups will permit refinement of these estimates.

¹ Consumption data for the special population groups were not available during this time period. However, previous studies have confirmed an above average consumption rate for these groups in the milk, fruit and vegetable categories (28).

Since farm families occupy a unique category among population groups they should be considered separately. Data have shown that farm families consume larger quantities of many foods (28). Their consumption of the ten foods considered most likely to be contaminated was 50 per cent higher than the average population. Whole milk consumption was also 28 per cent greater than the average rate of consumption. When these factors are considered along with the size of this group (13 million farmers) the situation demands a closer look. Table 4 gives rough estimates of the potential farm versus general population non-milk ^{131}I intake. As shown, ^{131}I intakes for farm families were 33 per cent greater than the average population. Other special groups could be similarly effected by their particular consumption patterns. More specific estimates of non-milk intake must be determined and the sources must be more clearly defined.

It must be recognized that these procedures seek to derive estimates for non-milk ^{131}I intake where data are not available. Therefore, the conditions implicit in such estimates are re-emphasized as follows:

- 1) The milk/pasture ratio as used must apply over varying geographic regions and under differing deposition rates.
- 2) The ratio between food plants and feed plants (pasture) has not been established and may vary widely from the 1:1 correspondence used for illustrative purposes.
- 3) The nature of the feeding program (high concentrates, little pasture, etc.) must also be considered in the final determination of the proper ratios used.

Summary

Non-milk ^{131}I intake could be a significant contributor to total ^{131}I intake under certain conditions, namely; a ready availability of freshly contaminated produce in relatively ample supply; short delay times between harvest and consumption; and significant consumption levels of contaminated products. There are several population groups meeting these criteria and since their numbers approach 50 million persons or more this could be an important matter. Data suggest the possibility of a 20 to 40 per cent ^{131}I contribution from non-milk sources among these groups. Increases of this magnitude would significantly alter dose estimations.

In future assessments of ^{131}I releases, it is strongly suggested that serious consideration be given to (1) identification of the contributing food products, (2) determination of the magnitude of their contribution among specific population groups and (3) establishment of procedures which would minimize the intake from such non-milk sources. Recognition of these problems is needed before further large ^{131}I releases occur so actions can be prompt and effective. Solving the problem of intake may prove to be as simple as converting to an all processed fruit, vegetable and milk diet during this period. However, harvesting, processing and other agricultural problems must be taken into account when major revisions of this type are recommended.

Table 1 Use of ^{131}I Milk Secretion Data to Estimate ^{131}I
Pasture Levels

	Secretion Rate	
	<u>1%/liter</u>	<u>0.5%/liter</u>
Observed ^{131}I in fluid milk (per liter)	40 pCi	40 pCi
Average milk production per cow (per day)	10 liters	10 liters
^{131}I in Milk (per day)	400 pCi	400 pCi
^{131}I intake per cow (daily)	4000 pCi	8000 pCi
Pasture Contamination (^{131}I /kg):		
10 kg consumption per day yields	400 pCi/kg	800 pCi/kg
20 kg consumption per day yields	200 pCi/kg	400 pCi/kg
30 kg consumption per day yields	133 pCi/kg	266 pCi/kg

Table 2 Use of Milk ^{131}I Levels to Estimate Pasture Levels,
Ten Major Milk Marketing Areas, June-September 1962.

<u>Milkshed</u>	^{131}I in <u>Milk¹</u> (pCi/liter)	Projected Pasture Contamination ²	
		<u>A</u>	<u>B</u>
		(pCi/kg)	
Salt Lake City	240 (10-580)	1200	2400
Laramie	150 (20-370)	750	1500
Spokane	125 (20-350)	625	1250
Kansas City	95 (30-240)	475	950
Omaha	65 (40-80)	325	650
Minot	65 (20-170)	325	650
Wichita	60 (20-130)	300	600
Des Moines	60 (20-120)	300	600
Oklahoma City	50 (10-160)	250	500
Cincinnati	<u>50</u> (20-80)	<u>250</u>	<u>500</u>
U. S. Average	35	175	350

¹ Average values with ranges in parentheses.

² Pasture contamination estimated from average milk ^{131}I levels using one per cent (A) and one-half per cent (B) secretion rates.

Table 3 ^{131}I Intake Estimates for Milk and Non-Milk Products, Special Consumer Groups in Ten Major Milk Marketing Areas, June-September 1962.

Milkshed	^{131}I Intake (pCi)				Percent Non-Milk	
	Milk ¹	Non-Milk ²		A	B	A
		A	B			
Salt Lake City	7403	3000	6000	29		45
Laramie	5063	1875	3750	27		42
Spokane	3237	1562	3124	32		49
Kansas City	3133	1187	2374	27		43
Omaha	2421	812	1624	25		40
Minot	2535	812	1624	24		39
Wichita	1862	750	1500	29		45
Des Moines	1950	750	1500	28		43
Oklahoma City	1828	625	1250	25		41
<u>Cincinnati</u>	<u>1649</u>	<u>625</u>	<u>1250</u>	<u>27</u>		<u>43</u>
U.S. Average	1170	437	874	27		43

¹ Milk ^{131}I intake calculated by using actual consumption data for each market (29). Delay times of 8 hours between collection and consumption were considered representative for these groups.

² Non-milk ^{131}I intake calculated by:

- Using pasture ^{131}I contamination estimates from Table 2 as an indicator of plant food contamination
- Determining actual consumption levels for ten plant foods considered most susceptible to ^{131}I contamination during the four month period (27).
- Assuming 65 per cent reduction factors for food preparation and processing.
- Assuming 12 hour delay time between harvest and consumption.

Table 4 Estimate of ^{131}I Intake, Farm Families vs General
Population, June through September 1962

	<u>Farm</u>	<u>General Population</u>
	(pCi ^{131}I)	
Milk	1498	1170
Non-Milk ¹	542-1086	388-776
Total	<u>2041-2584</u>	<u>1558-1946</u>

¹ Using estimated intake from the following most likely contaminated fruits and vegetables: broccoli, cabbage, cauliflower, escarole, grapes, kale, lettuce, peaches, snap beans, spinach and strawberries.

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b) Experimental I^{131} studies in pasture and food plants

Abstract: Green beans and pasture were grown in adjacent plots and contaminated with I^{131} solutions. A bean/pasture ratio of 0.03 to 0.05 was indicated. Removal rates for washing were calculated and averaged 40-50 per cent for field application studies. Laboratory studies indicate a similarity to field studies if similar air, light and temperature conditions are present. Increasing I^{131} concentration levels from 1 to 5 $\mu\text{Ci } I^{131}/\text{liter}$ did not influence removal rates under rinsing experiments.

Introduction

Food plants have not been considered important contributors of I^{131} to the human diet of most consumer groups. However, the possibility of a significant plant-based I^{131} contribution does exist for certain consumer groups (truck farmers, rural persons with gardens, etc.). Some effort is needed to clarify the potential role of I^{131} for these groups, particularly from an emergency standpoint.

Since plant-based foods have not been considered important I^{131} contributors to human diet, very little research efforts have been directed toward I^{131} levels in food plants. Most plant I^{131} research has been concentrated on pasture and the relationship between pasture and milk I^{131} levels. Pasture I^{131} levels might serve as indicators of other plant I^{131} levels if the specific interrelationships between these different plants were known. It may be possible to determine food plant/pasture ratios for the major food plants. These ratios would assist in determining plant-based I^{131} intake for human diets. A further knowledge of the edible portion versus total plant levels would complete the estimating procedure.

In the absence of observed data on I^{131} levels in plant foods and without knowledge of food plant/pasture ratios it has been necessary to initiate controlled studies to estimate the I^{131} food plant contribution. Data obtained from these studies can then be used to define critical groups or areas under emergency release conditions.

Methods

I^{131} plant contamination studies were planned to consider the following:

- a) Methods of applying I^{131} to growing plants.
- b) Amounts of I^{131} deposited on plant parts.
- c) Comparison of pasture I^{131} levels with adjacent growing food plants contaminated at same levels.
- d) Effect of normal washing or rinsing on I^{131} removal.

Control plots were established to conduct these experiments. Site location was in the middle of an eight acre established pasture at the Radiation Biology Field Laboratory. Factors considered in site selection were prevailing wind conditions, soil type and drainage as well as the restriction of public entry.

Plots were established to be representative of commercial operations in the area. An area 100 feet by 70 feet was fenced and divided into a pasture section and adjacent vegetable section. The existing stand of timothy was used for the pasture study since it had been established for several years. Green beans were selected as the vegetable crop because of their importance in total vegetable consumption. Soil preparation, fertilization, seeding and insect control measures followed standard practices for the area.

A variety of water spray application techniques were investigated before controlled dispensing rates and coverage could be established. Various spray heads and containers were used under positive pressure and gravity feed conditions to develop uniform coverage. Best results were obtained from a modified 5 gallon dispensing system equipped with round sprinkler heads. Sprinkler heads were designed to deliver a 12 inch spray over each row from a height of 24 inches. Two liters were dispensed per 55 foot row in a timed sequence (15 seconds). A maximum of three rows was sprayed before refilling the container (spray 6 liters then refill to 15 liter level).

Water solutions of I^{131} were then prepared and applied at various concentration levels to determine amounts needed for accurate counting of pasture, bean leaves, stems and pods. One microcurie of I^{131} was found to be adequate for good counting levels and formed the basic spray concentration level for the field experiments.

Counting was performed in a Tobor counter with vertically opposed NaI crystals (5 inch) and RIDL counting equipment. One quart wide-mouth glass bottles lined with plastic bags were used as sample containers. Samples were filled with water after weighing to insure constant geometry while being counted.

The standard washing and rinsing cycle involved emptying water from sample containers after they had been counted and refilling with uncontaminated water. Samples were then recounted to determine removal rates. This procedure was repeated for two or three successive trials, or until the additional removal rate was minimal.

The pasture plot was prepared for contaminating by mowing (lawn mower) access lanes adjacent to the one foot control pasture strips. I^{131} contaminated solutions were then applied with the spray container. Contaminated samples were cut with hedge shears, collected and placed directly into sample containers. Areas were marked and yields noted for each sub-plot.

The bean plot was contaminated in the same manner as the pasture plot. Contaminated beans were picked into sample containers right at the site. Data were collected on yield of pods, stems and leaves for the various sub-plots.

Field experiments were conducted during no wind conditions and with full sunlight. Protective clothing (gloves, boots, aprons, etc) and respirator were worn during all field work. Decontamination procedures were conducted after each experiment. All operations were monitored with portable counters.

Laboratory experiments were also conducted to determine the effects of I^{131} concentration levels on plant retention. Levels of 5, 3, 2 and 1 $\mu\text{Ci } I^{131}$ per liter were tested on green bean samples. Three liter containers were used for dipping the beans. Each bean pod was immersed in the solution and then placed in sample containers for counting. Various time delays between dipping and counting were then investigated. In addition, samples were also dipped and placed on screens for drying before counting. These screens were exposed to outside elements (light, temperature, air) for specified time intervals to simulate actual field conditions.

Results

Data from these experiments indicate a bean/pasture ratio of .03 to .05 when compared on a density basis (Table 1). This ratio could be used to estimate bean I^{131} levels when pasture levels were known. Determination of similar ratios for other major fruits and vegetables would facilitate estimates for the I^{131} intake from non-milk foods. Care must be taken to express this ratio on the basis of per gram contamination since a square meter or similar area basis does not compensate for differences in pasture or food plant density.

Measures to decontaminate the bean samples were employed throughout the study. Rinsing for several times, screen drying with rinsing and time delayed factors were considered in field and laboratory experiments. Results for field contaminated beans are shown in Table 2. It is evident that a 40 to 50 per cent removal rate was obtained with most samples. Repeated rinsings did not give significantly greater reductions and were discontinued.

Time delays in the field had a definite effect on removal rate, causing the rinsing to be about half as effective (averaging about 25 per cent removal). Part of the difference may be due to a greater absorption or adsorption capacity over the longer time interval (two hours versus a normal half hour period). Counting levels for these delayed samples were similar to normal samples (786 cpm for delayed versus 883 cpm for normal).

Laboratory tests of beans individually dipped into various contaminated solutions, then counted and rinsed gave further evidence of the time delay and I^{131} retention capability. Tests were conducted with solutions of 5, 3, 2 and 1 $\mu\text{Ci } I^{131}$ per liter. Table 3 shows the contamination level of these samples and the removal rates under container drying and screen drying experiments. Container dried refers to the practice of placing samples in glass containers and waiting until bean pods were dry before water was added for geometry standardization. Screen drying involved placing the beans on 4 ft by 6 ft raised screens and allowing to dry under normal weather conditions (outside light, temperature and air conditions) before counting.

Container dried samples had removal rates of 80 per cent or more at all concentration levels. This contrasts with a lower removal for screen drying (30 to 40 per cent) at all levels. The difference in removal rates is obviously a function of air circulation patterns along with some dependence on temperature and light. Apparently the bean pod can absorb and/or adsorb a certain fraction over the given time period and the rest of the I^{131} is available for loss through rinsing, vaporization, etc. In container drying experiments the dry appearance of bean pods would not

indicate the full extent of the I^{131} available for release when immersed and rinsed with water.

It is interesting to note the relatively constant rate of removal in both experiments despite the changes in I^{131} concentration rate. Again the possibility of a specific uptake or retention capacity for the bean pod is indicated. Removal rates were not influenced by changes in concentration level used in these experiments; perhaps much lower levels of I^{131} might show a concentration-removal rate dependency.

Comparison of the various plant part contamination levels indicated the following distribution pattern:

	<u>I^{131} (% activity)</u>	<u>Weight (% total)</u>
Beans	8	46
Leaves	75	31
Stems	17	23

The degree of protection afforded bean pods by the leafy cover is significant.

Discussion

Development of application techniques for I^{131} on control plot studies has permitted some preliminary investigations on relative contamination levels of bean plants. The nature and extent of their contamination as contrasted with pasture subjected to the same application rate has been studied. Data indicate that a bean pod/pasture ratio of 0.03 to 0.05 applies under the conditions of this study. Further investigation of this ratio under different growth conditions are needed as well as studies of other pasture mixtures. Development of this ratio and others similar to it will aid in determining I^{131} intake from specific food plants in the following manner:

<u>Pasture I^{131} Known</u>	<u>Pasture I^{131} Unknown</u>
1. Used food plant/pasture ratio to estimate food plant levels	1. Use milk I^{131} levels to estimate pasture levels
2. Food plant level plus consumption = intake	2. Use food plant/pasture ratio to estimate food plant levels
3. Correct intake for delay, washing, etc.	3. Food plant level plus consumption = intake
	4. Correct intake for delay, washing, etc.

Estimates of I^{131} intake determined in this manner will be particularly useful for special population groupings.

Removal rates of I^{131} from various food plants are other important factors to consider in emergency or high release situations. Controlled studies can be used to establish removal rates for the most important fruits and vegetables.

Table 1 Determination of I^{131} Bean/Pasture Ratios
Field Experiments -- Spray Application, 1967

	<u>Study I*</u>	<u>Study II*</u>
<u>Density:</u>		
Beans (g/sq ft)	108.8	108.1
Pasture (g/sq ft)	68.0	66.1
<u>I^{131} Counting:</u>		
Beans (cpm/gram)	2.8	3.2
Pasture (cpm/gram)	86.0	67.6
<u>Bean/Pasture Ratio:</u>	0.032	0.047

* Three samples were taken for each test.

Table 2. Removal of I^{131} from Beans Contaminated in the Field, 1967

<u>Test</u>	<u>Operation¹</u>		
	<u>One Rinse</u>	<u>Two Rinses</u>	<u>Three Rinses</u>
	(per cent removal)		
Spray - field dried - counted	49.6	52.9	55.5
	46.8	51.6	52.7
	47.5	52.9	
	39.7	42.9	
	44.4	50.1	
Spray - field dried - counted	47.4	50.5	49.8
	43.5		
	41.8		
	44.6		
	47.0		
Spray - field dried - counted	38.0		
	42.0		
	40.0		
Spray - field dried + 2 hour delay-counted	20.1		
	29.0		
	24.6		
	23.6		
	23.2		

¹ Rinsing means emptying water from sample container and replacing with new water.

Table 3. Laboratory Experiments on I^{131} Contamination and Removal Rates in Green Beans, 1967

		Removal Rate (%)		
Contamination Level ($\mu\text{Ci } I^{131}/\text{liter}$)	Standardized Net cpm/kg	First Rinse	Second Rinse	Third Rinse
<u>Container Dried:</u>				
5	83715	81.6	88.7	89.1
3	44256	78.5	85.4	86.1
2	31447	79.6	85.0	86.0
1	16622	78.6	85.1	86.1
<u>Screen Dried:</u>				
5	26071	37.1	43.4	42.6
3	18941	36.9	43.5	45.5
2	16435	30.2	30.0	32.6
1	8029	36.5	36.8	36.9

4. Practicality of Decontamination Processes as Related to
Food Technology
J. C. Thompson, Jr.

Abstract: Milk Sr^{90} levels were used to estimate Sr^{90} levels for the four major dietary sub-groups (Dairy, Grain, Fruits and Vegetables and Animal Protein Foods) in various countries of the world. These data were then modified on the basis of expected effects from standard food preparation and processing practices to determine the amount of Sr^{90} normally removed prior to consumption. Reductions in total dietary Sr^{90} intake were estimated to fall in the 10 to 30 per cent range. Direct correspondence between the degree of marketing services performed and the level of Sr^{90} removal were noted. Thus, the more developed countries could expect greater reductions and still retain the economic capability for achieving further reductions. Nearly 80 per cent of the dietary Sr^{90} intake could be eliminated in developed countries if maximal countermeasures were employed. Since the less developed countries cannot achieve this reduction without outside aid, it is necessary to examine these differences and consider appropriate emergency policies for improvement of these capabilities.

Introduction

Radioactive decontamination of foods has become an important consideration for emergency planning since the advent of nuclear weapons. Various processes have been studied for individual food products in order to determine the nature and magnitude of radionuclide removal. These processes range from simple washing, peeling and discarding of plant portions to complex ion-exchange reactions.

Since the dietary patterns of the world are so different, the measures designed for one country are not necessarily optimum for other countries. In order to examine the effects of these differences, the dietary intake patterns among various countries and areas need to be evaluated in terms of a series of decontaminating processes which are considered to be effective in reducing radionuclide intake.

Methods

Decontaminating practices and their effect on total dietary radionuclide intake would be important considerations in emergency planning during accidental or intentional large scale releases of fission products. Knowledge of potential reductions would permit shifts in distribution and marketing patterns, aid in dietary changes or substitution criteria, and provide a basis for action directives. Information of this type is needed for all countries of the world because of the widespread distribution of nuclear technology and the global pattern of radioactive fallout.

In order to determine the effect of decontaminating practices several factors must be known:

1. Quantities of various foods consumed.
2. Radionuclide levels in various foods.
3. Decontaminating factors for each food or food group.

Data on the consumption of each major food group are available from balance sheet statistics (production less waste divided by population) for many countries of the world (1). These data give a good indication of the general intake patterns for the four major dietary sub-groups (Dairy, Grain, Fruits-Vegetables and Animal Protein foods).

Radionuclide levels produced by past contamination are also not available for the major dietary sub-groups; in fact many countries can provide little or no usable information. Monitoring of radionuclide levels among many foods is generally confined to the developed countries of the world and few data are available for the less developed nations. Therefore, it is necessary to use estimates of radionuclide levels which are developed from analyses in adjacent or geographically similar countries. If these data are not available, intra-group associations offer one means of estimating the desired Sr^{90} levels. Since milk is the most commonly sampled product for radionuclides it should serve as the basis for these associative relationships. In many countries there are data available for radionuclides in milk but not in other foods.

Decontaminating or reduction factors have been developed for many foods and food groups and can be used to estimate the overall effect on dietary intake. The cost and conditions of use of remedial measures generally confine their application to the more developed nations of the world. However, it would be possible for many of the techniques and practices to be utilized in less developed nations if emergencies should develop that were local in nature (assistance programs from other nations, emergency aid, etc.).

Since no comprehensive worldwide data are available for total diet or dietary sub-group calcium intakes, it is impossible to utilize the well defined "Observed Ratio" concept to estimate diet sub-group Sr^{90} levels. In the absence of these data general estimates are presented based on milk Sr^{90} levels in the following manner:

If milk contains 10 pCi Sr^{90} per liter and a cow produces 10 liters per day the total Sr^{90} intake for the cow would be about 1000 pCi Sr^{90} . If pasture plants form the main feed and are ingested at a rate of 25 kg/day, the plant contamination level would be 40 pCi Sr^{90} /kg. This level of contamination could be used as an estimate for other plants growing in the same vicinity. Contamination levels for many human foods could then be estimated if the relationship between total plant Sr^{90} levels and edible food portions of the plant were known. Since these ratios are not well defined for the major food groupings, it is necessary to make estimates based on plant physiology and some limited data sets. Preliminary calculations based on this information indicate milk Sr^{90} levels can give some rough estimates of Sr^{90} levels in other major sub-groups by using multiples of the following nature:

	<u>Dairy</u>	<u>Grain</u>	<u>F & V</u>	<u>Animal Protein Foods</u>
Multiple	1.0	1.6	0.6	0.2

A grain/milk ratio of 1.6 conforms to observed data in the U.S. for 1963 (2). This ratio would apply for diets having considerable processing practices already incorporated into their grain products. Diets approaching a subsistence level would require ratios near 1.0 since little or no processing would occur to reduce Sr^{90} levels (no milling, little hulling or removal of outer parts). The same conditions would apply to the fruit-vegetable/milk ratio of 0.6 which relates primarily to average diets already benefitting from some standard removal conditions (washing, peeling, scraping, etc.). Subsistence diets would be closer to a ratio of 1.5 because of the lower levels of preparation utilized. Animal protein foods (meat, fish, eggs) would be similar for all groups because of the metabolic barriers inherent in animal production.

Results

Estimates of Sr^{90} levels in the major food groups are shown in Table 1 under normal and subsistence marketing conditions. They can be used with food consumption data to obtain estimates of total dietary Sr^{90} intake.

Since it is possible to reduce Sr^{90} levels in foods by various processing techniques some further consideration should be given toward this end. For example, it is possible to suggest that all grains be milled before consumption or that all fruits and vegetables be processed (frozen or canned) before use. These practices would materially aid in reducing Sr^{90} levels and would be economically feasible for many groups. The capability for use of these reduction techniques can be determined from selected economic indicators such as income (the higher the income the greater the demand for marketing services).

In the absence of personal income data for many countries it is necessary to relate other variables to the capability for financing increased marketing services. Animal protein consumption is a reasonable criterion of income since it serves as a good indicator of the standard of living. Animal protein intake also shows a direct response to income over a wide range and can be used as a good measure of the standard of living in most areas (3,4). Thus, animal protein intakes were used as the means for determining the extent of added reductions in Sr^{90} intake resulting from increased processing practices. The reduction factors were determined as follows:

Average daily animal protein(g)	6-17	18-19	30-41	42-53	54-65
Sr^{90} Reduction Factors (%)	10	20	30	40	50

Data from Table 1 were subjected to these reduction factors for the grain and fruits and vegetable categories. Total dietary reductions were then compared among the various countries according to animal protein intake in Table 2. Countries with the highest animal protein intake (and presumably highest income) had the greatest total Sr^{90} dietary reductions, ranging from 18 to 35 per cent. Low animal protein groupings had lowest reductions from 7 to 19 per cent. It must be emphasized that these reductions contrast the normal situation with improved marketing conditions (maximum milling, processing, etc.). They do not indicate results for subsistence conditions since the capability

to change or improve marketing under this level of consumption is minimal. Sr^{90} intake under subsistence levels would be much greater and the ability to obtain improved marketing would be much less than indicated in Table 2. Many persons exist on marginal diets without benefit of even the most basic preparation practices (washing, peeling, etc.). No estimates are made to compare these less developed areas with those of a developed nature but total Sr^{90} intakes would be much higher.

In order to examine the potential reductions under emergency conditions, diets were also examined with maximal reduction factors employed. Table 3 shows the typical reductions expected among well developed nations. Reductions in Sr^{90} dietary intake increased from the 25 per cent level to the 80 per cent level. Proportionately less reductions would be expected among other groupings as their economic capabilities declined.

The large reductions obtained are due to widespread changes incorporated into marketing patterns. The costs of these added services would be considerable but the reductions achieved would warrant consideration under emergency or large scale nuclear releases. The possibility of removing more than three-fourths of total dietary Sr^{90} intake might offset the costs involved in such a situation.

Since the practices utilized in this approach involve considerable costs they could only be visualized for developed areas. For example, the costs of milk decontamination, complete processing of all fruits and vegetables, food substitution measures and mass food allocation are within reach for well developed nations. Their costs would be prohibitive for smaller or less developed nations under most conditions. Therefore, Sr^{90} intake patterns under widespread releases will probably always be more critical for low income or less developed areas and their intakes will be much higher.

Discussion

It is evident that some consideration should be given toward the influence and impact of economic development on actual or potential Sr^{90} dietary intake. Data presented here indicate that less developed areas would be the most critical group in any large scale release or emergency. This situation may even extend to income stratification within a developed country in that special groups would experience much greater Sr^{90} intake than the average levels reported.

Further research is needed to determine accurately the nature and magnitude of these factors. As more data are made available, comparisons can be made between observed values and those derived from the use of milk Sr^{90} levels.

Table 1. Derived Sr^{90} Levels for Major Dietary Sub-Groups,
Normal and Subsistence Conditions, 1963

	(pCi Sr ⁹⁰ /kg)					
	<u>Dairy</u>	<u>Grain</u>	<u>Products</u>	<u>Fruits&Vegetables</u>		<u>Animal</u>
		<u>Normal</u>	<u>Subsistence</u>	<u>Normal</u>	<u>Subsistence</u>	<u>Protein</u>
						<u>Foods</u>
Belgium	21.3	34.1	213	12.8	32.0	4.3
Finland	36.7	58.7	367	22.0	55.0	7.3
France	39.6	63.4	396	23.8	59.4	7.9
Greece	25	40.0	250	15.0	37.5	5.0
India	11.0	17.6	110	6.6	16.5	2.2
Italy	37.2	59.5	372	22.3	55.8	7.4
Japan	10.8	17.6	108	6.6	16.2	2.2
Mexico	6.9	11.0	69	4.1	10.4	1.4
Netherlands	36.0	57.6	360	21.6	54.0	7.2
Soviet Union	33.0	52.8	330	19.8	49.5	6.6
United Kingdom	32.3	51.7	323	19.4	48.4	6.5
United States	23.5	37.6	235	14.1	35.2	7.1

Table 2. Derived Sr⁹⁰ Total Diet Intake - Normal versus Improved Marketing Conditions, Twelve Countries, 1963

Daily Animal Protein Intake High (45-65 g)	Estimated Sr ⁹⁰ Intake (pCi/yr)		Reduction
	Normal Marketing	Improved Marketing	
Belgium	9353	6767	28%
Finland	22064	18084	18%
France	18388	11952	35%
Netherlands	16798	12896	23%
United Kingdom	14396	11001	24%
United States	10354	7424	28%
<u>Medium (25-45 g)</u>			
Greece	12555	10413	17%
Italy	17532	14628	16%
Soviet Union	17121	14444	16%
<u>Low (<25 g)</u>			
India	4231	3936	7%
Japan	4709	3829	19%
Mexico	2589	2384	8%

Table 3. Examples of Maximum Dietary Sr^{90} Decontamination Practices, 1963.

	<u>pCi Sr^{90}/year</u>									
	Dairy ¹		Grain ²		F & V ³		MFE ⁴	Total		Net Re-
	A	B	A	B	A	B		A	B	ductions
Belgium	2535	254	3137	627	3328	666	353	9353	1900	80%
France	4673	467	6277	1255	6593	1319	845	18388	3886	79%
United Kingdom	5297	530	4239	848	4249	850	611	14396	2228	84%
United States	4018	402	2519	504	3342	668	475	10354	2049	80%

¹) A = Normal Intake B = 90 per cent reduction due to ion exchange process.

²) A = Normal Intake B = 80 per cent reduction if all grains milled.

³) A = Normal Intake B = 80 per cent reduction if all fruits and vegetables processed.

⁴) No significant reductions expected for Sr^{90} .

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4. FAO, Third World Food Survey, Rome, FAO, 1963.

5. Radionuclide Plant and Soil Relationships under Normal Cultivation Practices

J. C. Thompson, Jr.

Abstract: Soil and vegetable Sr^{90} and Cs^{137} levels show the effects of changes in fallout deposition rate during 1965 and 1966. Sr^{90} levels for cultivated sites continue to be significantly less than from uncultivated sites.

Introduction

Although considerable numbers of studies have been conducted on plant and soil aspects of fallout radionuclides, most of these efforts have been confined to the laboratory or to very limited experimental plots. Survey studies of soil levels have largely been restricted to soils that have been undisturbed for a considerable period of time (1, 2). These efforts permit a characterization of radionuclide behavior under conditions that may or may not represent normal patterns. Therefore, in order to gain a better understanding of radionuclide pathways under normal or operating conditions, studies were initiated in conjunction with the department of Vegetable Crops at Cornell University. These studies were designed to sample soil and food crops which were grown under normal cultivating conditions that were typical of commercial operations. Sampling over a period of years would thus permit a better understanding of radionuclide pathways under tilled conditions. In addition, emphasis could be placed on some of the major food crops that have not been adequately covered in past investigations.

Methods

The major vegetable crops that constitute about two-thirds of annual consumption were selected for study (cabbage, carrots, green beans, potatoes and tomatoes). Soil samples, taken with a standard sampling auger, were obtained at the growing site for each vegetable crop. Twenty soil cores, each representing the top 8 inches of soil, were taken in the immediate vicinity of the plants selected for sampling. Vegetable samples were collected from each site in sufficient amounts so that several experiments could be conducted on each vegetable crop (home preparation practices versus commercial practices, decontamination factors, etc.).

The original plan of alternate year sampling was changed in 1965 to permit annual collections. This approach should provide a better guide to time trends in deposition and soil accumulation.

Samples have been analyzed for Sr^{90} and calcium by commercial laboratories. All Cs^{137} and potassium analyses have been performed within the Department of Physical Biology.

Results

Soil Sr^{90} and Cs^{137} levels are shown in Table 1 for each vegetable sampled. A general increase in soil Sr^{90} levels was observed during the four year period (25 per cent). This contrasts with a 50 to 75 per cent increase being observed for uncultivated sites in the same area or geographic region (Table 2). The lower levels of Sr^{90} may be due to increased fertilization, liming, leaching or to the greater mixing that occurs when soils are plowed and tilled continuously. As more data become available, more precise estimates of the true difference will be determined.

Little data are available on soil Cs^{137} levels since it becomes relatively unavailable once it enters the soil profile. However, it is interesting to note the changes in soil levels as an indication of changes in deposition rates. The smaller inventory of Cs^{137} available for deposition after 1966 will be evident in subsequent samplings.

Tables 3 and 4 show similar patterns of decline for the Sr^{90} and Cs^{137} levels in specific vegetable crops. A general decline is evident for both radionuclides with Cs^{137} showing the effects of reduced plant availability as the deposition rate declines. The rate of decline in Sr^{90} levels appears to be lessening since 1966 data were quite similar to 1965.

Discussion

Changes in Sr^{90} and Cs^{137} levels among soil and vegetable samples provide an excellent indication of relative fallout levels. Time relationships and radionuclide pathways can be more closely examined under normal cropping and cultivation practices. Better estimates and assessments of radionuclides in the food chain can be made as more data become available.

Table 1. Soil Sr^{90} and Cs^{137} Levels from the Same Growing Sites, 1963 - 1966

<u>Site</u>	Sr^{90} (mCi/sq.mile)		
	<u>1963</u>	<u>1965</u>	<u>1966</u>
Cabbage	129	127	--
Carrot	87	78	117
Green Bean	134	81	153
Potato	95	151	160
Tomato	<u>122</u>	<u>140</u>	<u>137</u>
Average	114	116	142
Cs^{137} (mCi/sq. mile)			
Cabbage	--	--	--
Carrot	--	398	600
Green Bean	--	428	548
Potato	--	511	513
Tomato	--	<u>554</u>	<u>569</u>
Average		475	558

Table 2. Soil Sr^{90} Levels in Northeastern United States, 1963-1966

Site	1963	1964	1965	1966
	<u>(mCi Sr^{90}/sq. mile)</u>			
Cultivated Soils:				
Ithaca	116		116	142
Western New York		119		
Uncultivated Soils:				
Ithaca	110	141	156	
Maine	110			
Massachusetts	118	168	193	211
Rhode Island	172	195	234	
Vermont	132			

Table 3. Sr^{90} Levels in Major Vegetables from the Same Soils,
1963 - 1966

Crop	<u>(pCi Sr^{90} /gCa)</u>		
	1963	1965	1966
Cabbage	70.1	29.1	<u>a</u>
Carrot	166.0	66.2	37.4
Green Bean	75.4	67.9	69.0
Potato	281.0	57.4	55.1
Tomato	<u>74.5</u>	<u>18.9</u>	<u>36.8</u>
	133.4	47.9	49.6
Crop	<u>(pCi Sr^{90} /kg)</u>		
	1963	1965	1966
Cabbage	52.0	17.8	<u>a</u>
Carrot	33.0	12.3	16.6
Green Bean	43.5	19.1	41.4
Potato	10.1	3.3	8.4
Tomato	<u>14.5</u>	<u>2.2</u>	<u>9.2</u>
	30.6	10.9	18.9

a) No samples collected

Table 4. Cs^{137} Levels in Major Vegetable Crops Grown on the Same Soils, 1965 - 1966

<u>Crop</u>	<u>1965</u>	<u>1966</u>
	(pCi Cs^{137} /kg)	
Carrot	17.0	8.9
Green Bean	36.6	16.6
Potato	25.3	13.4
Tomato	13.4	10.2
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Average	23.1	12.3

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6. Climatological Aspects of Sr^{90} Levels in Milk

J. C. Thompson, Jr., R. S. Westman and F. L. Hiltz

Abstract: Development of a model to study the influence of climatological factors, radionuclide content of the atmosphere and time on the radionuclide content of milk has been continued into the stage of computer planning and programming. Because of the complexity of the program and the extensive computer analyses needed to complete this study it has been proposed as a separate project. A description of the scope and budgetary needs are included as Phase IV of the contract proposal.

Introduction

During the past year preliminary planning has been completed and diagrams of the computer program are shown in Figures 1a and 1b. The sequence of program development is then shown in Figure 2. The model has been reviewed by several outside agencies and their suggestions have now been incorporated into the structure. The basic aims and objectives of the project remain the same: a) to describe the interrelationship between radionuclide levels in milk and climatological variables, radionuclide levels and time, b) to determine these relationships for individual markets throughout the United States, c) to utilize new mathematical techniques in these analyses which would have application in other disciplines (oceanography, weather, etc.) and d) to develop the capacity of the model as an operating device for future use in fallout predictions of a localized nature.

Figure 1A. General Block Diagram of the Computer Program

Figure 1b. General Block Diagram of the Computer Program

AASP Algebraic Add-Subtract Package
ADP Algebraic Division Package
AMP Algebraic Multiplication Package
DF Density Function Subroutine
EFG Elementary Function Generator
FF Fourier Fit Subroutines
GSO Gramm-Schmidt Orthogonalization Routines
IIT Inverse Integral Transform Subroutine
NOF Non-orthogonal Function Set Fitting Routine
OFG Orthogonal Function Generator
PCS Parameter Control Subroutine
WFG Weighting Function Generator

Figure 1C. Abbreviations of programs shown in Figures 1B and 1A

Figure 2. Sequence of Program Development

C. Phase III - Neural and Behavioral Response to Radiation.

This section of the work was started as of October 1, 1967 under A.E.C. support. Because of previous background it has been possible to make considerable progress as evidenced by the following reports:

1. Olfactory X-ray Detection.
 - A. X-ray Detection: Interchangeability of Ozone and X-ray as Conditional Stimuli.
 - B. Modification of Stimulus-Response Curves.
2. Spinal Cord: Modification of its Labile Electrical Characteristics by X-irradiation.
3. Studies on Radiation-Induced Gustatory Avoidance Behavior.

1. Olfactory X-ray Detection
E. L. Gasteiger

Abstract: That rats detect X-ray by way of their olfactory systems is well established (e.g. ref. 1). Whether the detection is mediated by a primary process within the cell or by production of gaseous odorants in the nasal passage is still to be determined. Masking of X-ray detection by introduction of ozone (O_3) into the operant conditioning box supports the latter possibility (2). Further evidence involving O_3 in the mechanism has been found by establishing that O_3 and X-ray can be used interchangeably as conditional stimuli (3) and by succeeding in manipulating the stimulus-response curves for X-ray detection by introducing water vapor into the box. In the latter case the water vapor decreased detection efficiency, presumably by catalytically recombining atomic oxygen to O_2 . Since this effect occurred in a gaseous medium (the air in the nasal passages), it provides evidence that the X-ray detection is by way of a gaseous odorant rather than by direct action in the sensory cell.

1-A. X-ray Detection: Interchangeability of Ozone and X-ray
as Conditional Stimuli

Edgar L. Gasteiger and Sharon A. Helling

Abstract: To determine the possible role of ozone as mediator in X-ray detection, rats were conditioned to stop licking upon presentation of the odorants ozone or ethyl butyrate (pineapple odor). Under identical test conditions, X-irradiation for 10 sec. was then substituted for the odors. Rats conditioned to ozone readily transferred their conditional behavior to X-irradiation while those conditioned to ethyl butyrate did not. A reverse testing situation in which animals were first conditioned to the X-ray exposure and then presented with either ozone or ethyl butyrate gave additional support to the similarity of ozone and X-ray.

The essentiality of the olfactory system for detection of low doses of X-ray (e.g. 12 r/min) by rats has been demonstrated through removal of the olfactory bulbs¹⁻⁴. Currently, this detection is thought to be mediated by an X-ray produced odorant or by direct action of the radiation on the olfactory sensory cell. These possibilities are both indicated by experiments in which X-ray activation of olfactory cells was blocked by substitution of gases in the nasal cavities and by application of alcohol to the nasal mucosa⁵⁻⁸.

Active molecules of oxygen and nitrogen, which might act as odorant initiating X-ray detection, are known to be produced by ionizing radiation^{7,8}. On the assumption that an odorant, such as ozone, is produced and signals the presence of X-rays, we have tested the ability of rats to detect X-rays in the presence of various levels of this gas, and have found that ozone and other strong volatile oxidants, added to inspired air, blocked olfactory detection of X-rays⁹. For ozone, the degree of block was dependent on the amount present. Although this effect has been interpreted as indicating that ozone is an X-ray produced odorant which mediates detection, other interpretations are possible. Therefore, more definitive behavioral experiments were designed to test for the similarity of X-ray and ozone as olfactory stimuli¹⁰.

The ozone blocking might be explained as due to masking, adaption of the sensory cell, or reduction of intensity discrimination. If the mechanism is masking, it must be highly specific to pungent odors, since in our experiments there was detection of fruity odor in the presence of gaseous oxidants, and there was no effect on X-ray detection by minty, putrid and fruity odors. Adaptation is an unlikely mechanism since the

rats continue to detect X-ray or ozone over 15 sec periods, and Cooper and Kimeldorf⁶ reported continued increased discharge of olfactory units during X-ray exposures as long as 5 sec. On the basis that an ozone-like molecule in air or solution mediates X-ray detection, intensity discrimination remains as the most likely explanation for blockage by ozone. In this case the high level of ambient ozone would be expected, according to the Weber relation, to reduce the effectiveness of the X-ray-produced fixed increment of ozone-like molecules.

The uncertain role of ozone dictated the need for a more definitive behavioral measure of the similarity of X-ray and ozone. A suitable test would be successful transfer or generalization of a behavioral response from X-ray as a conditional stimulus to ozone, and vice versa. The behavioral response used was cessation of licking during presentation of the conditional stimuli.

The experimental methods were similar to those described for our earlier ozone experiments⁹. Sixteen male Sprague-Dawley rats learned to lick for 10% sugar water in a conditioning box after 23 hours daily deprivation of water. These animals were divided into 4 equal groups. Two of the groups were conditioned to stop licking during a 10 sec X-ray exposure and two during a 10 sec exposure to the odorants, ozone or ethyl butyrate (pineapple odor). The conditional behavior was established by shocking the animals if they licked during the 10 sec exposure periods. A correct response was scored if a rat refrained from licking throughout the exposure period.

X-ray exposure was effected by swinging out a 1.0 cm thick lead shutter after the beam had been activated for more than 30 sec; the radiations were generated by a 224 Kv machine with 0.50 Cu filter (HVL 0.93 mm Cu). Only the head region was exposed at a dose rate by sound insulation in the conditioning box, introduction of constant masking white noise, and interspersed sham exposures among the real exposures. Odorants were presented for 10 sec. near the drinking tube by means of a constant flow system (10 cc/sec) which was controlled by a servo-operated three way valve system. Ozone was supplied from a type II Sander Ozonizer set at its lowest level (manufacturer's rating of 2 mg/hr) which is near threshold for human detection. Performance of the animals was recorded by means of a lickometer and associated operant conditioning apparatus and by visual monitoring of closed circuit TV.

During a learning and stabilization period of 23 sessions (days) of 6 trials each, the animals achieved a detection level of 80% or above. At this time the transfer experiments were begun by exposing the animals to the novel stimulus of X-ray or odorant, without shock. As controls, trials with the initial conditional stimulus with shock were interspersed with the shock-free trials for the novel stimulus, thus increasing the number of trials to twelve per session.

The two groups of rats conditioned to X-ray continued to respond well to X-ray after transfer was attempted at the 24th session (Fig. 1). That group tested for transfer to O₃, responded at a high level as would be expected if X-ray and O₃ are very similar, while the other group showed no generalization from X-ray to pineapple odor. The rats conditioned

initially to pineapple odor performed well to this stimulus throughout the sessions, while the rats conditioned to O_3 responded with less certainty (50% level) at the time transfer was begun, recovering to control levels in three sessions. Transfer from O_3 to X-ray was initially at the 52% level, as though the animals were confusing the X-ray and O_3 stimuli. With time, discrimination between the stimuli appeared as evidenced by the recovery of the O_3 control curve and the fall of the X-ray curve. No generalization from pineapple odor to X-ray occurred. The differences in the responses to novel stimuli in sessions 24 and 25 were found to be highly significant as determined by the Mann-Whitney U test.

The failure of the animals to transfer to the novel stimuli could have resulted from their possible inability to sense these stimuli. That such was not the case was established by coupling shock with the novel stimuli after session 32. The two groups of animals initially conditioned to X-ray readily reached the 80% level when conditioned to the appropriate novel stimulus, O_3 or pineapple odor. Difficulty was encountered with the groups initially conditioned to O_3 and pineapple odor. When shocks were coupled with the X-ray after session 32, only slight improvement in detection level occurred. A significant increase in detection was produced by increasing the X-ray intensity to 19 r/min. The animals responded as though they had strongly habituated to the weaker X-ray stimulus.

It is not surprising that, although generalization from X-ray to O_3 and vice versa occurred, the rats did learn to discriminate between O_3 with shock and X-ray without shock. This is understandable since the stimuli were presented in markedly different ways and with unmatched intensities, the O_3 being the stronger stimulus. The common qualities of X-ray and O_3 as stimuli are demonstrated by the apparent confusion of the two upon transfer from O_3 to X-ray and by the high level of transfer from X-ray to O_3 . None of these relationships appeared for the pineapple odor.

These results strongly support the hypothesis that olfactory detection of X-ray is mediated by ozone or an ozone-like molecule. The earlier results for the "masking" experiments⁹ are best explained as resulting from reduction of intensity discrimination.

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Fig. 1. Training and transfer curves for rats avoiding shock by ceasing to lick during a 10 sec. X-ray exposure. The percent of correct trials for each group of 4 rats is plotted as a function of the daily session. With few exceptions each point was calculated from 24 stimulus presentations (6 per rat). At the vertical line, transfer from X-ray to O₃ (O—+—O) was successful, but not from X-ray to ethyl butyrate (Δ—+—Δ).

Fig. 2. As for Fig. 1, with the exception that transfer was successfully made at the vertical bar from O₃ to X-ray, (O—|—O) but not from ethyl butyrate to X-ray (Δ—|—Δ).

B. Modification of Stimulus-Response Curves.

Attempts were made to modify systematically the detection of X-ray in the threshold range by altering the conditioning environment so as to increase or decrease the amount of O_3 which might be produced.

In the previous studies, a conditioned aversion routine was used in which the rats were scored a correct response only if they ceased licking throughout the total 10 sec. irradiation period. This was an unnecessarily severe criterion, but was satisfactory for the type of question we were asking.

Such a method is relatively insensitive when the degree of detection as a function of X-ray intensity is desired (stimulus-response relation). Consequently, we changed our method, on the recommendation of Dr. James Smith, to the conditioned suppression routine of Hoffman and Fleshler(4).

This routine consists of using the 10 sec. X-ray exposure as a conditional stimulus which tells the animal it will be shocked at the end of the exposure regardless of its behavior during the exposure period. With this arrangement all the conditions for each trial are identical and the animal soon learns to stop licking while it waits to be shocked. Reduction of the stimulus intensity to a level at which the animal becomes uncertain about the presence of the stimulus gives a condition in which the animal licks apparently according to his degree of certainty--zero licks at a high dose rate of 6 - 10 R/min (definite detection) and about 50 licks in 10 sec. at less than 0.1 R/min (below threshold). Stimulus-response curves are obtained by plotting a suppression ratio against the radiation intensity in R/min., where the suppression ratio is

defined as $\frac{\#_C - \#_X}{\#_C}$, $\#_C$ = licks during 10 sec. preceeding irradiation and $\#_X$ = licks during 10 sec. irradiation.

Twenty-four rats were trained in this conditioned suppression routine and twelve of these were finally selected because of their consistancy and similarity in performance and detection. Two groups were formed by random selection and the groups were run under opposite conditions on the first day(session) with the conditions for the groups being reversed on the following day. Each animal was given 8 trials, two at each radiation intensity, for each session.

1. Increased O_2 concentration.

In the first experiment an attempt was made to shift the stimulus-response curves toward increased detection (higher suppression index) by introducing 100% O_2 into the conditioning box. The expectation that under these conditions more O_3 would be produced for a given amount of X-ray was based on the work of Koller (5) who found such an increase for U.V. produced O_2 .

The animals showed no difference in behavior or control counts in

100% O_2 as compared to air. Figure 1 contains the pooled data from two or more experiments in which comparison was made between detection in air and detection in 100% O_2 . The suppression ratio (detection efficiency) is plotted against the log of the X-ray intensity. The standard error is represented by the vertical bars. No difference is seen in detection under the two conditions, which reflects on the theoretical basis for the experiment or the methods used.

Since filling the box with 100% O_2 would raise the concentration in the nasal passages nearly five times without greatly increasing the O_2 dissolved in the blood, this approach hopefully should have answered the question of the site of O_3 production. The lack of effect on the stimulus-response curves argues for cell water being the site for O_3 mediation. However, greater knowledge of the O_3 production kinetics is necessary before accepting this view. Doubt rises since Koller's results were for U.V. and were obtained at O_3 equilibrium instead of for the early few moments of the O_3 production curve. Not only do high concentrations of O_2 enhance O_3 production but they also increase its destruction. Certainly the difference in O_3 concentration in the first 10 sec. of the growth curve must have been very small, perhaps too small to detect by this behavioral measure.

2. Increased H_2O concentration.

Increased humidity in the conditioning box should have a marked effect on the O_3 levels, since water is known to catalyze the recombination of atomic oxygen to O_2 . The previous experiments were run at 40--50% humidity. Addition of more drier to the gas flow system reduced the humidity to less than 30% or bubbling the air to the box through hot water increased the humidity to 80--90%. These two levels were used.

Again no behavioral difference was seen under control conditions for experienced animals in low and high humidity. Figure 2 contains the suppression ratio plotted against the log of the intensity for pooled data from five experiments. As predicted, the stimulus-response curve was shifted downward by increasing the water vapor, indicating that O_3 concentration was reduced. Another interpretation might be that the olfactory mechanism because less sensitive with increased humidity as has been found to be true in studies with other odors. In either case the results argue for detection via the gaseous route. Greater clarification could again be achieved by obtaining a direct measurement of O_3 production under these conditions.

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Fig. 1. Stimulus-response curves for X-ray detection by rats. The animals performed on a conditioned suppression routine with the operant conditioning box filled with air and with 100% O_2 . Vertical bars represent standard error for data pooled from two or more experiments.

Fig. 2. Difference in stimulus-response curves for rats detecting X-ray in low (less than 30%) and in high (80-90%) humidity. Vertical bars represent standard error for data pooled from five experiments.

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Gb-6. *Behavioral Evidence That Ozone Mediates X-ray Detection.* S. A. HELLING* AND E. L. GASTEIGER* (introduced by N. A. Buchwald), Department of Physical Biology, N. Y. State Veterinary College, Cornell University, Ithaca, New York.

Ozone, added to inspired air, blocks olfactory detection of low level X-rays (12 R/min.). Although this effect has been interpreted as indicating that ozone is an X-ray produced odorant which initiates detection, (Gasteiger and Helling, *Sci.* 154, 1039, 1966) the inhaled ozone might act as a masking substance. Therefore, behavioral experiments were designed to test for the similarity of X-ray and ozone as olfactory stimuli. After establishment of a licking routine in rats, they were conditioned to stop licking to avoid shock when presented with odorants for 10 sec. Ozone at just detectable levels or pineapple odor (ethyl-butyrate) was introduced into their nasal passages through cannulae chronically implanted in their frontal sinuses. An alternate method was to eject the odorant into the air near the drinking tube. In both methods a continuous flow of air was maintained. The animals performed this conditioned behavior at a high level. Transfer of the conditioned response to X-ray was tested by randomly interspersing 10-sec exposures to X-ray, without shock, among the exposures to the gaseous stimulants, with shock. The animals conditioned to ozone regularly stopped licking during X-irradiation while the animals conditioned to pineapple did not. (Supported by Defense Atomic Support Agency and U. S. Public Health Service)

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2. Spinal Cord: Modification of its Labile Electrical Characteristics
by X-irradiation
E. L. Gasteiger

Despite numerous studies (see ref. 1, 2, and 3) of acute responses of the spinal cord to ionizing radiation, few have involved the more plastic or labile mechanisms of the cord. When looking for subtle responses to low level irradiation (less than 500 r) which might account for behavioral effects, it is desirable to examine neural systems susceptible to plastic changes. Evidence that such low level effects exist comes from the spinal cord studies of Fedorova (ref. 3, p. 68) and Kudritakiz (ref. 1, p. 44) and the current work of Barnes (4).

Our approach has been to excite the spinal cord repeatedly with single shocks given once per $3\frac{1}{2}$, 5, or 8 seconds over long periods of time. In this way plastic elements of the cord reach a new state of decreased or increased excitability (5, 6). The cord was then irradiated and alteration of the new state was sought.

Methods

Five cats were anesthetized with ether, and the trachea and one carotid artery were cannulated for artificial respiration and blood pressure recording, respectively. The other carotid was ligated to reduce the circulation to the head. Spinalization was completed by occluding the vertebral arteries with a clamp, and transecting the spinal cord at C1. At this stage, ether was removed and the animal was placed on artificial respiration. A laminectomy was performed from S1 through L3 vertebrae, and roots L7 and S1 were isolated for recording. Ventral roots were cut. Surgical exposure was made of the popliteal region, and the sural nerve was prepared for stimulation. The cord and all roots and nerves were covered with warm mineral oil to prevent drying, and body temperature was maintained at $37.5 \pm 1.0^{\circ}\text{C}$ by means of a feedback controlled heating pad. Recording from the spinal cord was initiated five hours after removal of the ether. The following evoked potentials were displayed on an oscilloscope and recorded on FM tape for analysis on an average response computer: ventral root potential (VRP), dorsal root potential (DRP), and the cord dorsum potentials (CDP) from levels L4 and L7. In addition, the cord spontaneous slow waves (SEG) were monitored by paper recordings on a polygraph.

Results

Thus far our studies on the spinal cord can only be considered as pilot experiments used to resolve technical difficulties and to look for obvious effects. Despite early successes, we have had difficulty in producing long term modification of the evoked responses. However, some observations of the many cord potentials were made.

The experiments to date can be summarized as follows:

- Cat #1 -- no irradiation effected due to technical failures.
- Cat #2 and #3 -- no systematic modification of evoked potentials

obtained over 30 to 60 minutes of repeated stimulation of sural nerve at one stimulus per $3\frac{1}{2}$ sec. (cat #2), and one stimulus per 5 seconds (cat #3). No striking modification of evoked and spontaneous activity by irradiation of 300, 600, and 1,200 R total dose, and 640 and 1280 R total dose for cats 2 and 3, respectively. There was an indication that DR reflex and SEG were enhanced.

Cats #4 and #5: early experiments in which plasticity appeared as a systematic decrease of evoked potentials obtained in response to a stimulus repeated at once per 8 seconds. The potentials were restored to near control levels upon irradiation with 600 R total dose.

Discussion

As yet no conclusive results have been obtained. It appears that it will be difficult to use the long-term changes of the spinal cord to study radiation effects due to the large innate variability of the plastic changes. Consequently, additional experiments of this type must be performed. Attention will be given to the same evoked potentials already under examination, but, in addition, certain long-term aspects of the inhibitory processes will be studied (7).

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3. Studies on Radiation-Induced Gustatory Avoidance Behavior
D. N. Tapper

Abstract: These studies have been directed toward an understanding of (1) the nature and time course of the aversive stimulus which is induced by radiation exposure, (2) the time course of the developing avoidance in relation to radiation intensity, (3) the interaction of the aversive stimulus with the gustatory cue in forming a conditioned avoidance, and (4) the resultant strength of avoidance as a function of dose, gustatory stimulus intensity and time at which the gustatory cue is offered. In addition, the conditioned behavior has been exploited to study various aspects of gustatory physiology and behavior such as taste recognition thresholds, gustatory stimulus generalization and quantitative comparison of similarities of chemicals, with regard to gustatory stimulus quality.

An intermittent fluid presentation scheme has been developed whereby it has now become possible to offer and to simultaneously monitor the response of the animal to the flavor cue during and subsequent to radiation exposure. The method is useful for watching the avoidance develop and for subsequent testing of the strength of avoidance.

Development of avoidance was found to occur as early as 14 minutes after the start of exposure (200 R at 80 R/minute Cs^{137} gamma). When such fluid presentation scheme parameters as cycle time and initiation time after of exposure were varied it was found that the number of presentations (onset score) was the most invariant measure. Doses ranging from 50 to 200 R had little effect on onset score. Development of avoidance must be clearly distinguished from strength of established avoidance. While a clear and rapid development was obtained when the fluid presentations scheme was initiated 90 and 180 minutes after the start of exposure, little or no evidence of conditioned avoidance was established. The strongest conditioning was obtained when the presentation scheme was initiated 20 minutes after the start of exposure. Onset score was also markedly influenced by the chemical cue used. At highly preferred concentrations of d,l-alanine significantly greater numbers of fluid offerings than with sodium saccharin were needed before development started. Similar results were found with NaCl and glycine solutions. Substrain differences in terms of efficacy of establishing the avoidance and of onset scores were found.

Taste recognition thresholds were clearly established for sodium saccharin and d, l-alanine. The thresholds for sodium saccharin lay between 0.05 and 0.1 mM, and between 1 and 2 mM for d, l-alanine. Cross comparisons of these chemicals showed a large overlap in stimulus quality. Sodium saccharin was found to have qualitative similarities with d, l-alanine and glycine but little overlap with glucose; the animals could readily distinguish the latter three from each other.

A. Historical Perspective:

The first reports on radiation-induced gustatory avoidance by Garcia, Kimeldorf and colleagues showed that when a novel and distinctively tasting fluid, such as a solution of sodium saccharin, is offered to an animal during radiation exposure, the animal will reject the fluid even when under ordinary circumstances this flavor may be preferred. The radiation appeared to act as a noxious aversive stimulus which when paired with the novel taste cue led to a gustatory avoidance of the solution. It became evident with additional reports, however, that the order of pairing as well as the interval between exposure and presentation of the taste cue could be varied over a wide range and still effectively produce the avoidance. Thus, an unorthodox pattern emerged terms of the more widely held concepts of conditioning in which close temporal contiguity of the aversive stimulus - presumably the radiation - x and the cue with which it is associated was considered necessary. The studies of Garcia and Kimeldorf on their temporal relationships as expanded by Smith and colleagues and Scarborough *et al.* indicated that the strongest avoidance, as judged by a test at 24 hour after exposure, was obtained if the taste cue was presented 30 minutes to one hour after the initiation of the exposure, and that it was essentially ineffective when presented after four hours, and was intermediate in effect when given for a short time period at the start of exposure. These results suggest that the radiation is not the aversive stimulus, but rather, that the exposure initiates an alteration in physiological state which reaches a noxious level that constitutes an aversive stimulus with which the taste cue can be associated. Rather explicit evidence for such an alteration in physiology is beautifully demonstrated in the results of Hunt and Kimeldorf in which a humeral factor is involved.

Whereas it is now clear that temporal contiguity of exposure with the taste cue is not required, the nature of the internal aversive stimulus and the kinetics of its occurrence, the kinetics of the development of the avoidance in relation to radiation intensity and the gustatory chemical stimulus, and the mechanisms by which the association is made are all in need of further exploration.

While most studies have centered upon the radiation effects aspects of this conditioned behavior, the behavior appears to uniquely suitable for the study of gustatory behavior in animals. The conditioning paradigm allows the investigator to produce a strong and sustained avoidance to any one of a wide variety of chemicals at a single training session with relatively low exposure doses, thus readily enabling the study of gustatory stimulus generalization, taste stimulus recognition thresholds and quantitative chemical stimulus scaling.

The data reported below covers many of these aspects. The method developed to study the microstructure of the developing avoidance during and subsequent to exposure has been central to all the students.

B. Development of Gustatory Avoidance: Method of Study

One of the primary objectives of these studies was to understand the nature of the aversive stimulus that is initiated by, but not necessarily coincident with, radiation exposure. A method was devised whereby the flavor cue could be presented to the animal and the development of the gustatory avoidance could be concomitantly monitored during and subsequent to the exposure, that is, throughout the critical period up to several hours after the start of exposure. The basic assumption compelling this approach was that the pattern of development would reflect and closely follow the development of the internal noxious stimulus. The following criteria were used to design the method:

(1) The period of radiation should be as short as possible to minimize any error in timing due to length of exposure and to clearly separate the period of exposure from that of earliest possible development. This criterion was important, also, because the minimum dose which is effective in initiating an aversive stimulus is as yet uncertain. Long exposures at low dose rate obscure the point of zero time and smear the period of exposure with that of development.

(2) The animals should maintain high and steady drinking levels throughout the critical period of interest.

(3) There should be a concinual monitor not only of the animal's drinking interest in general so that the specificity in the avoidance toward the flavor can be established at the time of development.

(4) The parameters of the method should be subject to controlled variation so that the influence of these factors can be independently examined.

The method may be divided into three periods: training, development, and testing.

I - Training

Male Sprague-Dawley rats weighing 150-200 gms. were used. The training regimen started with an adjustment period of approximately one week during which their usual drinking fluid, distilled water, was offered to them for only one hour per day. Body weights and fluid consumption was measured daily, the latter by the lick counting method. The resumption of growth was used as the criterion of adjustment. The animals were then trained to an intermittent fluid presentation schedule whereby the distilled water was offered to the animal every 40 seconds for a 20-second period. The endpoint of this phase of training was reached when sustained and high level of licking was obtained through 40 consecutive fluid presentations (usually one week). At this stage a steady drinking activity was obtained at uniform intervals for 27 minutes. The duration of the drinking activity could then be extended beyond two hours by gradually increasing the interval between fluid presentations.

Throughout the training period and most of the subsequent development and testing periods, the animal remains in his home cage. The cage fits into a specially designed fluid-presentation apparatus which is drawn in Figure 1. Fluids can be presented through one of two portals. The animal gains access to the portals through a window in the front wall of his cage. The portal is narrow and limits entry to the animal's tongue only. Fluid consumption is monitored by the photocell lickmeter circuit shown in the figure or by the conventional high impedance circuit. Parameters of the fluid presentation scheme such as span of fluid offering, interval between offerings and selection of sequence of appearance at the portals, are all independently and electronically controlled.

II - Development

On the day of development the fluid presentation scheme is modified such that the usual distilled water offering is alternated with that of a novel flavored solution (such as 5mM Na saccharine, 0.2 M d,l-alanine, 0.2 M. glycine, 0.2 M. NaCl, 0.1 M KCl, etc.). The animal is usually irradiated while in the fluid-presentation apparatus by use of Cobalt-60 or Cesium-137 gamma sources. The fluid presentation sequence may be initiated at any time from the start of the exposure. When higher dose rates are required than can be readily obtained by exposure in the presentation apparatus, the animals are irradiated outside the device and then returned to it. At the dose rates and total doses customarily used, however, the minimum initiation time is 10 minutes after the start of exposure.

The exposure, presentation, and measurement parameters are graphed in Figure 2. The beginning of radiation is taken as zero time. The initiation time is the time at which the fluid presentation begins relative to the onset of exposure. Cycle time refers to the interval between offerings of the flavored solution or of the distilled water. The number of flavored solution offerings until avoidance develops is called the onset score and the time from first presentation of the flavor to the start of development is designated the onset time. The time between start of exposure and the onset of development is called the post exposure time. The number of flavor offerings between the start of development and its completion is called the development score.

Examples of characteristic developments are shown in Figures 3 and 4 in which 0.1% Na saccharin solution was used as the taste cue. In the first example, a dose of 100 r at 10 r/minute was used. A 10 minute cycle and 0 initiation time was used. An onset score of 8 corresponding to onset and post-exposure times of approximately 75 minutes and an onset score of 3 were obtained. In the second example, a dose of 100 r at 80 r/minute was used. The fluid presentation sequence was initiation at +10 minutes with a cycle of 1.33 minutes. An onset score of 2 and a development score of 2 were obtained. Avoidance started approximately 12 minutes after the start of exposure and 1.3 to 2.7 minutes after initiation of the presentation sequence. In both examples consumption of the familiar distilled water continued unabated accentuating the specificity of the avoidance in this instance, to the novel sodium saccharin solution. In a later section of this report, the influence of initiation and cycle times on onset and post-exposure times will be

considered in detail. (Section C-2)

III - Testing

As will be demonstrated later, a clearly defined development pattern is not a measure of the strength of the established avoidance. Such a measure must be independently determined and is conveniently done on the next and subsequent days by use of the 1.33 cycle fluid presentation cycle in which distilled water is alternated with the novel and distinctively flavored solution.

For purposes of scoring consumption of the flavor relative to distilled water, consecutive flavor-water or water-flavor offerings are grouped as presentation pairs and a fluid consumption score is calculated as the percent of aversive fluid to total fluid of the pair consumed, i.e.

$$\text{Consumption score} = \frac{\text{aversive fluid}}{\text{aversive} + \text{water}} \times 100.$$

After much testing experience we have accepted this parameter as valid only when at least 80 water licks per pair is registered. This precaution was inserted to assure us that the animal was capable and willing to drink, that is, that the animal's general physiological state was adequate for the procedure and that the avoidance was specific to the primary averting flavor and those to which it may be generalized* rather than to any fluid.

An example of twenty presentation pair tests of the strength of avoidance is shown in Figure 5. The animal received an exposure dose of 200 r at 10 r/minute. A development scheme of 10 minute cycle and an 0 initiation time was used. The onset score equalled 8. On day 1 the animal took only a few licks of the primary averting fluid and had a mean consumption score of less than 10 percent. By Day 4, however, consumption of the flavored solution was high until the 6th flavor presentation at which time, the avoidance became evident and a very little flavor consumption followed. Not until the 11th day was the flavored solution accepted equally with the distilled water. The pattern of extinction is a typical one. Its time course is dose dependent.

At doses of 200 r and above, a measure of the strength of avoidance may be had by using fewer presentation pairs and incorporating a dilution series into the procedure. Thus a solution may be tested for 5 pairs at the primary averting concentration and then for 8 pairs each at two lower concentrations of the same flavor. This test procedure and modifications thereof serve as a basis for determination of taste recognition thresholds (see Section D-1).

* Gustatory chemical generalization studies are reported in Sections D-2.

C. Parameters Affecting Development of Gustatory Avoidance:

Radiation Dose, Dose Date, Initiation Time and Cycle Time, Strength of Avoidance, The Gustatory Stimulus, Substrain Differences.

C-1. Radiation Dose In the earliest experiments to test this method 12 Sprague-Dawley male rats were used. The objectives were (1) to determine whether clear definition of the start of development could be obtained, (2) to determine whether a sufficient span of the critical development period could be covered, and (3) to obtain preliminary information on the influence of total dose on the time at which the avoidance developed.

The rats were trained to a 10-minute cycle schedule in order to span at least the first two hours after the start of exposure. Using a zero initiation time and 5 mM sodium saccharin solution as the averting taste cue, three animals received an exposure of 40 r, four of 100 r and five of 200 r at a dose rate of 10 r/minute. The longest irradiation was, then, 20 minutes.

The results of this preliminary experiment are given in Table 1.

Table 1

Dose	Onset Score	Development Score
50 r	8,8,7	2,4,2
100 r	6,7,8,-	3,2,3,-
200 r	7,7,4,6,7	1,1,3,6,3

Eleven of the twelve animals demonstrated reasonable well-defined development patterns. The pattern illustrated in Figure 3 of the preceding section was one of this group. Little difference in the onset score was seen among the groups; the median of the 11 values was 7. This corresponds to onset and post-exposure times of approximately 70 minutes. Once development started, it was completed, on the average, within the next 2 to 3 flavor presentations.

C-2. Influence of Initiation and Cycle Times. Whereas the preliminary results showed that the method clearly defined the development of the avoidance, no information was gained concerning the influence on the time of development of the methodological variables. A large number of experiments were conducted to determine whether the time of development was independent of the initiation time and the cycle time, i.e., whether it was determined solely by the radiation exposure and ensuing internal changes. Since the least variability was attained by use of an exposure dose of 200 r and the strength of the subsequently tested avoidance was the largest at this dose, 200 r was used in all subsequent experiments. While most effective in eliciting the response, a 200 r exposure at 10 r/minute required 20 minutes to deliver and this made it impossible to study the earliest time period at short cycle times. A dose-rate of 80 r/minute was adopted. The 200 r could be administered in 2.5 minutes. To obtain this dose rate it was necessary to expose the rats in small lucite boxes rotated very close to a single cesium-137 source. This limited our earliest initiation time to +10 after the start of exposure.

Next, two experiments involving 8 and 7 animals respectively were done.* In the first study the animals were trained on a 6-minute cycle schedule and the fluid presentation was initiated at 10 minutes after the onset of exposure. In the second study a 1.33 minute cycle and an initiation time of 20 minutes were used. Five (5) mM sodium saccharin was the flavored solution. If the time were independent of the methodological variables, it would be anticipated that the onset score in the first experiment would be approximately 10 and that in the second experiment few if any of the animals would show development. Those that did would have high onset scores.

The results of these and the previous experiment are summarized in Table 2.

Table 2

Expt.	N	Duration		Initiation	Curve	Post-Exp.	Onset	Devel.
		Dur.	Rate	Time		Time	Score	Score
I	5	20	10	0	10	57(6.6)*	6.2(0.6)	3.2(0.4)
II	8	2.5	80	10	6	43(4.6)	6.3(0.7)	3.2(0.5)
III	7	2.5	80	20	1.33	27(1.7)	5.7(1.2)	2.7(0.5)

* Mean (Std. error)

* At this time, we changed our supplier of the Sprague-Dawley strain from Blue Spruce to Charles River since a larger percentage of these animals were found to develop the avoidance than either those from Blue Spruce or Holtzman (see section on substrain differences)

It is at once clear (1) that the post-exposure time is not an independent measure of the onset of an internally produced noxious stimulus and (2) that the invariant element appears to be the onset score, that is, the number of flavored solution presentations. One may conclude from this data that the quantity of flavor offered in the 6 flavor presentations constitutes a minimum taste stimulus with which the aversive stimulus need be paired to develop the avoidance. The data clearly illustrate that the avoidance can develop as soon as 27 minutes after the start of exposure under these dose-metric conditions.

Additional studies were performed using the 1.33 cycle at initiation times of 10, 20, 90 and 180 minutes. The results of these experiments are shown in Table 3.

Table 3

Expt.	N	Initiation Time	Post exp. Time	Onset Score	Devel. Score
IV	7	10	15(0.5)*	4.4(0.3)	4.0(0.3)
V	12	20	28(1.0)	5.8(0.7)	4.0(0.5)
VI	8	90	92(0.5)	1.9(0.5)	3.1(0.3)
VII	8	180	182(0.5)	1.8(0.5)	3.5(0.4)

5mM Na Saccharin, 200 r, 8 r/minute, 1.33 minute cycle. CR 150-200 gms.

* Mean (Std. Error)

The results of this series amplify those of the former ones. The post exposure time depends on the initiation time and cycle. In addition, small but highly significant differences in onset score with initiation time were observed. Development can occur within 15 minutes after the start of exposure; the noxious stimulus had developed by this time. Again, the development score appears to be independent of the experimental conditions.

C-3. Strength of Avoidance As indicated in B-III, Testing, a test of the avoidance can be conveniently made on the days after the development day by using the 1.33 cycle in which the primary averting flavor is alternated with water. The strongest avoidances show up during the initial presentation pairs. Weaker avoidances become evident only after several pairs and the weakest emerge only toward the end of a 20-pair sequence. The strength of avoidance was measured for several initiation times on Day 1 after exposure. A 5-pair test was run using the primary averting solution. Histograms of onset and mean consumption score (with

standard errors) are shown in Figure 6 for 10-, 20-, 90- and 180-minute initiation times. Only mean consumption scores for the 10 and 20 minute initiation times are significantly different from a water-water control (see later sections on taste recognitive thresholds). Clearly, the development of avoidance during the period just after exposure should not, under these conditions, be interpreted in terms of subsequent strength of avoidance. There is a suggestion, however, of an inverse relationship between onset score and mean consumption score. The data here would only describe the boundaries of this relationship. No data are presently available for mean consumptions in the region of 30 percent. Should this relationship be correct one might then conclude that another factor determining the strength of the avoidance would be the quantity of flavored solution that the animal consumes before it starts to reject the solution, or, that the strength of the avoidance subsequently developed depends on the stimulus strength of the flavored solution. The appropriate scale for stimulus strength is as yet uncertain.

The results on strength of avoidance agree well with those previously reported, namely the avoidance is strongest when the primary association is made at about 20 minutes, is least effective at three hours and may be slightly less than maximally effective when the association is made with the onset of the exposure.

C-4. The Gustatory Stimulus All of the above work has described radiation-avoidance produced by use of sodium saccharin solution as the primary averting flavor. In Section D a detailed report on gustatory stimulus recognition thresholds and stimulus generalization will be given. From these data, it has become clear that the onset score and post exposure time is also influenced by the nature of the taste cue, that is, it appears to be related to the stimulus strength of the flavor used. Table 4 summarizes the results of two experiments in which rats of the same batch were conditioned to avoid d,l-alanine or sodium saccharin.

Table 4*				
Expt.	Primary Averting Chemical	N=	Onset Score	Devel. Score
CR 161	Na Sacch.	11 ⁽¹⁾	6.4(0.7)**	3.9(0.7)
	d,l-alanine	10 ⁽²⁾	9.9(0.6)	3.5(0.6)
CR 186	Na Sacch.	12	7.3(0.9)	3.3(0.4)
	d,l-alanine	9	12.4(1.1)	2.8(0.5)

* 200 r, 80 r/minute, 150-200 gms., Na Sacch. (5 mM), d,l-alanine (0.2M), initiation time = 20, 1.33 minute cycle.

** Mean (STD error)

- (1) 1 animal did not avert
- (2) 2 animals did not avert

The sodium saccharin scores do not vary significantly from that reported above. In both experiments, the d,l-alanine scores are significantly larger. Strong avoidances, as determined on subsequent days (see page), were obtained in all animals of both studies.

Onset and development scores for NaCl, glucose, and glycine are given on Table 5. With all of these solutions, the onset scores are significantly greater than those obtained for sodium saccharin solution.

Table 5

Expt.	Primary Averting Chemical	N	Concentration	Onset Score	Devel. Score
CR 78	NaCl	6	0.2 M	10.5(1.2)*	2.8(0.5)
CR 107	glucose	8	0.5 M	12.3(1.4)	2.1(0.4)
CR 48	glycine	3	0.2 M	11.7(0.7)	2.3(0.7)

200 r, 80 r/minute, 20 minute initiation time, 1.33 minute cycle

* Mean (STD. error)

C-5. Substrain Differences The percentage success in producing the avoidance varies remarkably depending upon the source of the Sprague-Dawley Strain rats. The earlier experiments were done with a Blue Spruce supply. While onset scores were similar to those obtained by use of the Charles River supply, the percentage success was approximately 50 percent. In about 95 percent of the Charles River animals a strong avoidance can be established. The problem is still under study. For our latest studies of this matter the Holtzman source was used. Table 6 summarizes the result from 10 animals.

Table 6

Animal	Initiation Time	Onset Score	Devel. Score	Mean Consumption Score
H 57	10	11	5	6 $\pm$ 3
58		14	4	20 $\pm$ 6
59		13	2	11 $\pm$ 7
50	20	10	5	57 $\pm$ 3
54		10	3	8 $\pm$ 4
55		12	4	6 $\pm$ 3
56		—	—	45 $\pm$ 4
51	90	14	2	49 $\pm$ 1
52		—	—	50 $\pm$ 1
53		8	1	47 $\pm$ 1

A comparison of these data with those of Table 3 indicates that the Holtzman substrain is a much different animal. The onset scores are much higher, even at +90 minutes and the mean consumption scores taken from 6-pair tests shows that less than very strong avoidances were established. We have had similar results with this substrain when using other flavors.

D. Studies of Taste in Animals by Use of the Conditioned-Avoidance Paradigm

It has been postulated that chemical gustatory stimuli may be ordered along several sets of neural and behavioral continua that reflect the important groupings for taste quality. Taste matrices derived from responses of many gustatory neurons to a variety of chemical stimuli have been constructed on this basis. There is, however, a limited behavioral literature on how animals classify chemicals according to qualitative gustatory similarities and there is need of further behavioral evaluation of these concepts.

Conditioned avoidances procedures by which the motivation is independently attached to a chemical stimulus have been employed for determining qualitative comparisons. Avoidance to gustatory cues are most readily and firmly established when non-somatic, or visceral, aversive stimuli are used. The toxic chemical, lithium chloride, has been used both as the noxious stimulus and the taste stimulus but the subsequent comparisons are limited to those chemicals which are qualitatively similar to it. In contrast, the radiation-induced gustatory avoidance procedure appears to be a uniquely suitable one. A strong and sustained avoidance to any one of a wide variety of chemicals can be developed at a single training session with relatively low exposure doses. Therefore, this paradigm was exploited to study gustatory stimulus generalization at various dilutions of a single chemical, i.e., an evaluation of recognition threshold, and generalization across chemicals, i.e. qualitative chemical classification.

These studies were based on the following assumptions, some of which apply to the use of conditioned avoidance methods in general and some to this specific radiation-induced paradigm.

- (1) Whereas the degree of flavor rejection depends on the induced avoidance motivation we have not independently measured this motivation.
- (2) Nevertheless, we can establish boundaries on the motivation, namely, when tested, the animals will strongly reject the flavored solution at the primary averting concentration but will still drink water freely. (The criterion of strength of rejection will be spelled out below).
- (3) The stronger the established avoidance, the lower will be the dilution of the primary averting chemical that the animal is motivated to reject. At the strongest motivational level within the boundaries set by (2), the animal will fail to reject the flavored solution only when it is so dilute that the animal can no longer recognize the quality of the solution.
- (4) When the animal is tested for rejection of chemicals other than the primary averting chemical, the magnitude of rejection of such chemicals will vary according to the similarity in sensory stimulus, i.e. quality, that these flavors have with the primary averting chemical. The quantitative relationships are concentration dependent.

D-1. Taste Recognition Thresholds Detailed studies on taste recognition thresholds were run for two substances, D, L-alanine and sodium saccharin. In all experiments Sprague-Dawley rats from Charles River were used. They received a 200 r exposure at 80 r/minute and the fluid presentation scheme was initiated 10 minutes or 20 minutes after the start of exposure. On the first or second day following development, the animals were tested first at the primary averting concentration on a 5-pair test and then, in descending order at two or three lower concentrations. In all but the first sodium saccharin experiment each animal was run the day before development in a water-water simulated test experiment to obtain internal

controls. In all instances, the mean of the flavor consumption scores were tested against 45 percent, a lower boundary of water-water intake.

Na Saccharin: Two experiments were run. In the first, 8 animals were used. They were conditioned to avoid 5 mM Na saccharin solution when presented on a 6-minute cycle initiated at +10 minutes. Their mean onset and development scores were 6.6 (.8) and 3.4 (.5), respectively. On the second day after exposure they were tested at four concentrations of sodium saccharin in consecutive 5-pair tests. Figure 7A illustrates the results of this experiment. The filled circles represent animals whose mean consumption score was significantly different from 45 percent as determined by Student's t test. The open circles represent animals whose scores were not. At the highest concentration, 5 mM, seven out of eight animals showed strong and highly significant rejection of the solution. The same seven still rejected a 0.5 mM solution, however, the dispersion in the values has increased. At this lower concentration some of the animals do not reject the flavor as strongly; an apparent gradation in response to stimulus intensity. With the 0.05 mM and 0.005 mM solutions only two out of the eight animals continue to show significant rejection. It appears that at some concentration between 0.5 and 0.05 mM lies the transitional zone where a majority of the sample fail to recognize the solution.

In the second experiment 12 animals were tested at the primary averting concentration and at 0.05 and 0.1 mM, levels in the transitional zone. The results are graphed in Figure 7b. Again strong avoidances were established at a 5 mM concentration. Nine of the twelve animals were still aversive at 0.1 mM and only one out of 12 rejected 0.05 mM sodium saccharin. One may conclude from these results that greater than half of male rats from this source can recognize 0.1 mM sodium saccharin. Included on this graph are internal water-water controls.

A characteristic behavior pattern has emerged from this data. By use of the avoidance paradigm, all animals are placed into a single population which strongly reject the primary flavor at its averting concentration. With dilution, this original population is divided into those who don't distinguish the flavor from a distilled water solution and those who still reject, hence recognize, the flavor. With further dilution, there is a shift from the recognizing to non-recognizing populations. Furthermore, with the population that still recognizes the flavor, there appears a tendency for the animals to consume larger amounts of the flavor at the lower concentrations; perhaps larger amounts at concentrations at which the stimulus magnitude per unit consumption is diminished.

d, l-alanine: An experiment similar to that described above was conducted using 0.2 M d,l-alanine as the primary averting solution. Water-water controls were obtained on the day prior to exposure for a 5-, 8-, 8-pair series. The development was conducted at 200 r, 80 r/minute, 1.33 minute cycle at a 20-minute initiation time.

The mean onset and development scores for the 12 animals were 7.6(0.7) and 3(0.4) respectively. The animals were tested on Day 1 after exposure on a 5-, 8-, 8-, 5-pair descending series. The results are shown in Figure 8. Those mean consumption scores that are significantly

different from 45 percent are shown in filled circles, those that were not shown in open circles, and the water-water control values are plotted as crosses. A pattern similar to that seen with sodium saccharin was obtained with d,l-alanine. All the animals were strongly conditioned to avoid 200 mM alanine but as the dilution increased the population divided with increasing numbers jumping into the non-recognizing class. The steepest transitional zone appears to be between 1 and 2 mM. Again, in those animals that still recognized the flavor, the consumption tended to increase as the concentration was reduced.

D-2. Comparison of chemicals according to taste quality. Generalization..

Studies were initiated whereby animals were conditioned to strongly avoid one substance and were then tested against other substances, some of which might be thought to be similar and others that were thought to be different. At first several scanning-type of experiments were done in which many chemicals were tested at a single concentration. These experiments tended to give gross information concerning similarity. In later experiments, the influence of concentration of the test chemical solution on the response is evaluated.

(1) Nine rats were used. After an exposure of 200 r at 80 r/minute they received a bottle of either 5 mM sodium saccharin, 0.1 M glycine or 0.1 M KCl in their home cage 20 minutes after the start of exposure. On day 1 post-exposure all animals were tested on 5-pair tests for acceptance of the primary averting chemical and the other two solutions. The results of this survey experiment are shown on Table 7. In all cases, the animals were strongly conditioned to avoid the primary aversive solution. In the case of the sodium saccharin animals this was generalized to some extent to the glycine solution but not to the KCl solution. Similarly, the glycine avoidance was generalized to the saccharin but not to the KCl solution. As expected KCl did not generalize to either glycine or sodium saccharin.

(2) Eight rats were conditioned to avoid 5 mM sodium saccharin by exposure to 200 r at 80 r/minute using a two-minute initiation time on a 6-minute cycle. They were then tested against 5 mM sodium saccharin, 0.1 M d,l-alanine and 0.1 M glucose. The results of this experiment are shown in Table 8. All animals were strongly conditioned to avoid the sodium saccharin solution. In half of the animals this avoidance was generalized to d,l-alanine at 0.1 M but none of the animals associated 0.1 M glucose with the primary aversing solution.

(3) Six animals were conditioned to strongly avoid 0.2 M d,l-alanine. They were tested with a 5-pair test on day 1 after exposure for acceptance of 0.2 M alanine, 1 M glucose and 0.2 M glycine. The results of this experiment are shown in Table 9.

Table 7

<u>Averted to</u>	5-Pair Tests*		
	Sodium Saccharin	Glycine	KCl
Sodium Saccharin	11(4) ⁺	20(7) ⁺	52(.4)
	7(4) ⁺	43(2)	49(1)
	9(4) ⁺	17(7) ⁺	21(11)
Glycine	6(2) ⁺	0(0) ⁺	56(4)
	28(5) ⁺	0(1) ⁺	50(.2)
	32(11)	9(1) ⁺	43(6)
KCl	52(6)	51(2)	5(4) ⁺
	49(2)	39(10)	1(.7) ⁺
	52(6)	56(.6)	10(6) ⁺

* Mean (STD error)

⁺ Significantly different from 45% $P < .05$.

Table 8

5-Pair Test - Generalization from Sodium Saccharin Avoidance*

Sodium Saccharin	d,l-alanine	glucose
10(6) ⁺	14(10) ⁺	23(20)
0(0) ⁺	20(9)	36(3.5)
.4(.4) ⁺	53(.5)	39(3.2)
1(1) ⁺	51(2)	29(12)
0(0) ⁺	1(.6) ⁺	42(7)
1(1) ⁺	0(0) ⁺	47(6)
0(0) ⁺	3(3) ⁺	42(1)
1(.3) ⁺	48(1)	48(1)

* Mean (STD error)

⁺ Significantly different from 45% P<.05.

Table 9*

d,l-alanine	glucose	glycine
3(1) ⁺	27(10)	50(2)
2(2) ⁺	51(.4)	51(1)
0(0) ⁺	50(1)	48(.6)
7(5) ⁺	51(1)	30(5) ⁺
3(2) ⁺	52(1)	52(.6)
1(.6) ⁺	49(2)	23(8)

* Mean (STD error), N=5

⁺ Significantly different from 45%, $P < .05$.

Whereas the animals were strongly conditioned to avoid d,l-alanine, they had no trouble distinguishing either glucose or glycine from the alanine. One animal marginally associated the alanine with the glycine. It is of considerable interest that both alanine and glycine are classified by the animals as similar to sodium saccharin but are readily distinguished one from the other.

(4) Sixteen animals were conditioned to avoid 5 mM sodium saccharin. Eight of the animals were tested on a 5-, 8-, 8-pair test sequence at three descending concentrations of sodium saccharin solution and eight were similarly tested with three d,l-alanine solutions. The results are graphed in Figure 9. With 5 mM sodium saccharin only four of the eight animals rejected the solution. With dilution to 0.1 mM two of these remained strongly conditioned and by 0.05 mM none showed any avoidance - a result affirming that seen above (). Seven of the eight animals tested on the 200 mM d,l-alanine showed a marked rejection of the flavor. Two of these continued to reject both the 20 and 2 mM solutions. Also illustrated are the water-water controls measured one day prior to exposure. The concentrations used in this experiment span the transitional zones for the recognition thresholds of each chemical. This interchemical comparison was further explored in the next set of experiments.

(5) Twenty-four animals were used in each of two replicate experiments. Twelve animals were conditioned to avoid 5 mM sodium saccharin and 12 to avoid 200 mM d,l-alanine. Six animals from each avoidance group were tested against d,l-alanine and sodium saccharin at three concentrations of solution on descending 6-pair tests. In the first replicate water-water controls were determined the day before the

Table 10

Conditioned to Avoid	SUBSTANCES TESTED		
	Na Saccharin		
	5 mM	0.5 mM	0.1 mM
Na Saccharin P 0.05 n.s.d.	2.4(.8), n=12 —	11.5(5.6), n=5 56.1(3.4), n=7	3(3), n=2 52(4), n=10
d,l-Alanine P 0.05 n.s.d.	7.3(3), n=10 46, n=1	14.8(4.8), n=5 49.7(1.5), n=6	— 57.3(4.1), n=11
	d,l-Alanine		
	200 mM	20 mM	2 mM
Na Saccharin P 0.05 n.s.d.	4.3(1.9), n=11 —	7(4.4), n=6 50.6(2.1), n=5	3.5(2.6), n=4 46.3(3.7), n=7
d,l-Alanine P 0.05 n.s.d.	5.1(1.5), n=12 —	5.6(2.9), n=5 44.4(5.8), n=5	14(9), n=2 51.4(3), n=10

exposure and the chemical tests were made on day 1 after exposure. In the second experiment, water-water controls were measured day 1 after exposure and the flavor tests were made on day 2 after exposure. The results of the first experiment of this series are shown in Figure 10 and the last in Figure 11. A composite summary of the two experiments is found on Table 10. There is little difference in the acceptance of the flavors as influenced by the primary averting chemical used. The small differences that are evident at the lowest concentrations of the two chemical solutions are not statistically significant. These data indicate d,l-alanine and sodium saccharin overlap markedly in terms of qualitative categorization by these rats. On a concentration basis, however, sodium saccharin appears to be 10 times as strong in evoking the same quality of stimulus.

Figure 1. Fluid presentation apparatus. Animal in home cage is placed in apparatus. Window in forward wall of cage allows animal access to fluid presentation area. A solenoid-driven fan permits the animal to drink from one or the other portal. Duration of fluid offering and sequence of portal presentation is electronically controlled. Fluid consumption is measured by counting licks.

Figure 2. Parameters of the conditioned-avoidance development scheme.

Figure 3. Development of gustatory avoidance of 0.1% sodium saccharin. Onset score = 8, development score = 3, post exposure and onset times = 75 minutes, initiation time = 0 minutes.

Figure 4. Development of gustatory avoidance to 0.1% sodium saccharin. Onset score = 2, development score = 2, post exposure time = 16 minutes, onset time = 6 minutes, initiation time = 10 minutes.

Figure 5. Test of strength of conditioned avoidance to sodium saccharin solution at an exposure of 200r. A gradual increase in the number of accepted presentations of the sodium saccharin is seen from Day 1 through Day 7 post exposure.

Figure 6. A. Relationship of initiation time to onset score. Sodium saccharin avoidance, 200r at 80r/min., 1.33 cycle development scheme. Means and standard errors. B. Initiation time vs. mean consumption score.

Figure 7. Taste recognition threshold of sodium saccharin.
A. Animals tested on a descending series of 5-pair consumption tests. Transitional recognition zone between 0.5 and 0.05 mM examined in detail in second experiment shown in B.

Figure 8. Taste recognition threshold of d, l-alanine. Transitional recognition zone between 1 and 2mM.

Figure 9. Stimulus generalization of sodium saccharin avoidance to d,l-alanine.

Figure 10. Stimulus generalization comparison of sodium saccharin and d,l-alanine.

Figure 11. Stimulus generalization comparison of sodium saccharin and d,l-alanine.