A TRACER TECHNIQUE FOR THE DETECTION OF RESIDUAL DIANISYLHEXENE IN THE EDIBLE PORTIONS OF TREATED BROILERS

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A TRACER TECHNIQUE FOR THE DETECTION OF RESIDUAL DIANISYLHEXENE IN THE EDIBLE PORTIONS OF TREATED BROILERS

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INTRODUCTION

The presence of estrogenically-active compounds within the animal system has long been demonstrated to exert a profound physiological effect by promoting growth rate, feed efficiency and increased carcass quality. Due to the sex-hormonal action of these compounds, the final deposition in any potential food system must be ascertained as a prerequisite to the commercial application of synthetic estrogens in the production of meat.

The implantation of pellets or paste which contain diethylstilbestrol or related compounds has been widely applied in the production of beef. Subcutaneous administration of this type is economically feasible where the number of animals involved is small and the per animal value is relatively high. In the field of commercial poultry production, these economic requirements are non-prevalent, and the handling of the large number of animals involved for subcutaneous injection is not feasible. A more practical approach for the poultry industry is achieved with oral administration by incorporating the estrogenic compounds in the feed normally provided to the animals. Of the various stilbestrols, dianisylhexene is one of the more estrogenically-potent compounds when administered orally to poultry.

Numerous attempts have been made to identify and establish the residual level of stilbestrol compounds which might be retained in the edible portions of animals treated by implantation or oral methods. The majority of these studies has been directed toward the detection

of residual levels of diethylstilbestrol following subcutaneous implantation. It is questionable to attempt an extension of the results obtained from trials involving one class of animal to that of another class. In addition, the metabolic behavior of the various stilbestrols may vary considerably in relation to the method of administration. It becomes imperative that determinations be made for each specific estrogenic compound, for each class of animal and for each method of administration. In view of the high estrogenic potency of dianisylhexene when administered orally to poultry, it would be desirable to obtain additional information on the amounts of residual dianisylhexene retained in the edible portions of broilers.

With the advent of the pile-produced radioisotope, a host of new techniques became available to the researcher. The tracer technique appeared to be particularly adapted to a study of micro-determinations as would be encountered in a study of residual hormones. Due to the molecular structure of dianisylhexene, a tracer study involving radioisotopes would be limited to the isotopes carbon-14 and hydrogen-3, or tritium.

Carbon-14 has been widely applied as a labeling isotope in both the physical and biological sciences; however, the cost of carbon-14 may become prohibitive if a lengthy or difficult synthesis is involved. Hydrogen-3, or tritium, may be obtained at a fraction of the cost of carbon-14, thereby allowing the synthesis of large quantities of labeled compounds for the experimental procedure. Difficulties incurred in the analysis of tritium have restricted the application of this radioisotope in biological studies where low activity levels might be encountered. It would be desirable to further study the possible uses

of tritium as a low-activity tracer in the animal system and to extend the technology available to the biological researcher.

This investigation was designed for the purpose of establishing the following:

1. A synthesis for incorporating a specific tritium label within the molecular structure of dianisylhexene.
2. To study the application of tritium as a low level biological tracer.
3. To identify the maximum quantity of estrogen retained in the edible portions of broilers following oral administration of dianisylhexene.

REVIEW OF LITERATURE

General Applications

The value of diethylstilbestrol, a synthetic, female sex hormone, in promoting fat deposition and growth rate and in improving feed efficiency has been shown by many workers. Lorenz (1943), using three-week old cockerels, first demonstrated a profound increase in the deposition of fat within various tissues following subcutaneous administration of diethylstilbestrol implants. Analysis of breast and leg muscle and liver tissue for fat content showed a three-fold increase over controls as a result of diethylstilbestrol treatment. The quantity and fat content of abdominal fatty tissue were strikingly increased as a result of this treatment.

Jaap and Thayer (1944) found 0.5 of a milligram of diethylstilbestrol per day to be very low in estrogenic potency when administered orally in tablet form to chickens. The addition of 23 milligrams of diethylstilbestrol per pound of feed gave an estrogenic response, but was considered too expensive for the results obtained. These workers found the dimethyl ether of diethylstilbestrol, hereafter referred to as dianisylhexene, to be a potent estrogen when administered orally to chickens.

Thayer et al. (1945) found estrogenic compounds to be more potent when added to the feed as an oil solution than when given in tablet form. For fattening poultry, dianisylhexene was found to be a more

efficient estrogen than diethylstilbestrol. These workers observed an improvement in market quality, skin texture and fat distribution as a result of estrogenic treatment. An optimum level of 40 to 50 milligrams of dianisylhexene per pound of feed was identified for a three to four-week fattening period.

After testing 17 different estrogenic compounds, Jaap (1945) reported dianisylhexene and dianisylhexane to have a high relative potency when administered orally to chickens. This worker reported a high estrogenic activity for dianisylhexene when given orally to turkeys, but under similar conditions, failed to obtain a response from dianisylhexane. Munro and Kosin (1946) studied the efficiency of various synthetic estrogens following the addition of these compounds to the diet at different levels. These workers found dianisylhexane and dianisylhexene to be the most efficient and diethylstilbestrol to be the least efficient of the estrogens under the test conditions.

Lorenz (1954) reviewed the literature pertaining to the effects and applications of the synthetic estrogens to the poultry industry. This author reported a definite improvement in the market quality of birds which had received estrogenic treatment. This improvement in market quality represented a summation of improved fat deposition and distribution in the muscle tissues, increased tenderness of the skin and a decrease in the occurrence of pinfeathers. The convenience and labor-saving advantages of oral administration by incorporating the active estrogen within the feed were pointed out by the author. With feeding techniques, dianisylhexane and dianisylhexene were evaluated to have a higher estrogenic efficiency than that displayed by several other common estrogens, including diethylstilbestrol. Lorenz (1954),

after reviewing the problem of hormone residues in treated birds, expressed the need for more extensive data, particularly in the case of oral administration of the dimethylethers of the stilbenes. This author emphasized restrictions imposed on the applications of estrogen preparations intended for oral administration.

Residual Determinations

In an early attempt to determine the quantity and location of residual hormones in cockerels, Bird et al. (1947) included various estrogenic compounds in the diet for a period of 12 days. The cockerels were then sacrificed and the tissues ether-extracted to remove fat-soluble materials. The ether extracts were then administered to ovariectomized rats and the vaginal smear response was employed as the estrogenic test. These workers found muscular tissues to be very low in estrogenic potency, whereas adipose tissue displayed a high relative potency. Gowe (1949) utilized the estrogen, dienestrol diacetate, in a study of the residue of this hormone following a prolonged feeding period. Ethanol and ether extracts of the tissues were combined and administered to rats and the vaginal smear test was employed to establish the estrogenic potency of these extracts. This worker found that muscle and skin tissue gave a positive response while other tissues failed to show estrogenic activity.

Jones and Deatherage (1953) utilized fat-soluble pellets and aqueous pasty suspensions containing 15 milligrams of diethylstilbestrol as a means of administering the estrogen subcutaneously. An extraction procedure was employed for removal of the residual hormone from the tissues to be tested. Aliquots of the condensed extracts

were added to antimony pentachloride and the color development read in a colorimeter. As a preliminary test, known amounts of diethylstilbestrol were added to various tissues and an 80 percent recovery was obtained by this procedure. These workers reported that no significant amount of diethylstilbestrol was found to be present in the extracts from liver, breast muscle and abdominal fat for either method of estrogen administration.

Swift (1954) utilized subcutaneous methods by implanting diethylstilbestrol pellets with potencies which ranged from 15 to 120 milligrams of the active estrogen. Tissue samples were processed with an extraction procedure which removed and concentrated the residual hormone. A spectrophotometer with blue-sensitive phototube was used for the quantitative determination of the diethylstilbestrol present in the tissue extracts. Preliminary tests indicated a 65 to 90 percent recovery of known amounts of diethylstilbestrol in similar tissues. This worker reported that breast, back and leg muscle did not contain a detectible quantity of the residual hormone. Detectible quantities of the estrogen were measured in skin and liver samples.

In a study of diethylstilbestrol, Stob et al. (1954) made a direct comparison of the vaginal smear and the uterine weight response as an analytical test for the detection of residual estrogen. These workers reported the uterine weight response to be four times more sensitive than the vaginal smear when employed as a measure of estrogenic activity. Tissues were collected from steers from treatment and control groups and were incorporated into the diets provided to ovariectomized mice. These results were compared to a standard dose-response curve which was established by feeding known amounts of

diethylstilbestrol to the test mice. These workers reported that detectible amounts of residual estrogens were found to be present in the meat of these steers.

Stob et al. (1954), using subcutaneous implantation techniques, administered diethylstilbestrol to cockerels for various lengths of time. Tissue samples were collected and incorporated into the diets of castrated female mice and the uterine weight response was employed as the estrogenic test. Detectible quantities of residual estrogen were reported to be present in both liver and muscle tissue for all time intervals tested.

In a study of residual diethylstilbestrol, Merker et al. (1955) made subcutaneous implants in cockerels and collected tissue samples at graded time intervals following treatment. An extraction procedure which had previously been tested to give a 50 percent yield of known amounts of added estrogen was used to remove the hormone residues from the tissues. The concentrated extracts were injected subcutaneously into castrated mice and the estrogenic activity was measured by the uterine weight response. They reported that extracts from the tissues of treated birds did not display significantly different estrogenic activity from control tissues.

In a study of estrogenic residues in the tissues of beef cattle following oral administration of diethylstilbestrol, Turner (1956) and Preston et al. (1956) included the treatment tissues in the diets of ovariectomized mice. The uterine weight response was compared to the measured response obtained by adding known amounts of this hormone to the meat tissues fed to test mice. Turner (1956) reported detectible amounts of estrogenic residues to be present in lung and

kidney tissues, while other tissues did not show an estrogenic response. Preston et al. (1956) reported that no detectible estrogenic residues were found in any of the tissues tested.

Umberger et al. (1959) employed an analytical design of graded response in order to test for estrogenic residues in the tissues of chickens following subcutaneous injection of stilbestrol in different preparations and at different activity levels. Control and treatment tissues were added to the diets of immature female mice and the uterine weight response was employed as the estrogenic test. Measurable amounts of estrogenic activity were detected in the edible tissues of treated birds. These workers reported a decrease in residual activity as the post-injection time increased. Umberger and Gass (1959) employed these same analytical techniques in a study of residual dienestrol diacetate following the inclusion of this estrogen in the chick rations. They reported measurable quantities of the hormone to be present in liver, kidney and intestines whereas other tissues failed to display an estrogenic response.

Tracer Applications

After the application of an extraction-identification technique for the measurement of estrogenic residues, Swift (1954) pointed out the possibility of a tracer application in order to more accurately determine the fate of administered estrogens in the animal body.

With the advent of the pile-produced radioisotope, and with the simultaneous development of instrumentation and methodology, numerous workers have utilized the tracer technique in a wide variety of experiments ranging from those of a purely physical nature to those of

physiological and biochemical processes. Comar (1955), Kamen (1957), Siri (1949) and many other authors have reviewed and presented the theoretical aspects involved in the tracer technique.

Among the primary principles of tracer methodology, the following must be considered:

1. The availability of a radioactive form of an isotopic element which is a normal constituent of the molecular structure of the compound or reaction under study.
2. The availability or development of a means or synthesis which will allow the incorporation of the radioactive isotope as an integral part of the compound which is to be identified and followed.
3. The availability or development of instrumentation and analytical procedures which, after experimental dilution of the labeled compound has occurred, will allow measurement of the emitted radiation within the allowable experimental error.
4. The change in isotopic mass must be recognized when applying a tracer technique, and the possibility of an isotopic effect must be considered in the final conclusion.
5. The integrity of the label position must be assumed or proven, as the case may be. In most biological studies involving complex metabolic processes, data should be obtained to support the integrity of the label position.

Due to the molecular structure of the common synthetic estrogens, the selection of an isotopic tracer would necessarily be limited to either carbon-14 or hydrogen-3. Both of these isotopes are readily available through the Oak Ridge National Laboratory. Carbon-14,

with a 5,000 year half-life and a β -emission of 0.155 mev, would normally be preferred to hydrogen-3, which has a relatively short half-life of 12.4 years and a β -emission of only 17.9 kev.

Diethylstilbestrol, which had been synthesized with a carbon-14 label in the methylene position, was utilized by Hanahan et al. (1951) in a study of the metabolism, distribution and excretion of this hormone. These workers did not publish details of the synthesis involved in establishing the specific label position for carbon-14. In a similar study, Twombly and Schoenewaldt (1951) utilized diethylstilbestrol which had a carbon-14 label on the number-5 carbon position. These workers presented the detailed steps involved in the synthesis, which was accomplished with considerable difficulty. A final yield of 73 milligrams of labeled diethylstilbestrol was obtained with a specific activity of 1.86 microcuries per milligram of product.

Numerous other workers have used carbon-14 labeled products in both chemical and biological studies. In the majority of the biological applications, small numbers of laboratory animals were utilized and minimum quantities of the labeled product were required. In the event of massive doses, or where large numbers of animals might become involved, considerable quantities of isotopically labeled products would be required. The cost of carbon-14 could become prohibitive and the researcher would be forced to consider other labeling isotopes, particularly hydrogen-3, or tritium. The Oak Ridge National Laboratories (1959) listed carbon-14 at $28.00 per millicurie while tritium was listed at $2.00 per curie.

The application of tritium as a biological tracer had been rather limited when this research project was activated. There was no

literature available which would indicate the previous application of tritium as a labeling isotope in a low-activity study as would be encountered in a determination of residual hormones. However, Thompson (1954), in a review article, presented evidence to the validity of tritium as a labeling isotope on organic molecular structures which are to undergo biological activity. The author stated that, with improved counting techniques which overcome analytical difficulties resulting from the low energy β-emission, the use of tritium as a biological tracer should increase very rapidly.

PROCEDURE

A group of 12 six-week old New Hampshire cockerels which had been randomly selected from a large broiler house was transferred to the Radioisotopes and Radiations Laboratory where individual cages were available. Facilities were provided for artificial lighting and ventilation and for individual feeding and watering. An acclimation period was maintained for seven days to allow the birds to become accustomed to the environmental change and feeding procedures. Throughout the 24-day experimental period, feed and water were provided ad libitum and feed consumption records were maintained on a 12-hour basis.

The experimental design which was used in this experiment is presented in Table I. EX-52, a standard experimental broiler ration of the Oklahoma State University Poultry Science Department was provided throughout the experimental period for the control and treatment groups. Corn oil was added to this ration at the rate of 10 grams per pound of feed. Dianisylhexene was dissolved in the corn oil before it was added to the rations of the treatment groups as outlined in Table I.

The dianisylhexene which was used in this experiment was synthesized in the Chemistry Section of the Radioisotopes and Radiations Laboratory (Hodnett and Gallagher, 1959). A tritium label was established on the number two carbon atom with a specific activity of 5.016 millicuries per gram of the active estrogen.

TABLE I

EXPERIMENTAL DESIGN

Treatment number	Ration	Estrogen feeding schedule			Number birds per replicate	Number replicates
		1-3 days	4-21 days	22-24 days		
Control	EX-52 + corn oil	None	None	None	1	4
One	EX-52 + corn oil	Dianisylhexene 50 mg./lb. feed	Dianisylhexene 50 mg./lb. feed	None	1	4
Two	EX-52 + corn oil	None	Dianisylhexene 50 mg./lb. feed	Dianisylhexene 50 mg./lb. feed	1	4

Upon termination of the experimental period, one milliliter blood samples in duplicate were removed from the wing veins of each bird. A separate needle and syringe were used for the collection of each sample. The blood was quickly transferred from the syringes to pre-weighed glass vials and allowed to clot before freezing.

Each bird was weighed and then slaughtered by cutting the jugular vein. After complete bleeding, the carcass was dipped in hot water and the feathers were removed with a feather picking machine in the usual manner. The carcasses were grouped by treatment and the collection of samples was in order of the anticipated activity to be encountered. In every case, the order of sample collection was (1) the control group, (2) treatment group one and (3) treatment group two.

In order to control possible cross-contamination among samples, the equipment used for sample collection was changed and discarded for each tissue processed. The dissecting equipment was never allowed to contact more than one sample of one specific tissue. During the collection of the tissue samples, glass plates were utilized as a working surface and scalpels, forceps and glass rods were used for the dissection and aliquoting of the various tissues.

After removal of the feathers, the skin was dissected clear of the carcass, wrapped in aluminum foil and frozen. All traces of adipose tissue from the muscle areas and abdominal cavity were removed and placed in pre-weighed glass vials. The breast muscles were dissected clear of the carcass and placed on glass plates. A grid system was utilized in connection with the glass plates to estimate muscle size and to facilitate collection of representative aliquots of whole muscle. In this manner, four aliquots of approximately one gram

each were removed from the entire muscle and placed in pre-weighed glass vials. The thigh and leg muscles were dissected clear of the carcass, combined, and processed in the manner described for the breast muscle. The last tissue to be removed was the liver, which was processed in the same manner. The tissues collected may be summarized as follows:

skin	one sample from each bird
adipose tissue	one sample from each bird
blood	2 - one milliliter samples from each bird
breast muscle	4 - one gram samples from each bird
thigh-leg muscle	4 - one gram samples from each bird
liver	4 - one gram samples from each bird

Immediately after sample collection, each vial was backweighed and the tissue aliquot weight was determined. Each vial was then sealed and the contents were frozen and maintained in this state until drying procedures could be completed.

A Vitris freeze drying unit with a capacity of 12 samples was used for drying the majority of the tissue samples. A vacuum of less than 0.05 millimeters was maintained on the unit and a dry ice-acetone slurry was used as the refrigerant. Each sample to be dried was placed in the slurry for at least 15 minutes before being attached to the drying unit. After the sample was attached to the unit and the vacuum applied, the vial and contents were allowed to come to room temperature slowly as the drying process continued. Room temperature was normally achieved after about eight hours of drying time. Preliminary trials had indicated that a nearly constant weight was reached for similar samples after 12 hours of drying time. To insure complete removal of moisture, a 24-hour drying time was allowed for each sample. After drying, the vial and contents were transferred to a vacuum desiccator for an additional 24 hours at room temperature. The vial and contents

were then weighed and returned to the vacuum desiccator for storage until analysis could be completed.

It was felt desirable to test the removed moisture from the tissue samples for any traces of tritium activity which might be present as a test of the integrity of the label position. A second freeze-drying unit of glass construction and one-sample capacity was used for this purpose. The same drying procedure as detailed above was followed for each tissue to be tested. The condensed moisture was transferred to a glass ampoule which was sealed and stored until tritium analysis could be completed.

A separate grinding device was provided for each sample because of the possibility of cross-contamination which could result from the multiple use of conventional mincing devices. A solid pyrex rod of six millimeters diameter was broken into lengths of about six inches. One end of each rod was fire-polished and fitted with a firm tygon bushing, while the opposite end was left with a rough finish. The tygon bushing was secured in a small, variable-speed motor which was mounted on a ring stand. With the rod secured and rotating at a slow speed, the glass vial containing the dried tissue was slowly pressed into position. About ten minutes were required to grind each tissue to a fine state. After each sample was ground and mixed, the glass rod was discarded and a new one installed for the next sample.

Due to the weak β-emission of tritium, internal gas counting techniques were used for the analysis of this radioisotope. The method of Wilzbach et al. (1953) was used for the conversion of the solid tissue samples to a gaseous state. Corning Pyrex 1720 glass tubing was utilized in the construction of a reaction tube which was 17

centimeters long and had a break tip on one end. Exactly 1.5 grams of forty-mesh granular zinc (Baker and Adamson) and 0.1 grams of nickelic oxide (Baker's analyzed, gray-black powder) were added to the reaction tube. With the use of a micro-balance, a 5 to 10 milligram sample of the prepared tissue was weighed into a procelain weighing boat and the boat and contents were then added to the reaction tube. A small glass ampoule with a break tip on one end was made from three-millimeter diameter pyrex tubing. Approximately five milligrams of water was added to the ampoule by heating the ampoule over a micro-burner and then inserting the open end into a 6 λ drop of water. After the water was drawn into the ampoule, the open end was sealed and the ampoule and contents were added to the reaction tube.

An oxygen-methane hand torch was used to make a constriction in the reaction tube at about ten centimeters distance from the break tip. The reaction tube was then attached to a high-vacuum line and evacuated to less than 5 microns of pressure. After the proper vacuum was obtained, the reaction tube was sealed at the point of constriction with a hand torch. The reaction tube was agitated until the water ampoule was broken and the contents mixed and evenly distributed. The prepared tubes were placed in a horizontal position in a furnace at $640 \pm 10^{\circ}C$. for a period of three hours after which the tubes were removed and cooled. Each tube was then weighed in order to apply volumetric corrections for the gaseous transfer.

The combusted reaction tube and an internal ionization chamber of the Borkowski (1947) type were attached to a high vacuum line and the entire system was then evacuated to less than one micron of pressure. The tube was broken and a pre-determined aliquot of the gaseous mixture

of methane and hydrogen was expanded from the broken reaction tube into the ionization chamber. The chamber was then charged to atmospheric pressure with methane counting gas.

The tritium analysis was accomplished by ion current measurements as outlined by Wilzbach et al. (1954). After it was charged, the ionization chamber was coupled to a vibrating reed electrometer (Applied Physics Corporation, Model 30) which was equipped with a recording potentiometer (Brown Elektrovik strip chart recorder, Model 153). A potential of 180 volts, negative with respect to ground, was applied to the shell of the ionization chamber. A preliminary counting time of 20 minutes was allowed for the dissipation of extraneous charges such as those generated by insulator strain.

The two methods which were used in this study for the ion current measurements were the rate of drift method and the static counting system. When large quantities of tritium activity were encountered, the static counting system was utilized. With this system, a counting time of 20 minutes was recorded and corrected for extraneous radiation events. For the conversion of the millivolts reading to tritium activity, the relationship $V = IR$ as expressed by Ohm's law was applied. This counting system allowed the direct inclusion of the resistance R which had a value of 0.99×10^{12} ohms. The value of the current flow I was determined by multiplying 3.7×10^{10} disintegrations per second curie by 3.09×10^{-17} coulombs per disintegration for tritium. This latter value represents an average of 3.04×10^{-17} coulombs per disintegration as reported by Jenks et al. (1949) and 3.14×10^{-17} coulombs per disintegration as reported by Jones (1951). The relationship of volts to activity may be expressed as follows:

$$V = IR$$

by substitution: $$V = \left(3.09 \times 10^{-17} \frac{\text{coulomb}}{\text{disintegration}}\right) \left(3.7 \times 10^{10} \frac{\text{disintegrations}}{\text{second curie}}\right) \left(0.99 \times 10^{12} \text{ ohms}\right)$$

then: $V = 11.318670 \times 10^{5}$ volts per curie

and finally: 1 millivolt = 0.0008835 microcuries.

The readings which were obtained from the sample assays were corrected and converted to activity values as follows:

$$A = \left(\frac{R - B}{W}\right)\left(F\, e^{-kt}\right)\left(\frac{Cv + (Lv - \frac{Tw}{D})}{Cv}\right)$$

where: A = activity, microcuries per milligram

R = millivolts reading

B = background

W = sample weight in milligrams

F = 0.0008835 microcuries per millivolt

e^{-kt} = decay constant

Cv = chamber volume

Lv = volume, vacuum line

Tw = total weight, reaction tube

D = density of glass.

When extremely small quantities of tritium activity were encountered, the rate of drift method was utilized. With this method, a counting time of one hour was recorded and corrected by subtracting the activity produced by extraneous radiation events. All instantaneous charges in excess of 0.9 millivolts have been regarded as extraneous. The activity was then expressed as millivolts charge per unit of time. These values were then processed as follows:

$$M = \left(R-B\right)\left(e^{-kt}\right)\left(\frac{Cv + (Lv - \frac{Tw}{D})}{Cv}\right)$$

where:

M = millivolts charge per unit time, corrected

R = millivolts charge per unit time, recorded

B = background

e^{-kt} = decay constant

Cv = chamber volume

Lv = volume, vacuum line

Tw = total weight, reaction tube

D = density of glass.

With the application of the rate of drift method, the resistance R was deleted and the relationship V = IR could not be directly applied. Therefore, a standard curve was used for the conversion of millivolts charge to units of tritium activity. This curve was established by the dilution of a known, standard tritiated source and subsequent analysis by the rate of drift method. A least squares fit was applied to the data and an equation was established for the curve as presented in Figure 1.

The data obtained from the tritium analyses were processed by computer methods which utilized an IBM-650 digitial computer and related equipment. The equation for the standard tritiated source which was derived by a least squares fit was incorporated into the computer program and, through this equation, all measurements of millivolts were converted to tritium activity values. An analysis of variance and orthogonal comparisons were applied to these activity values as a test for significant differences. The analysis of variance for the adipose tissue was computed separately from the analysis of variance for the

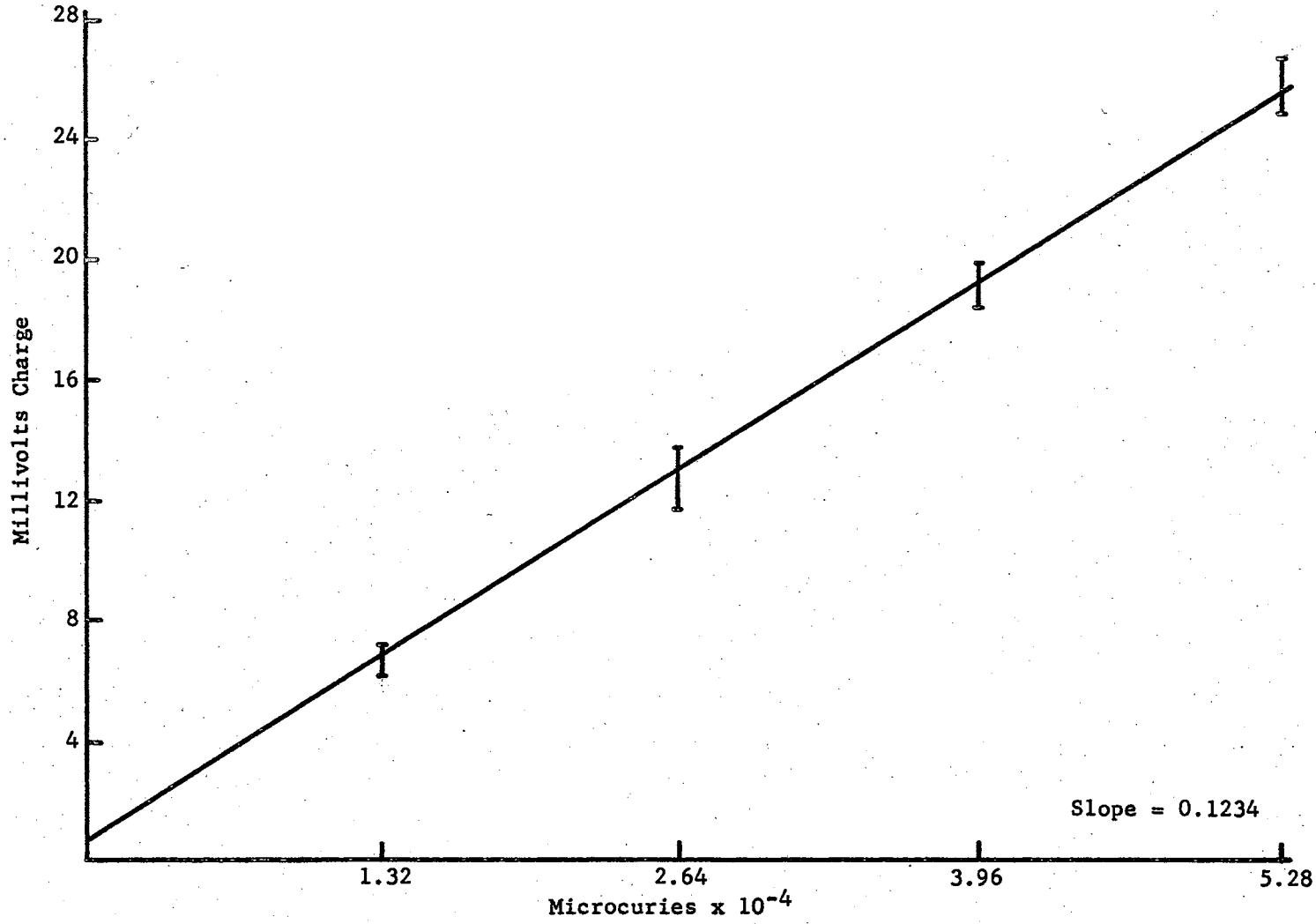

Figure 1
Standard Curve with Maximum and Minimum Values

other tissues, due to a difference in the number of samples available for tritium analysis. The results obtained from these statistical tests and recommendations pertaining to analytical procedures based on these findings will be discussed in the following sections.

RESULTS AND DISCUSSION

Tritium Procedures

Tritium labeled dianisylhexene was used in this experiment in the application of a tracer technique for the detection of estrogenic residues in the edible portions of broilers. 3,4-dianisyl-2-t-3-hexene which had a specific activity of 5.016 millicuries per gram was incorporated into the diet of growing broilers. Tissues from these birds were assayed for tritium and the residual estrogen level was determined from these analyses.

Tritium was selected as the labeling isotope for this study because of the quantity of labeled estrogen required for the feeding trials. The cost of a carbon-14 synthesis was prohibitive in view of the quantity and high specific activity of the product which was needed. A step-wise synthesis was used in order to establish a tritium label on the number two carbon atom in preference to either the random labeling as produced by the recoil reactions as outlined by Rowland and Wolfgang (1956) or by the tritium exchange reaction as presented by Wilzbach (1957).

The analysis for tritium is quite laborious due to the weak β emission of this isotope. This analysis becomes extremely difficult when activities of 20 disintegrations per second or less are to be measured. If activities of this magnitude are to be measured, it is essential to utilize the higher efficiency of gas counting techniques in

preference to the lower efficiency of scintillation detectors or counting systems which require the sample to be placed external to the sensing device. In order to use gaseous counting systems, all solid or liquid samples must be converted to a suitable gas for analysis. Several methods have been presented in the literature for the conversion of biological tissues to a gaseous state. However, the majority of these methods require a series of procedures and reactions. The method of Wilzbach et al. (1953) was used in this study because it provided a one-step method for the conversion of organic compounds to a mixture of methane and hydrogen gas. For the tritium analysis, the ion current measurement technique as developed by Wilzbach et al. (1954) was used because this analytical method had been proven to detect as few as 10 disintegrations per second in a gaseous volume of 250 cc.

Though time consuming, especially where large numbers of samples are involved, these methods were found to be acceptable for the detection of tritium in biological samples at an activity level of less than 3×10^{-10} curies. Variation in analytical results begins to increase as the activity level decreases below 3×10^{-10} curies. However, if enough samples are assayed, acceptable results can be obtained. As the activity level approaches an infinitely small value, the measured analysis becomes quite variable due to poor disintegration statistics and instrument variation and is eventually lost in the normal variation encountered in background determinations.

In this study, the activity assayed in the control tissues was so low as to eliminate the possibility of making any real distinction between the activity of the control tissues and the normal ionization chamber background. In the analyses of the treatment samples, it was

generally possible to make a clear distinction between these assays and those of the control tissues. However, in view of the low activity which was encountered, more accurate results could have been obtained by increasing the specific activity of the labeled estrogen. A study of the variation which was encountered in this experiment would indicate that, for similar types of studies, a decrease in the counting time for each assay and an increase in the total number of samples assayed would bring about an improvement in the analytical results.

With the application of a tracer technique, some evidence should be obtained to support the integrity of the label position. Since tritium was used as the labeling isotope in this study, activity would have been detected in the body water of the birds if the label position had been broken. An analysis of moisture samples which were collected during the drying procedures failed to show any tritium activity. This supports the basic assumption that the tritium label remained intact while the dianisylhexene was undergoing biological activity.

A cross-contamination between samples was encountered during preliminary trials and a study was conducted to isolate the source of this contamination. The procedure for preparing the samples for conversion to a gaseous state initially involved weighing the sample into a porcelain combustion boat. The porcelain boat and contents were then added to the reaction tube and the tube was then completed as previously described. After the gaseous sample had been transferred to the ionization chamber, the porcelain boat was recovered from the broken reaction tube. The boat was placed in an acid solution for at least twenty-four hours, washed several times in distilled water and flamed to a cherry-red using an oxygen-methane torch. After proper

cooling, the boats were reused for weighing out additional samples. These clean-up procedures were found insufficient and the use of the porcelain combustion boats was discontinued. Expendible weighing boats pressed from sheet zinc were used to replace the porcelain boats. Zinc weighing boats were later obtained from the Department of Chemistry of the University of Kansas.

Excessive variation within samples was again encountered during the preliminary trials. This variation was traced to residual contamination occurring in the vacuum line as a result of the multiple use of this equipment by other workers. Normal clean-up procedures failed to remove the residual contamination and it became necessary to provide a new vacuum system which was isolated from other projects involving radioisotopes.

Improper combustion of the reaction tubes occurred during the analysis of the last few samples. This condition was characterized by an extremely high number of the reaction tubes exploding in the furnace and by an unsilvered, dark appearance of those tubes which did not explode. The use of fresh quantities of zinc and nickelic oxide and an increased combustion time had no effect on this condition. The condition was finally corrected by raising the temperature of the furnace to 650-670°C. for the first fifteen minutes of the combustion period and then slowly reducing the temperature to that normally used for the remainder of the reaction time.

The vacuum system which was used in this study is presented in Figure 2. With this system the vacuum was applied at V and measured with a tilting McLeod gauge at M. The sample to be assayed was introduced in the swivel tube S for expansion into the ionization

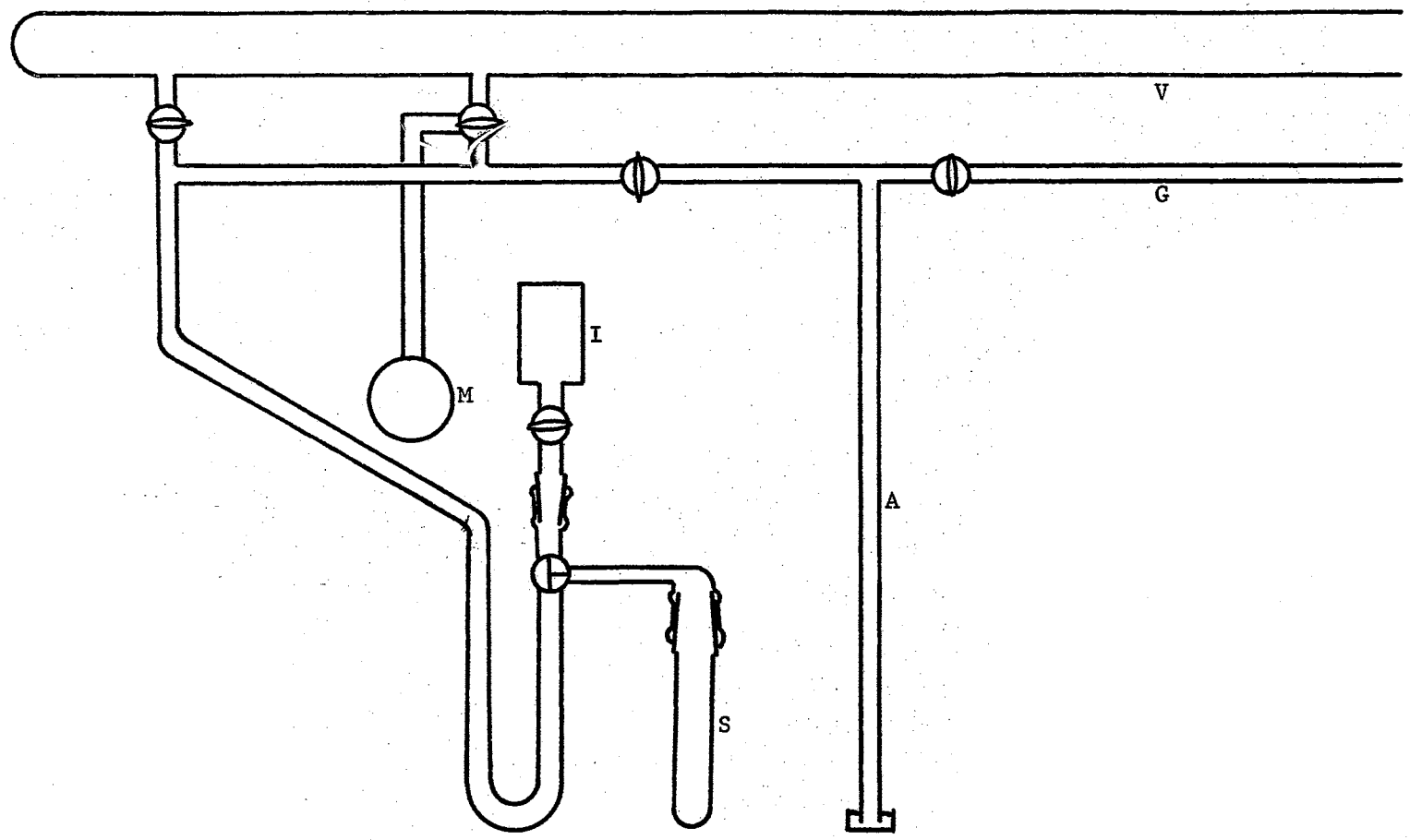

Figure 2

Vacuum System for Sample Transfer

chamber I. The counting gas was then admitted at G and atmospheric pressure measured at A. This vacuum system was found to be convenient, versatile and easy to clean and maintain.

Analytical Results

The response of the broilers to the application of dianisylhexene was typical. The male characteristics of those birds which received the tritium labeled dianisylhexene were surpressed and the quantity of adipose tissue was considerably greater and more widely distributed. As compared to the controls, the blood from the treated birds contained increased quantities of lipoid substances. However, no attempt was made to identify either quantitatively or qualitatively the lipoid fractions which were present.

There was some variation encountered in the individual consumption of dianisylhexene. In treatment one, the maximum and minimum consumption by individuals were 200.60 mg. and 176.00 mg., and the overall treatment average was 190.06 mg. In treatment two, the maximum and minimum consumption by individuals were 195.00 mg. and 169.75 mg., and the overall treatment average was 181.10 mg. The difference in the overall consumption of dianisylhexene by treatment groups is believed to be due to chance alone. Therefore, an increase in the number of birds per treatment might have decreased the magnitude of this variation.

The moisture content of the various tissues is presented in Table II. The moisture content of the breast, thigh and leg, and liver tissues did not show a definite increase due to estrogenic treatment as was anticipated. The moisture content of the blood decreased from 85.8 percent for the control to 84.5 and 83.1 percent for treatments one and

TABLE II

RESIDUAL ESTROGEN CONTENT OF TISSUES BY TREATMENTS

Treatment	Tissue	Percent moisture	Estrogen, ppm.	
			Dry weight	Wet weight
Control	Breast	73.8	0.00	0.00
	Thigh	76.0	0.00	0.00
	Liver	70.5	0.00	0.00
	Blood	85.8	0.00	0.00
	Skin	37.2	0.00	0.00
	Adipose	76.9	0.00	0.00
One	Breast	74.5	1.10	0.25
	Thigh	75.6	0.65	0.16
	Liver	70.7	2.74	0.80
	Blood	84.5	0.66	0.10
	Skin	43.2	2.64	1.50
	Adipose	81.3	2.19	0.41
Two	Breast	73.9	1.15	0.30
	Thigh	75.4	0.86	0.21
	Liver	71.2	1.85	0.53
	Blood	83.1	1.75	0.30
	Skin	42.3	4.18	2.42
	Adipose	80.4	4.08	0.80

two, respectively. In this case, the decreased moisture content of the blood by treatments might represent the reciprocal of the lipoid content of these same samples. The moisture content of the skin increased from 37.2 percent for the control to 43.2 and 42.3 percent for treatments one and two, respectively. In addition, the fat content of the skin was increased in those birds which received the estrogen-containing diet. These results were anticipated and logically support an improvement in the skin texture of birds as a result of estrogenic treatment. The moisture content of the adipose tissue was 76.9, 81.3 and 80.4 percent for the control and treatments one and two, respectively. These values appear to be high; however, due to the method of sample collection which was used, large quantities of membranes and supporting tissues were included in these samples. Therefore, these values should not be interpreted as representing the moisture content of nearly homogenous adipose tissue.

The tritium activity values of the various tissues which were obtained by data processing with the digital computer have been converted to estrogen equivalents and listed in Table II as parts per million of estrogen on both a dry tissue and wet tissue basis. The values which were obtained from the analysis of the control tissues have been treated as an estimate of zero activity and have been expressed in this manner in Table II. The estrogenic contents for treatments one and two as listed have been adjusted accordingly.

From the values presented in Table II, it is readily apparent that the inclusion of dianisylhexene in the diet under the conditions of this experiment resulted in a retention of this synthetic hormone in the edible portions of treated birds. Furthermore, the provision

of a three-day depletion period prior to sacrifice has had little effect in reducing the quantity of this residual estrogen.

An analysis of variance, as presented in Table III, shows the variation between treatments, tissues and treatments x tissues to be highly significant. An orthogonal comparison of treatments over all tissues, as presented in Table IV, identifies the principle source of treatment variation as existing between the control and the treatment groups. No significant difference was found in a comparison of treatment groups one and two.

An orthogonal comparison of tissues over all treatments, as presented in Table V, was made in order to examine more closely the tissue variation. Highly significant differences were found when skin was compared to breast, thigh-leg, liver and blood; when muscle tissues were compared to liver and blood; and when liver was compared to blood. No significant differences were found in a comparison of breast muscles to thigh-leg muscles.

In the case of adipose tissue, an analysis of variance, as presented in Table VI, shows the variation between treatments and between animals within treatments to be highly significant. An orthogonal comparison of treatments, as presented in Table VII, shows this variation to be highly significant between the control and treatment groups and also between treatment groups one and two. In the analysis of the adipose tissue, one bird in treatment group two had an exceptionally high tritium activity. Additional analysis of this tissue upheld these results; therefore, it is felt that this was a real variation in the case of this one particular bird. It is felt that the high assay values obtained from this one bird has altered the analysis of variance and

TABLE III

ANALYSIS OF VARIANCE BASED ON TRITIUM ACTIVITY VALUES

	df	SS	MS	F
		Experimental Terms		
Treatment	2	9520.740	4760.370	9.16**
Tissue	4	4997.505	1249.376	8.45**
Treatment x Tissue	8	4462.455	557.807	3.78**
		Error Terms		
Birds in Treatments (For Treatments)	9	4678.980	519.887	------
Birds x Tissues in Treatments	36	5309.160	147.477	------
		Technique Terms		
Samples Within Birds x Tissues in Treatments	60	3047.865	50.798	------
Duplicates in Samples in Birds x Tissues in Treatments	120	2952.750	24.606	------
Readings	3360	1838.415	0.547	------

**Significant at the 1 percent level of probability

TABLE IV

ORTHOGONAL COMPARISON OF TREATMENTS OVER ALL TISSUES

	Control	Treatment one	Treatment two	Difference	SS	F
Average over all tissues	9.9806	12.9862	13.7474	------	--------	-----
	-2	+1	+1	6.7724	9173.080	17.64**
	0	-1	+1	0.7612	347.655	0.67

TABLE V

ORTHOGONAL COMPARISON OF TISSUES OVER ALL TREATMENTS

	Breast	Thigh-leg	Liver	Blood	Skin	Difference	SS	F
Average over all treatments	11.2164	11.1067	13.2253	11.6037	14.0381	------	--------	-------
	+1	+1	+1	+1	-4	9.0003	2916.194	19.77**
	+2	+2	-2	-2	0	5.0118	1130.316	7.66**
	+1	-1	0	0	0	0.1097	4.332	0.03
	0	0	+1	-1	0	1.6216	946.651	6.42**

**Significant at the 1 percent level of probability

TABLE VI

ANALYSIS OF VARIANCE FOR ADIPOSE TISSUE

	df	SS	MS	F
			Experimental Terms	
Treatment	2	241.476	120.738	101.80**
Between Animals Within Treatments	8	325.531	40.691	34.85**
			Error Term	
Between Samples Within Animals Within Treatments	11	13.046	1.186	-------

TABLE VII

ORTHOGONAL COMPARISON OF TREATMENTS FOR ADIPOSE TISSUE

	Control	Treatment one	Treatment two	Difference	SS	F
Average over all samples	0.634	4.806	8.400	------	------	-------
	-2	+1	+1	11.937	42.378	35.73**
	0	-1	+1	3.594	47.470	40.03**

**Significant at the 1 percent level of probability

that the three-day depletion period provided to treatment group one did not make a truly significant reduction in the quantity of residual estrogen. In view of these results, it is recommended that the number of experimental animals be increased in any similar studies.

There is little information in the literature to support the analytical methods which were used in this experiment as applied to a biological system at activity levels of less than 3×10^{-10} curies. Wilzbach et al. (1954) had reported an analytical time of one hour for an accuracy of $\pm$ 1 percent, where extremely low activities were to be measured. In biological work where individual variation cannot be controlled, an analytical accuracy of $\pm$ 1 percent may be less meaningful in the final conclusion than that which might be obtained by assaying more samples with a slight decrease in the precision of the analytical procedures. This experiment was designed in order to test statistically the procedures used and to make recommendations for the design of similar types of experiments in the future.

In this experiment, readings were taken every 240 seconds until a recording time of one hour was obtained. The variation in these readings was regarded as a measure of (1) the variation encountered in the decomposition of small numbers of radioactive atoms and (2) the fluctuations occurring in the performance of the vibrating reed electrometer during the measurement of extremely small currents. In the analysis of variance, as presented in Table III, a Mean Square value of only 0.547 for readings was obtained as compared to an error term of 147.477. Since the variation in readings was so small, it is recommended that the recording time be decreased from one hour to a period of approximately 15 minutes and that only one cumulative

observation should be recorded for this period. A Mean Square of 24.606 for Duplicates in Samples within Birds x Tissues in Treatments was relatively small as compared to an error term of 147.477. Since this variation was small, the use of duplicate analyses of samples could be discontinued with a considerable saving in the time required for analytical work. In testing Samples within Birds x Tissues in Treatments, a Mean Square value of 50.798 was found as compared to an error term of 147.477. This value represents a considerable portion of the variation which was encountered. In view of these results, it is recommended that a portion of the time which could be saved by the elimination of duplicate analyses and by decreasing the analytical time should be applied to the collection and analysis of additional samples.

SUMMARY

3,4-dianisyl-2-t-3-hexene which had a specific activity of 5.016 microcuries per milligram was synthesized and included in the diet of growing broilers at the rate of 50 milligrams per pound of ration. An experimental design was used which provided for individual maintenance of four birds each in a control and two treatment groups. Treatment group one received the estrogen-containing diet for 21 days followed by a three-day depletion period, while treatment group two received the same diet for 21 days with no depletion period. Tissue samples of breast, thigh-leg muscle, liver, blood, skin and adipose were collected and freeze-dried. These tissues were converted to a gaseous mixture of hydrogen and methane and ionization current measurement techniques were used for the tritium analysis. Digitial computer methods were utilized for data processing and statistical analysis.

Estrogen residues were found to be present in all tissues tested. The provision of a three-day depletion period resulted in a slight but not significant decrease in the quantity of residual estrogen.

The analytical methods used for tritium analysis gave good results at activity levels of less than 3×10^{-10} curies. Data indicate the integrity of the label position to be sound while undergoing biological activity. Based upon the results of this study, it is recommended to modify the experimental procedures by reducing the analytical time, eliminating duplicate analyses, increasing the number of samples assayed, and, if possible, to increase the number of animals in the experiment.

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VITA

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