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STUDIES WITH RADIOACTIVE PROTEIN
HORMONE PREPARATIONS

by

Leon Kraintz

A THESIS
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INTRODUCTION

In the book, One Family: Vitamins, Enzymes, Hormones (1950), Harrow points out the relationships between vitamins, enzymes and hormones and also emphasizes the similarities of these important biological compounds. All of these substances are effective in minute quantities; they probably all act as biocatalytic agents, and play an important role in the homeostasis or regulatory mechanisms of the organism. The mode of action and biochemical mechanisms entered into by many of the vitamins and enzymes has been clearly established, but less progress has been made with the study of hormones.

Some hormones, such as the tropic hormones of the anterior pituitary, exert their effects on specific glands. For example, thyrotropic hormone affects the thyroid; the adrenocorticotropic hormone specifically affects the adrenal cortex; gonadotropic hormones affect the tissues of the gonads. Glands so affected by pituitary hormones are referred to as target organs. Other hormones have both local and general effects. Estrogens and androgens affect genital structures and secondary sex characteristics. The term "target tissue" is sometimes used to refer to these tissues that selectively respond to a hormone. Thyroid hormone and insulin essentially affect all of the cells in the body and it would be difficult to specify target tissues for these hormones.

There are two generally accepted concepts of hormone action. One view regards the degree of stimulation of the target organ or tissue as a function of the concentration of the circulating blood level of the specific hormone. The other concept, sometimes referred to as the target organ
localization theory, assumes that the hormone affecting the target organ or tissues is selectively localized in those tissues in order to produce its effects. These concepts are hypothetical as classical endocrinological techniques have contributed mainly to the cause and effect relationships of hormones, and little knowledge has been accumulated about the site and mode of action.

Because small amounts of hormones liberated into the blood stream produce striking changes in the organism, knowledge of the fate and metabolism of these hormones should give some information about their action. However, in order to detect the presence of these substances in body fluids and tissues by conventional techniques, it is necessary to administer large amounts of hormones; therefore, much of the information obtained in this manner should be considered pharmacological rather than physiological evidence. The primary objective in the study of the fate of a biological compound is to observe that substance "in vivo" under experimental conditions which would cause a minimal physiological disturbance. The most fruitful of the known methods is the isotopic tracer technique in which one or more atoms of the compound studied is labeled with an isotope.

The use of isotopes as tracers in biological systems was initiated over thirty years ago by Hevesy (1923) in studies of the localization of radioactive lead in tomato plants. It was not until 1933 that Hevesy and Hofer did the first animal experiments with a naturally occurring metabolite, water labeled with deuterium. Schoenheimer and Rittenberg (1935) were the first to investigate the use of deuterium as a label in the study of intermediary metabolism of lipids. Later Keston, Rittenberg and Schoen-
heimer (1939) extended the work to the use of nitrogen 15 as a label for
the study of nitrogen and amino acid metabolism. More biological studies
with radioisotopes followed shortly after isotopes were made readily avail-
able as the result of wartime research. Considerable knowledge was gained
using isotopic techniques in the fields of biochemistry and physiology that
could not have been obtained by classical methods. The use of radioactive
isotopes as a tracer for hormones naturally followed since it allowed the
detection of minute quantities of hormone and also enabled the investiga-
tor to follow the metabolism of the hormone studied.

A general review of radioactive hormone preparations is not available,
therefore, a survey of the pertinent literature regarding the preparation
of radioactive hormones and information obtained with these labeled hor-
mones in experimental studies will be presented. Unfortunately, the termin-
ology used to describe radioactive hormones is inconsistent. For the sake
of clarity, it is necessary to redefine these terms in a somewhat arbitrary
manner. The general term radioactive hormone will refer to any hormone
preparation containing a radioactive isotope. Labeled hormones will refer
only to those preparations that contain radioactive atoms in place of stable
atoms without altering the structure or composition of the hormone molecule.
Chemically tagged hormones will refer to preparations obtained by reacting
the hormone with a radioactive element or compound resulting in a radioac-
tive derivative of the hormone. From these statements it appears that there
are several methods of preparing radioactive hormones and these will be con-
sidered in the following order:

1. Labeled hormones can be synthesized chemically using a radioactive
   element or compound as one of the reactants in the synthesis.
2. Labeled hormones can be produced biosynthetically by providing radioactive nutrients or precursors to a living organism or tissue which will incorporate the radioactivity into the desired compound which is later extracted and purified.

3. A chemically tagged radioactive hormone can be produced by reacting the hormone with a radioactive compound or element which forms a stable derivative and supposedly retains the biological activity of the original preparation.

**Synthetically Produced Hormones Labeled with Radioactivity**

Any hormone that can be prepared synthetically could conceivably be made available in radioactive form by substituting a radioactive reactant in the proper step of the synthesis. Thyroxine, epinephrine and many of the steroid hormones have been prepared synthetically and, up to the date of this writing, most of them have been prepared labeled with a radioactive isotope.

The first radioactive hormone prepared synthetically was DL-thyroxine, labeled with I 131 in the 3 and 5 position of the diiodotyrosine component (Joliot et al. 1944). DL-thyroxine containing C 14 in the 1 carbon position was synthesized by Wang, Hummel, and Wirnack (1952). Schayer (1952) prepared epinephrine labeled in the beta position with C 14. Turner (1947) prepared the first C 14 labeled radioactive steroid hormone. He synthesized testosterone labeled in the 3 position of ring A. Turner's work initiated other successful attempts at labeling steroid hormones. Riegel and Prout (1948) prepared C 14 progesterone in which the radioactive carbon was in the 21 position. Heard and Ziegler (1950) labeled progesterone with C 14 using (4)
Turner's technique for labeling the 3 or 4 carbon in ring A. Heard, Saffran, and Thompson have synthesized C\textsuperscript{14} labeled estrone (Cited by Twombly, 1951) and Nicholas et al. (1950) have prepared 17\textsuperscript{C} C\textsuperscript{14} labeled methyl estriol. Fukushima et al. (1951) have prepared cortisone labeled with tritium in positions that were said not to be labile during metabolism. Cortisone and hydrocortisone tagged with C\textsuperscript{14} in position 4 have been prepared under the direction of the National Institutes of Health with the cooperation of several private laboratories (Reported in Tracerlog, 1953). As yet no radioactive peptide or protein hormone has been prepared synthetically, however, du Vigneaud, Ressler, and Trippett have reported the chemical structure of oxytocin peptide (1953) and, in another publication the synthesis of this peptide (du Vigneaud et al., 1953) was reported. The substitution of a labeled amino acid in this synthesis should be a relatively simple procedure if radioactive preparations were desired for animal studies.

**Biosynthetically Prepared Radioactive Hormones**

Biosynthetic methods have been used extensively to provide labeled amino acids and a host of other labeled metabolites utilizing the ability of bacteria and plants to incorporate elements or relatively simple substances into biologically important compounds.

Perlman and co-workers (1941) took advantage of the fact that the thyroid has the ability of concentrating administered iodide and subsequently incorporating iodine into the thyroxine molecule. They were thus able to extract radiiodine labeled thyroxine from the thyroid glands of rats that had been given radioactive iodide. Haines (1952) has shown that radioactive compound F, dehydrocorticosterone, can be isolated from the perfusate of isolated beef adrenals which have been perfused with C\textsuperscript{14}
carboxyl labeled acetate. The only radioactive protein hormone prepared biosynthetically was reported by Pettings and Rice (1952). This group prepared S 35 labeled insulin by incubating foetal beef pancreas with S 35 labeled methionine.

Chemically Tagged Radioactive Hormone Preparations

Most of the work done with radioactive hormones, particularly the protein hormones, has been done with chemical tagging. This is due in part to the availability of the isotopes used and the relative simplicity of the method. In addition, some of the hormones, especially the protein hormones, cannot be tagged in any other way. Halogenated steroids have been prepared, therefore, it was possible to label estradiol and estrone with radiiodine and radiobromine. Albert et al. (1949) have prepared iodo-alpha-estradiol using I 131 as the tracer. Twombly et al. (1948) have prepared radioactive dibromestrone. Sodium estrone sulfate has been prepared with radioactive sulfur by the drug firm of Ayerst, McKenna, and Harrison (Cited by Twombly, 1951).

It has been known for some time that certain biologically active proteins can be reacted with substituent groups and still apparently retain their biological activity. This is particularly true of antibodies and antigens which have been coupled with diazo compounds or iodinated, providing only a small number of substituent groups, usually less than 6, per molecule have been introduced. The first protein to be tagged with radioactivity in this manner was insulin reacted with diazotized p-iodoaniline containing radiiodine as reported by Reiner, Keston, and Green (1942). Ferreebe et al. (1951) have also reported tagging insulin with I 131, but they used a method involving direct iodination of the protein. More recently Stadie et al.
(1952) have prepared insulin sulfated with S 35 sulfuric acid to form a stable complex that retained the original biological activity. Sonenberg et al. (1951) prepared iodinated ACTH using I 131 as the tagging agent. Ferrebee et al. (1951) also prepared I 131 ACTH and reported that its biological activity was retained. Cox (1951) and Sonenberg et al. (1951a) have independently reported the preparation of iodinated prolactin tagged with I 131 that retained the biological activity. Kraintz and Talmage (1952) tagged relaxin with I 131 by iodination. Sonenberg has extended his tagging to growth hormone and luteinizing hormone (1952), and in addition, his group has prepared S 35 tagged thyrotropic hormone by coupling it with diazotized benzenesulphonic acid labeled with S 35 (1952).

Results Obtained with Radioactive Hormones in Animal Studies

While numerous methods have been developed and published for producing radioactive hormones, little has been published concerning the use of these radioactive hormones in physiological studies. This is particularly true of the radioactive steroids. Since this thesis is concerned primarily with radioactive protein hormones, only a brief review will be presented concerning the results obtained with other radioactive hormone preparations.

(1) Radioactive Thyroxine and Epinephrine

Gross and Leblond (1947) did the first complete study on the distribution of I 131 labeled thyroxine in the tissues of the rat. They determined the distribution of radioactivity in female rats at varying time intervals up to 24 hours and found that 80% of the administered dose could be recovered in the feces at the end of 24 hours, while 11% was recovered in the urine in the form of inorganic iodide. The route of entry of thyroxine
into the gut was shown to be mainly via the bile. Taurog et al. (1950, 1951, 1952) at the University of California have published a series of papers on
the biochemical nature of the iodinated products eliminated via the bile after administration of I 131 thyroxine. Klitgaard et al. (1953) using 1-C
14 labeled thyroxine showed that the alanine portion of thyroxine was cata-
bolized since 10% of the radioactivity administered to rats could be recovered
in the expired air after 12 hours. They also showed that the bile was the
major route of elimination for thyroxine. Albert et al. (1952, 1952a, 1953)
studied the metabolism and pathways of elimination of thyroxine using I 131
labeled material and concluded that there was an enterohepatic circulation
of thyroxine. Schayer et al. (1952, 1953) used C 14 epinephrine labeled in
the beta position and also in the methyl position. They presented a tenta-
tive summary of the fate of epinephrine in the rat. About 50% of the epine-
phrine administered is inactivated by the loss of methyl amine, presumably
through the action of amine oxidase. The remaining radioactivity is ex-
creted via the urine as epinephrine and at least two other unidentified meta-
bolites.

(2) Radioactive Steroid Hormones

The results of studies completed with radioactive steroids up to
1950 have been reviewed by Twombly (1951). Iodo-alpha-estradiol tagged with
I 131 was studied by Albert et al. (1949) in female rats. There appeared to
be no selective uptake of radioactivity in any of the target organs while
considerable radioactivity was found in the thyroid, indicating that the
iodine was metabolized. Similar findings, except for the uptake in the thy-
roid, were obtained by Twombly et al. (1948) using radiobromine labeled di-
bromestron. Studies with radioactive sodium estrone sulfate also revealed
no selective localization of radioactivity in any target tissue (Twombly, 1951). However, since this was labeled with S 35 as the sulfate, there is little possibility that the sulfate would remain with the steroid. Riegel, Hartop, and Kittinger (1950) carried out observations on mice and rats injected with progesterone-21-C 14 intraperitoneally and reported that selective absorption of radioactivity occurred in the pituitary and adrenals of mice, but only in the pituitary of rats. Gallagher et al. (1951) and Barry et al. (1952) reported studies with radioactive testosterone and progesterone in mice and rats and found no localization of radioactivity in any organ or tissues other than those concerned with the excretion of the hormone or its metabolites. Evidence was presented to indicate that the majority of the radioactivity administered was eliminated with the feces. Bradlow, Dobriner, and Gallagher (1954) reported the fate of cortisone labeled with tritium and, as in other studies in animals given radioactive steroids, no selective uptake in any target tissue was demonstrated. Studies concerned with the distribution of radioactivity after administration of labeled steroids to animals have failed to reveal any significant localization in any tissues effected by these steroids. Since the metabolism of steroid hormones represents a separate field of study, a review of the use of radioactive steroids in relation to this subject is not presented.

(3) Radioactive Protein Hormones

The first radioactive protein hormone to be used in biological or medical studies was insulin tagged with I 131. Reiner et al. (1942, 1943) and Root et al. (1944) studied the absorption and distribution of
insulin in normal and diabetic subjects. As the result of their work they concluded that insulin resistance was due in part to a delayed absorption at the site of injection. Stadie (1954) used insulin tagged with both I 131 and S 35 in studies concerning the binding of insulin to tissues in vitro. A firm association or binding of isotopic insulin could be demonstrated with diaphragm, mammary tissue and adipose tissue of the rat. The exposure of rat diaphragm for as little as ten seconds to isotopic insulin resulted in binding that was not affected by prolonged washing. The bound insulin exerted its customary action on the metabolism of the tissue (an increase in glycogen synthesis). The increase in glycogen synthesis was directly proportional to the amount of insulin bound. By utilizing this technique, as little as .01 micrograms of insulin could be detected.

Sonenberg and his co-workers were the first to apply the isotopic tagging technique to pituitary hormones. They reported studies on the distribution of radioactivity in rats given I 131 ACTH intravenously (1951). From this study they concluded that there was selective localization of radioactivity in the adrenal cortex within a minute after the injection and that the ACTH had a half-time disappearance from the blood in 5.5 minutes. In another publication Sonenberg (1952) reported that exposure to cold stress for four hours prior to the injection of the I 131 ACTH increased the uptake of radioactivity in the adrenals. This indicated that the adrenal utilized more ACTH as the result of the stress.

Cox (1951) reported that I 131 prolactin given to animals localized in the mammary tissue. In a more thorough study, Sonenberg's group (1951a) administered I 131 prolactin to rats and found no significant localization
of radioactivity in mammary tissue of normal or pregnant rats, but they did
find significant concentrations of radioactivity in the corpora lutea of
the rat ovary 30 minutes after intravenous injection. Recently Williams
and Turner (1954) reported that I 131 tagged lactogenic hormone injected
into the mammary ducts of pseudo pregnant rabbits resulted in localization
of radioactivity in the particulate fractions of mammary cells, the mito-
chondria and the microsomes. Sonenberg extended his studies to I 131
growth hormone and I 131 luteinizing hormone (1952). He found that radio-
activity appeared in the pancreas in significant concentrations after the
injection of tagged growth hormone, and that localization of radioactivity
occurred in the ovary after administration of I 131 luteinizing hormone
to female rats. Sonenberg's group (1952) also studied thyrotropic hor-
mone tagged with S 35 and claimed that selective localization occurred in
the thyroid 24 hours after giving this preparation to day old chicks.
Kraintz and Talmage (1952), using I 131 relaxin, showed that concentra-
tions of radioactivity above that of muscle appeared in the ovaries and
adrenals of female rats, however, no claims of selective localization were
made. In all of these experiments with I 131 tagged protein hormones,
control studies were carried out using the tracer alone or I 131 tagged
bovine serum albumin and in no instance did the concentration of radioac-
tivity in the target organs approach that obtained with the tagged hormones.

In summary, it can be stated that there is little evidence to demon-
strate selective uptake in any target tissue after the administration of
radioactive non-protein hormones, such as thyroxine, epinephrine, and the
steroid hormones. Admittedly, target tissue would be more difficult to
define for these hormones. On the other hand, evidence has been presented that the tropic protein hormones of the anterior pituitary may selectively bind with target tissue. Insulin has also been shown to bind to tissue; but since all tissues are affected by insulin, there is no selectivity in regard to the tissue.

In the study presented in this thesis, an attempt will be made to further investigate the specificity of localization of protein hormones and to point out valid objections against the present interpretation of such studies. The simplicity of the tracer technique in biological work has many advantages, however, the difficulties of interpretation can lead to erroneous conclusions.
DESCRIPTION OF THE RESEARCH PROBLEM

The general thesis of the work presented in this investigation is concerned with the feasibility of adding radioactivity to protein hormones by chemical reactions (tagging), or by incorporating radioactivity into the actual protein molecule (labeling), and determining the usefulness of such radioactive preparations in biological studies. Since this work is a continuation of studies reported in a thesis submitted for a Master's degree (Kraintz, 1952) it is necessary to summarize those findings in order to present an integrated description of the problem.

Adrenocorticotrophic hormone and prolactin hormone preparations were tagged with I 131 and distribution studies done in rats injected intravenously with these tagged preparations. In addition, a similar study was done with I 131 tagged relaxin. The results obtained with the tagged ACTH and prolactin were in agreement with those reported by Sonenberg et al. (1951, 1951a). This group reported localization of radioactivity in the adrenal within a minute after the administration of tagged ACTH, and localization of radioactivity in the corpora lutea of the rat ovary 30 minutes after the administration of the tagged prolactin. The criteria of localization was based on several factors: (1) The radioactivity in the target organ was greater than that appearing in an equal weight of indifferent tissue, such as muscle. (2) The radioactivity in the target organ was greater than that resulting from the administration of bovine serum albumin tagged with I 131 or I 131 alone. (3) The radioactivity in the target organ could be shown to be firmly bound since very little could be removed from the tissue by thorough perfusion with physiological saline.
The uptake of radioactivity by the adrenal following injections of tagged ACTH fulfilled the above criteria, however, it was never greater than that present in an equal weight of blood. On the other hand, the localization of radioactivity in the corpora lutea of the ovary after administration of I-131 prolactin was more striking; for in addition to satisfying these same criteria, the concentration of the radioactivity in the whole ovary exceeded the amount found in an equal weight of blood. However, similar concentrations of radioactivity occurred in the adrenals after I-131 prolactin administration; a finding that was not sufficiently emphasized by Sonenberg (1952).

The studies reported in the Master's thesis (Kraintz, 1952) demonstrated that I-131 tagged ACTH or I-131 tagged prolactin could not be utilized in isotopic dilution studies for assay procedures: that is, the addition of "carrier" non-radioactive hormone to the tagged preparations prior to injection into rats had no influence on the uptake of radioactivity in the respective end organs. It was also suggested that the localization of radioactivity in the adrenals might not be specific in the studies with I-131 ACTH. Sonenberg's suggestion, that the paradoxical finding of radioactivity in the adrenal in the I-131 prolactin studies might be due to ACTH contamination (actually less than 1% contamination in the prolactin preparations used), would seem untenable since this uptake was greater than that obtained with I-131 ACTH and persisted in the adrenal for a longer time period. The possibility that prolactin itself may localize in the adrenal because of some functional relationship between this hormone and the adrenal must be considered, since a variety of metabolic effects that may be related to adrenal cortical function have
been ascribed to prolactin by Riddle and Bates (1939). Another possibility is that the uptake of radioactivity in the adrenal in the prolactin studies is non-specific and is related to the physical and chemical properties of the iodinated protein rather than to its biological properties. If that would indeed be the case, then neither radiiodide alone nor I 131 tagged bovine serum albumin would be adequate reference controls for distribution studies. Iodide would be utilized by the thyroid and little radioactivity would be found in other tissues. Studies with I 131 bovine serum albumin provide only a control for blood volume since albumin is a protein native to the circulation. Protein hormone preparations are essentially tissue extracts, therefore, their retention in the circulation and subsequent removal would differ considerably from that of a blood protein.

It appeared necessary to use a tissue protein of non-pituitary origin, one that presumably has no relation to adrenal function, for control studies. For this reason, relaxin, a protein hormone of the female reproductive tract was chosen since its molecular weight (approximately 10,000) is closer to the value of ACTH (20,000) than is the molecular weight of serum albumin (70,000). A preparation of relaxin was iodinated with I 131 as a tracer and administered to rats intravenously. Distribution studies with this preparation revealed that localization of radioactivity occurred in the adrenal in as high or higher concentrations than occurred when I 131 ACTH was administered. In this case, as with the I 131 prolactin studies, the radioactivity was retained in the adrenal for a longer time period. This observation provided additional evidence that the uptake of radioactivity in the adrenals after the administration of I 131 ACTH may not be specific. It is possible, however, that the metabolism of I 131 ACTH by
the adrenal is specific since ACTH localizes and disappears from the adrenal very rapidly compared to the results obtained with tagged prolactin or relaxin where the concentrations remain high in the adrenal for as long as 30 minutes.

These studies pointed out that the mere localization of radioactivity in adrenal tissue, in experiments with I 131 tagged protein hormone preparations, is not necessarily due to a target organ relationship and may be non-specific. In contrast, the uptake of radioactivity in the corpora lutea of the rat ovary, in experiments with tagged prolactin, appeared to be more specific since the degree of uptake was greater than that obtained in the studies with other tagged protein hormones.

In view of the findings obtained in the initial studies, the following questions were raised: (a) Would studies with other tagged protein hormones provide any information about the site of action of these respective hormones? (b) Would protein hormones other than ACTH tagged with I 131 also show localization in the adrenal? (c) Would "in vitro" studies with adrenal slices and radiiodine tagged ACTH clarify the specificity of the binding of ACTH to the adrenal? (d) Would the tagging of a protein hormone with another radioactive isotope substantiate the results obtained with I 131 tagged preparations? (e) Could an anterior pituitary hormone preparation be labeled biosynthetically?

In an attempt to answer the questions posed, the following experimental studies were undertaken:

1. Another non-pituitary hormone preparation, parathyroid extract, was tagged with I 131 and distribution studies were undertaken, to determine whether radioactivity might localize in the target
tissue, such as bone and kidney, and also to determine whether this hormone, like I 131 relaxin, would localize radioactivity in the adrenal.

2. Several protein hormone preparations, ACTH, prolactin, and insulin, were tagged with I 131 and incubated with surviving rat adrenals to determine whether there was any selective binding of ACTH to the adrenal when compared with prolactin and insulin.

3. Attempts were made to tag a prolactin preparation with radioactive chromium in order to compare results already obtained with I 131 prolactin. Prolactin was chosen for this study because of its availability in relatively pure form and also because its localization in the ovary was relatively high.

4. As the result of the problems encountered with chemically tagged hormones, studies regarding the feasibility of producing a radioactive prolactin preparation biosynthetically were undertaken.

The first three problems will be considered under the heading Studies with Chemically Tagged Radioactive Protein Hormones. The last problem represents a major effort in a relatively unexplored field of investigation and will be presented as a separate problem entitled The Biosynthesis of Radioactive Labeled Prolactin.
STUDIES WITH CHEMICALLY TAGGED RADIOACTIVE PROTEIN HORMONES

I. RADIOIODINE TAGGED PARATHYROID EXTRACT

The site of action of parathyroid hormone has never been clearly established. It is, of course, recognized that the hormone has an effect on the metabolism of phosphorus and calcium. There is general agreement that the hormone has a direct effect on the excretion of phosphorus by the kidney, thereby lowering the serum phosphorus. However, a sharp divergence of opinion regarding the other sites of action of this hormone exists. The view expressed by Albright and Reifenstein (1948) suggests that changes in calcium metabolism are secondary to the phosphorus changes; that is, serum calcium rises when serum phosphorus is lowered by the simple relationship of these two ions to the solubility product of calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$). Evidence that parathyroid hormone may have a direct effect on calcium metabolism has been presented. Talmage et al. (1952) showed that parathyroid extract is effective in maintaining and raising the serum calcium in parathyroidectomized-nephrectomized rats. Studies with radioiodine tagged parathyroid extract might conceivably provide additional evidence as to the site of action of the hormone. In addition, these studies would provide data regarding the non-specific localization in the adrenal with non-pituitary proteins. Therefore, the following studies were undertaken with a parathyroid preparation. Experiments were designed to demonstrate whether the extract contained more than one protein component. The amino acids present in the protein were investigated prior to iodination studies.
The preparation was tagged with radiiodine and distribution studies done in normal and parathyroidectomized rats.

MATERIALS AND METHODS

A. Chemical

1. Electrophoresis

Paroidin, a lyophilized preparation of parathyroid extract prepared by Parke-Davis company containing 10 U.S.P. units per milligram of protein, was used throughout this study. A 2% solution of this preparation was prepared in distilled water and studied with the paper electrophoresis method of Kunkel and Tiselius (1951) and the continuous filter paper electrophoresis method of Durrum (1951) to determine the homogeneity of the preparation.

2. Qualitative Amino Acid Analysis

In order to determine the amino acids present in the extract, 10 milligrams of the preparation were placed in a glass tube and 1 milliliter of 6 normal hydrochloric acid added. The tube was then evacuated, sealed, and placed in an oven at a temperature of 120 degrees Centigrade for 24 hours. After hydrolysis was complete, the contents of the tube were placed in a small beaker and evaporated to dryness on a steam bath. This material was then redissolved in distilled water and an aliquot placed on the edge of a 9x12 inch sheet of Whatman #4 filter paper, dried, and subjected to two dimensional (phenol-lutidine) chromatography. Later the paper was sprayed with ninhydrin for the identification of the amino acids.

3. Iodination

Two milliliters of a 2% solution of Paroidin in a bicarbonate-carbonate buffer, pH 8.5, were iodinated by the addition of 2 milliliters
of an iodinating mixture containing 0.38 milligrams of sodium iodide, 0.16 milligrams of iodine and 5 millicuries of I 131. This mixture was added dropwise to the hormone preparations with constant stirring for a period of 4 hours at a temperature of 5 degrees Centigrade. At the conclusion of this procedure the material was dialyzed against 4 changes of 20 liters of tap water for 72 hours. The pH of the mixture was adjusted to 8.5 with 0.1 normal sodium hydroxide prior to the injection since parathyroid extract is not soluble between the pH range of 4-8. The volume was then adjusted to obtain a concentration of 4 milligrams per milliliter for injection purposes.

B. Biological

A preparation of parathyroid extract similarly iodinated was assayed by G.D. Buchanan of this department. The criteria for biological activity was the ability of the extract to produce a phosphaturia in anesthetized dogs. This procedure is described in detail by Buchanan (1954).

The studies reported here were done on male rats weighing between 200 and 250 grams. For distribution studies, rats were injected intravenously with 0.5 milliliters of the tagged extract containing 2 milligrams of protein. The rats were sacrificed at varying time intervals up to 3 hours in order to determine the relationship of the uptake in tissue to the time expired. Sixteen normal and sixteen parathyroidectomized rats were used in these experiments. The rats were parathyroidectomized by the method of Richter and Birmingham (1941) and used 24 hours later. Four rats from each group were killed at each time period by exsanguination while under ether anesthesia. Representative portions of tissues or whole organs were
removed, weighed, and placed in vials containing 10% sodium hydroxide in order to digest the tissue.

Radioactivity measurements were made by the use of a scintillation type gamma counter permitting the direct counting of fluid and tissue samples. All samples were counted for at least 5000 counts giving a counting error of plus or minus 2.25%. All determinations are expressed as percentages of injected dose per gram of sample and also as tissue/blood ratios.

RESULTS

A. Chemical

1. Electrophoresis

Paper electrophoresis by the paper strip method at various pH's only revealed that the parathyroid extract must consist of numerous unidentifiable fractions, since it appeared as a long "smudge" without definitive zones such as those obtained with serum. However, the continuous paper electrophoresis technique showed more definitive zones of proteins. As many as 6 different components can be demonstrated in the parathyroid extract by this latter method. Plate I is a reproduction of the filter paper curtain stained with brom phenol blue dye. The conditions of the separation are listed on the plate, and the "zones" are indicated by the penciled borders. A pure protein would form a single narrow band.

2. Qualitative Amino Acid Analysis

The amino acids found to be present in the Paroidin after acid hydrolysis and subsequent paper chromatography are as follows: alanine, aspartic acid, arginine, glutamic acid, glycine, lysine, phenylalanine, tyrosine, proline, valine, and methionine.
PLATE I

PHOTOGRAPH OF PARATHYROID EXTRACT FILTER PAPER CURTAIN

(METHOD OF DURRUM, 1890)

Protein stained with brom phenol blue dye

Electrolyte - 0.1 M acetic acid

1000 volts - 8 milliams

Duration - 48 hours
B. Biological

A preparation of parathyroid extract similarly iodinated with stable iodine was found to retain its ability to produce a phosphaturia in anesthetized dogs.

The relationship between the uptake of radioactivity in various tissues and the time after administration of I $^{131}$ parathyroid extract to rats is presented in Table 1. Significant amounts of radioactivity above that of blood occurred in the kidney, liver, spleen, thyroid and adrenals during the first 30 minutes. At the end of 3 hours the radioactivity in the adrenal and spleen dropped below that of blood while the radioactivity in the thyroid increased, indicating that iodine was being released from the protein. There appeared to be no significant difference between the normal and the parathyroidectomized animals except for the uptake of radioactivity in the thyroid.

DISCUSSION

Commercial parathyroid extract can be shown to be a relatively inhomogeneous protein hormone preparation containing at least 6 components by the continuous paper electrophoresis technique of Durrum. This technique has been shown to be applicable to the separation of serum proteins. Other studies done by L'Heureux et al. (1947) emphasized the inhomogeneity of parathyroid extract preparations. Ross and Wood (1942) pointed out that even in the highly purified preparations of parathyroid extract prepared by their group, at least two components were revealed in ultracentrifugation studies. One component had a molecular weight range of approximately 15,000-25,000, while the other was in the range of 500,000-1,000,000.

(22)
Table 1

DISTRIBUTION OF RADIOACTIVITY IN NORMAL AND PARATHYROIDECTOMIZED RATS AFTER INTRAVENOUS ADMINISTRATION OF I 131 PARATHYROID EXTRACT

<table>
<thead>
<tr>
<th>Time After Injection</th>
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<th>30 min.</th>
<th>1 hr.</th>
<th>3 hrs.</th>
</tr>
</thead>
<tbody>
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<td>% Gm t/b</td>
<td>% Gm t/b</td>
<td>% Gm t/b</td>
</tr>
<tr>
<td>Blood</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>N</td>
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<td>0.79 1.00</td>
<td>0.97 1.00</td>
<td>1.18 1.00</td>
</tr>
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<td>1.06 1.00</td>
<td>0.87 1.00</td>
<td>0.82 1.00</td>
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<td></td>
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<tr>
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<td>0.86 1.11</td>
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<td>0.52 0.44</td>
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<td>0.96 1.14</td>
<td>0.63 0.52</td>
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<td></td>
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<tr>
<td>N</td>
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<td>0.38 0.47</td>
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<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>8.56 6.30</td>
<td>5.97 7.55</td>
<td>2.38 2.49</td>
<td>1.91 1.63</td>
</tr>
<tr>
<td>PTX</td>
<td>10.50 7.23</td>
<td>4.72 4.49</td>
<td>3.92 2.91</td>
<td>1.45 1.74</td>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>N</td>
<td>6.74 5.01</td>
<td>3.13 3.97</td>
<td>3.07 3.17</td>
<td>1.35 1.15</td>
</tr>
<tr>
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<td>4.49 4.17</td>
<td>2.55 2.88</td>
<td>0.93 1.04</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.96 1.45</td>
<td>1.67 2.12</td>
<td>0.79 0.82</td>
<td>0.72 0.63</td>
</tr>
<tr>
<td>PTX</td>
<td>2.12 1.44</td>
<td>1.34 1.23</td>
<td>0.93 1.08</td>
<td>0.65 0.53</td>
</tr>
<tr>
<td>Thyroid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>4.69 3.51</td>
<td>9.35 11.80</td>
<td>45.4 47.2</td>
<td>86.0 71.0</td>
</tr>
<tr>
<td>PTX</td>
<td>6.77 4.77</td>
<td>11.68 10.86</td>
<td>11.76 13.80</td>
<td>80.0 73.0</td>
</tr>
<tr>
<td>Muscle</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0.21 0.16</td>
<td>0.17 0.21</td>
<td>0.24 0.24</td>
<td>0.22 0.19</td>
</tr>
<tr>
<td>PTX</td>
<td>0.26 0.18</td>
<td>0.23 0.22</td>
<td>0.19 0.24</td>
<td>0.15 0.18</td>
</tr>
</tbody>
</table>

Each figure is an average of the values obtained with 4 animals

\[
\% \text{ Gm} = \frac{\text{counts per gram of tissue}}{\text{injected counts}}
\]

\[
T/B = \frac{\% \text{ per gram tissue}}{\% \text{ per gram blood}}
\]
There can be no doubt about the inhomogeneity of the commercial preparations. Obviously the amino acid composition of this mixture of proteins would reveal little about the nature of the hormone, since some of these amino acids may be present in the inert protein, and, at this time, there is no way to distinguish the inert protein from the biologically active protein. The study of the amino acids was done primarily to demonstrate the presence of tyrosine, which would have to be present in order to successfully iodinate the preparation.

The retention of biological activity by the extract, after iodination that results in less than one iodine group per theoretical molecule of protein, gives no indication whether the specific molecules containing the radioactivity are biologically active, since the tagging rates of each component of an inhomogenous protein preparation may vary widely. It is possible that the inert contaminants were tagged with the iodine while the biologically active constituents were left unreacted. These factors should be considered in interpreting any studies with this preparation.

It would be very tempting to interpret the localization of radioactivity in the kidney as functional localization of parathyroid hormone. Unfortunately, almost all foreign proteins tagged with I\(^{131}\) show a similar or higher localization of radioactivity in the kidney. It was found in other investigations with I\(^{131}\) tagged proteins (Master's thesis, Kraintz, 1952) that the kidney, liver and spleen are common sites of localization after the administration of tagged protein hormone preparations. Any differences in the uptake of radioactivity by the thyroid of these two groups is of little significance since thyroid function was undoubtedly
Table 2

COMPARISON OF RADIOACTIVITY IN THE ADRENAL AFTER ADMINISTRATION OF I $^{131}$ PROTEIN HORMONE PREPARATIONS

<table>
<thead>
<tr>
<th>Time After Injection</th>
<th>ACTH* % Gm T/B</th>
<th>PROLACTIN* % Gm T/B</th>
<th>RELAXIN* % Gm T/B</th>
<th>PARATHYROID EXTRACT % Gm T/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>15'</td>
<td>0.48 0.74</td>
<td>1.24 1.48</td>
<td>1.17 0.64</td>
<td>1.57 1.18</td>
</tr>
<tr>
<td>30'</td>
<td>0.50 1.19</td>
<td>1.20 1.54</td>
<td>1.22 0.60</td>
<td>0.86 1.11</td>
</tr>
</tbody>
</table>

*Data taken from Master's Thesis (Kraintz, 1952)

\[
\% \text{ Gm} = \frac{\text{counts per gram of tissue}}{\text{injected counts}}
\]

\[
\text{T/B} = \frac{\% \text{ per gram of tissue}}{\% \text{ per gram of blood}}
\]
altered slightly by the parathyroidectomy procedure.

The only really significant finding was of a negative nature and concerned the uptake of radioactivity in the adrenal. In this instance we have an inhomogenous protein preparation that could hardly localize in the adrenal as the result of some target gland relationship. If one compares the data obtained from other studies with I \( ^{131} \)I tagged protein hormone preparations, it becomes evident that uptake of radioactivity occurs in the adrenal after the administration of tagged ACTH, prolactin, relaxin, and parathyroid extract (see Table 2). The binding ability of adrenal tissue is not limited to \( ^{131} \)I ACTH alone.

**SUMMARY**

A parathyroid extract preparation, Paroidin, was tagged with \( ^{131} \)I and distribution studies done in normal and parathyroidectomized rats. Little significance was attributed to the localization of radioactivity in the various tissues studied in view of the inhomogeneity of the preparation. In addition, no differences in distribution of radioactivity was noticed between the two groups in studies up to three hours. The appearance of radioactivity in the adrenals in concentrations above that of blood was interpreted as additional evidence of the non-specific localization of radioactivity which has consistently occurred in studies with \( ^{131} \)I labeled protein hormone preparations.
II. IN VITRO STUDIES WITH RADIOIODINE TAGGED PROTEIN HORMONE PREPARATIONS

Interpretation of studies of localization of radioactivity in the adrenal following injection of tagged ACTH is complicated by the inhomogeneity of the ACTH preparation used. The fact that all commercial protein hormone preparations are tissue extracts, and probably are not identical to the circulating hormone, might result in distribution patterns which bear little resemblance to the localization and metabolism of the naturally occurring material. Since the kidney, liver, and spleen actively partake in the removal of foreign proteins in vivo, incubation of isolated target tissue with tagged preparations of protein hormones might reveal the selectivity of the binding in a more dramatic fashion.

As mentioned in the review, Stadie and co-workers (1952) demonstrated that tagged insulin can be used to study the binding of that hormone to tissues. Saffron and Bayliss (1953) showed that ACTH added in vitro stimulates the formation of corticoids by rat adrenal slices in direct proportion to the amount of ACTH added. They claimed that this effect of ACTH on the adrenal persisted after washing, but did not state whether or not the ACTH was actually bound by the adrenal tissue. Stadie et al. (1952) did not report additional control studies with boiled tissue or other tagged hormones, since they were interested only in the correlation of the binding of the insulin with its ability to increase glycogen synthesis.

The study presented here was not designed to disprove the concept that binding of I 131 ACTH occurs with adrenal tissue, but rather to point out that this binding is a property of other protein hormones as well. The experiment was exploratory in nature and if the supposed
dramatic selectivity of ACTH for the adrenal had been demonstrated, more
detailed studies would have been undertaken.

MATERIALS AND METHODS

The following commercial preparations were used in this study: ACTH
(ACTHAR, Armour), prolactin (Squibb), crystalline zinc insulin (Lilly),
and bovine serum albumin (Armour).

I 131 tagged ACTH, prolactin, insulin, and serum albumin were pre-
pared by a method described in the previous section. Each preparation
contained about 10 microcuries of radioactivity per milligram and 1 or less
iodine groups per molecule of protein.

Five male rats of the Sprague–Dawley strain, weighing approximately
200 grams each, were used as donors for the adrenal glands. The animals
were killed with a blow on the head and the pair of adrenals from each
rat removed. One adrenal from each rat was bisected with a razor blade,
weighed and placed in a Warburg flask with tagged protein or I 131 as io-
dide in Krebs Ringers Glucose solution at pH 7.4. The contralateral ad-
renal was first boiled in physiological saline and then treated in the
same way. All the preparations were incubated for 30 minutes at 37 de-
grees Centigrade. At the end of this time the tissue was removed from the
flask and washed 3 times with physiological saline.

The radioactivity in the adrenal was determined by means of the scin-
tillation cup type gamma counter. An aliquot of the incubation mixture
was taken as a standard for each preparation prior to this experiment.
The radioactivity bound is expressed as a percentage of the total radio-
activity present in each incubation mixture. Each value is the percentage

(26)
for 10 milligrams of wet tissue.

RESULTS AND DISCUSSION

Table 3 shows the results obtained in a single experiment. As can be seen from the figures, no dramatic binding of ACTH over the control substances, albumin and iodide, is apparent. In addition, the other two protein hormones, prolactin and insulin, show a binding which is as high or higher than that of ACTH. It will be noted that all the preparations, except the albumin, bound to the boiled adrenal in much greater concentrations than to the surviving slices.

Although extensive data has not been obtained or presented, the demonstration that protein hormones other than ACTH, similarly tagged with I<sup>131</sup>, also bind with adrenal tissue implies that further in vitro work in this vein would show very little concerning the affinity of adrenal tissue for a particular protein hormone. The fact that the boiled adrenal tissue shows an even greater binding capacity than the surviving slices may indicate that this binding phenomenon is of a physico-chemical nature rather than a biological mechanism involving enzymes.
Table 3

The Binding of Radiiodine Preparations with Rat Adrenal In Vitro

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>I131 Preparation in Medium</th>
<th>Amount Prep. in Medium in mgms.</th>
<th>Radioactivity Bound % per 10 mgms adrenal Surviving Adrenal</th>
<th>Boiled Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACTH</td>
<td>.04</td>
<td>0.482</td>
<td>1.230</td>
</tr>
<tr>
<td>2</td>
<td>Prolactin</td>
<td>.04</td>
<td>0.438</td>
<td>1.425</td>
</tr>
<tr>
<td>3</td>
<td>Insulin</td>
<td>.02</td>
<td>0.825</td>
<td>5.450</td>
</tr>
<tr>
<td>4</td>
<td>Albumin</td>
<td>.04</td>
<td>0.372</td>
<td>0.355</td>
</tr>
<tr>
<td>5</td>
<td>Iodide</td>
<td>.000006</td>
<td>0.300</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Each of the protein preparations contained one or less iodine groups per theoretical molecule of protein.
III. STUDIES WITH RADIOACTIVE CHROMIUM TAGGED PROLACTIN

The use of an isotope other than radioactive iodine for tagging protein hormones would in part answer some of the objections raised to studies with I 131 tagged preparations. Sonenberg et al. (1952) used S 35 for the tagging of a thyrotropic hormone preparation, but they did not report any comparative studies on preparations tagged with I 131 and S 35. Actually there would be similar objections raised to studies with S 35 tagged preparations, since one would have to interpret these findings in relation to sulfur metabolism in general. An ideal tagging agent would be an element which does not normally occur in the body so that problems such as isotope dilution would not be encountered in biological studies.

The application of radioactive chromium 51 as a biological tracer was first reported by Gray and Sterling (1950). They demonstrated that trivalent chromium 51 could be used to tag serum albumin and serum globulin preparations. Although the nature of the binding of the chromic ion to the protein was not established, it was suggested that the chromic ion reacted with the terminal amino groups of lysine or other basic groups of proteins to form a chromium amino complex. Physico-chemical analysis of chromium tagged albumin (1 mole of Cr to 1 mole of albumin) did not disclose any alterations from the native state. In view of Gray and Sterling's findings on the tagging of blood proteins, the use of chromium as a tagging agent for protein hormones was considered.

At the time this problem was being investigated, no other biological studies with radioactive chromium had been done. It was necessary to do distribution studies of radioactivity in animals injected with trivalent
radioactive chromium in order to determine the pattern of localization
in various tissues. These studies were first reported by Kraintz and
Talmage (1952) and have been confirmed in subsequent studies by Visek,
et al. (1953). Unfortunately the specific activity of the chromium 51
was such that initial studies with this isotope were limited to the demon-
stration that certain anterior pituitary protein hormone preparations,
ACTH, TSH, and prolactin, could be tagged. Distribution studies in ani-
mals with these tagged preparations were not possible because of the
enormous dilution factor encountered in animal experiments. A prepa-
ration of high specific activity chromium 51 became available at a later
date and was utilized for biological studies with chromium tagged pro-
lactin and chromium tagged albumin.

Filter paper strip electrophoresis, using the method of Kunkel and
Tiselius (1951), of the tagged preparations was attempted. However, it
was only applicable to studies with chromium tagged albumin and prolac-
tic, since the TSH and ACTH could not be characterized by this method.

MATERIALS AND METHODS

The chromium 51 was obtained from Oak Ridge as the trivalent chro-
mium chloride in acid solution. The specific activities of the prepa-
rations used in the studies reported here were 200 Millicuries per gram
for lot #1, and 1000 Millicuries per gram for lot #5. The preparations
of ACTH, prolactin, and bovine serum albumin were the same as those used
in the previous studies. The TSH was a preparation of Armour Laboratories.

A. Measurements of Radioactivity

The problem of detecting the radioactivity had to be considered.
Chromium 51 is a soft X-ray emitter with a half life of 26.5 days. It emits an X-ray of 4.92 Kev plus a few percent of .237 Mev gamma rays. Gray and Sterling (1950) used an X-ray and window Geiger Muller counter for the detection of radioactivity, however, considerable self-absorption occurs in this method. Because it would facilitate the counting of samples without any involved preparations, the chromium was counted by gamma counting using the Texas Co. toroidal type gamma counter and a cup type scintillation counter.

No attempts were made to standardize the values for a microcurie of chromium 51 in an absolute fashion, since Abbott Laboratories and the Oak Ridge National Laboratories did not agree on the values for the same shipment, (Tabern, personal communication). Using the values supplied by Oak Ridge, relative standardization was performed.

B. Tagging of Proteins with Trivalent Radioactive Chromium 51

The method used for the tagging of the protein preparations was essentially that of Gray and Sterling (1950). Radioactive Cr 51 in the form of chromium chloride in acetate buffer (0.15 molar) at a pH of 4.5 was added to a series of 2% protein solutions. The amount of chromium added was 2 moles of chromium to every theoretical mole of protein. After 4 hours of mixing in the cold (5 degrees Centigrade), the mixtures were placed in Visking dialysis bags and dialyzed against 20 liters of isotonic saline, changed daily, for 3 days. The radioactivity of each preparation was determined before and after dialysis in order to calculate the amount of chromium bound.

The experiment designed to demonstrate the possible tagging of proteins with chromium 51 was done with the low specific activity preparation (30)
(lot #1) and could not be used for biological studies. The preparation of high specific activity chromium 51 (lot #5) enabled the tagging of prolactin and albumin for biological work.

C. Distribution Studies

No distribution studies were done with TSH or ACTH since they were not tagged with the high specific activity preparation of chromium 51.

Distribution studies were carried out in female rats of the Sprague-Dawley strain weighing 200-250 grams with chromium 51 chloride, chromium 51 tagged albumin, and chromium 51 tagged prolactin injected intravenously. Twelve rats were used in these studies. One group of rats received only chromium 51 chloride in saline, the second group received chromium 51 tagged albumin, and the third group received chromium 51 tagged prolactin. Each of the rats received approximately .006 milligrams of chromium. The rats that were injected with the chromium tagged albumin received 2 milligrams of protein each, as did the ones that were injected with the chromium tagged prolactin.

The rats were killed at 15 minutes, 30 minutes, 1 hour, and 2 hours after the administration of the tagged materials by exsanguination under ether anesthesia. Tissues were removed, weighed, and placed in vials for direct counting. A diluted standard of each of the injected preparations was counted at the same time.

D. Electrophoresis

Preparations of chromium tagged albumin and chromium tagged prolactin were subjected to paper strip electrophoresis in 0.1 molar veronal buffer at pH 8.8. Radioautography of the paper strips using no-screen X-ray film was used to demonstrate the binding of the radioactivity to the protein.

(31)
RESULTS

A. Measurements of Radioactivity

One microcurie of chromium 51 gave approximately 5,000 counts per minute with the Texas Co. counter and approximately 50,000 counts per minute with the scintillation counter. The efficiency of the scintillation counter over the Texas Co. counter was about 10 times for the detection of the gamma radiation from chromium 51. Since the radioactive chromium solution was in a glass vial and the walls of both counters are made of metal, there is little chance that any of the low energy X-rays emitted by this isotope were detected.

B. Tagging of Proteins with Trivalent Radioactive Chromium 51

Table 4 shows the binding of radioactive trivalent chromium 51 (lot #1) by the various protein preparations. As can be seen from the data, binding occurs with all the preparations studied. Between 30% and 60% of the radioactivity added to the various preparations was retained after prolonged dialysis. By assuming average molecular weights for the proteins, ACTH, TSH, prolactin and bovine serum albumin, the number of chromium groups bound to one molecule of protein can be determined. No bioassays of these preparations were made to determine whether they retained their biological activity.

Table 5 shows the results obtained with high specific activity chromium 51 (lot #5) tagging of prolactin and bovine serum albumin. The percentage of chromium bound compared to the amount added is similar to that obtained in the initial experiments. Each milligram of prolactin and albumin contained about the same amount of chromium, however, the number of chromium groups per molecule of albumin is much higher because of its
greater molecular weight. These tagged proteins were prepared to contain similar amounts of chromium and radioactivity rather than the same number of chromium groups per molecule, since they were to be used in biological studies.

C. Distribution Studies

The distribution of radioactivity in various tissues for different time intervals after the administration of the tagged preparations and the trivalent chromium 51 chloride alone is presented in Tables 6, 7 and 8. As can be seen from these data, there is marked difference in the distribution pattern of these three substances. Using the criteria that the appearance of radioactivity, in greater concentrations than in equal amounts of blood, in a particular tissue is of significance, values over 1 were obtained only in the kidney at the end of 2 hours with chromium chloride and with the chromium tagged albumin. On the other hand, similar tissue-blood ratios for the chromium tagged prolactin show that values greater than 1 are obtained in liver, kidney, and adrenals by 15 minutes, and in bone, ovaries, and spleen by 30 minutes. By the end of 2 hours all the tissues except muscle showed this positive uptake of radioactivity in the prolactin study.

D. Electrophoresis

Subsequent studies with paper electrophoresis and radioautography revealed that the chromium 51 was firmly tagged with serum albumin. However, if we look at the results obtained with the chromium 51 tagged prolactin, it can be seen from Plate II that while the chromium was bound to the protein most of the radioactivity did not move with the major component of the prolactin preparation.
Table 4

Low Specific Activity* Chromium 51 Tagging of Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total Mgms</th>
<th>Cr Added</th>
<th>Cts./Min. Added</th>
<th>Cts./Min. Bound</th>
<th>%Bound</th>
<th>Mole Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>M.W. 20,000</td>
<td>20</td>
<td>0.10</td>
<td>100,000</td>
<td>32,310</td>
<td>32.8</td>
</tr>
<tr>
<td>TSH</td>
<td>M.S. 10,000</td>
<td>20</td>
<td>0.20</td>
<td>200,000</td>
<td>91,420</td>
<td>45.7</td>
</tr>
<tr>
<td>Prolactin</td>
<td>M.W. 30,000</td>
<td>20</td>
<td>0.07</td>
<td>70,000</td>
<td>42,200</td>
<td>60.3</td>
</tr>
<tr>
<td>B.S. Albumin</td>
<td>M.W. 70,000</td>
<td>20</td>
<td>0.03</td>
<td>30,000</td>
<td>19,400</td>
<td>64.1</td>
</tr>
</tbody>
</table>

*The specific activity of this chromium 51 preparation was 200 Millicuries per gram of chromium. This value was obtained from Oak Ridge National Laboratories.
Table 5

High Specific Activity* Chromium 51 Tagging of Prolactin and Bovine Serum Albumin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total Mgms.</th>
<th>Cr Added</th>
<th>Cts./Min. Added</th>
<th>Cts./Min. Bound</th>
<th>%Bound</th>
<th>Mole Ratio</th>
<th>Mgsm Cr. Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin M.W. 30,000</td>
<td>20</td>
<td>0.10</td>
<td>5,000,000</td>
<td>3,300,000</td>
<td>66%</td>
<td>2.4:1</td>
<td>.0033</td>
</tr>
<tr>
<td>Albumin M.W. 70,000</td>
<td>20</td>
<td>0.10</td>
<td>5,000,000</td>
<td>2,980,000</td>
<td>59%</td>
<td>4.4:1</td>
<td>.0030</td>
</tr>
</tbody>
</table>

*The specific activity of this chromium 51 preparation was 1000 Millicuries per gram of chromium. This value was obtained from Oak Ridge National Laboratories.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>15 Min.</th>
<th>30 Min.</th>
<th>1 Hr.</th>
<th>2 Hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm. T/B</td>
<td>gm. T/B</td>
<td>gm. T/B</td>
<td>gm. T/B</td>
</tr>
<tr>
<td>Blood</td>
<td>5.00 1.00</td>
<td>5.28 1.00</td>
<td>5.20 1.00</td>
<td>2.70 1.00</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.55 0.11</td>
<td>0.77 0.15</td>
<td>1.28 0.25</td>
<td>0.67 0.25</td>
</tr>
<tr>
<td>Liver</td>
<td>0.31 0.06</td>
<td>1.24 0.24</td>
<td>1.36 0.26</td>
<td>0.97 0.36</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.27 0.45</td>
<td>3.41 0.65</td>
<td>4.34 0.84</td>
<td>3.24 1.20</td>
</tr>
<tr>
<td>Bone</td>
<td>0.59 0.12</td>
<td>0.88 0.17</td>
<td>0.97 0.19</td>
<td>0.83 0.31</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.09 0.02</td>
<td>0.17 0.03</td>
<td>0.17 0.03</td>
<td>0.10 0.04</td>
</tr>
<tr>
<td>Adrenal</td>
<td>1.40 0.28</td>
<td>1.44 0.27</td>
<td>1.23 0.24</td>
<td>0.86 0.32</td>
</tr>
<tr>
<td>Ovary</td>
<td>2.10 0.42</td>
<td>1.19 0.23</td>
<td>2.12 0.41</td>
<td>1.70 0.63</td>
</tr>
</tbody>
</table>

*counts per gram of tissue*

* injected counts

*gm* = per gram tissue

*T/B* = per gram blood
Table 7

DISTRIBUTION OF RADIOACTIVITY AFTER ADMINISTRATION OF PROLACTIN LABELED WITH CHROMIUM 51

Each rat received 2 mg. prolactin containing .0066 mg. chromium (6.6 mc)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time After Administration of Radioactivity</th>
<th>15 Min.</th>
<th>30 Min.</th>
<th>1 Hr.</th>
<th>2 Hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%gm. T/B</td>
<td>%gm. T/B</td>
<td>%gm. T/B</td>
<td>%gm. T/B</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>1.52</td>
<td>1.00</td>
<td>0.40</td>
<td>1.00</td>
<td>0.32</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.79</td>
<td>0.52</td>
<td>3.55</td>
<td>8.87</td>
<td>4.27</td>
</tr>
<tr>
<td>Liver</td>
<td>3.94</td>
<td>2.59</td>
<td>6.58</td>
<td>16.45</td>
<td>8.05</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.21</td>
<td>2.12</td>
<td>1.18</td>
<td>2.95</td>
<td>0.44</td>
</tr>
<tr>
<td>Bone</td>
<td>0.41</td>
<td>0.27</td>
<td>0.60</td>
<td>1.50</td>
<td>0.38</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.17</td>
<td>0.11</td>
<td>0.06</td>
<td>0.15</td>
<td>-</td>
</tr>
<tr>
<td>Adrenal</td>
<td>1.70</td>
<td>1.12</td>
<td>2.45</td>
<td>6.12</td>
<td>1.46</td>
</tr>
<tr>
<td>Ovary</td>
<td>0.76</td>
<td>0.50</td>
<td>0.87</td>
<td>2.17</td>
<td>0.54</td>
</tr>
</tbody>
</table>

%gm = counts per gram of tissue injected counts
T/B = % per gram tissue

% per gram blood


Table 3

DISTRIBUTION OF RADIOACTIVITY AFTER ADMINISTRATION OF CHROMIUM 51 IN SALINE

Each rat received .006 mgs. chromium 51 in 0.5 ml. saline (60 μC)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>15 Min. %gm. T/B</th>
<th>30 Min. %gm. T/B</th>
<th>1 Hr. %gm. T/B</th>
<th>2 Hrs. %gm T/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.46. 1.00</td>
<td>2.35 1.00</td>
<td>2.16 1.00</td>
<td>2.29 1.00</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.42 0.12</td>
<td>0.32 0.14</td>
<td>0.38 0.18</td>
<td>0.35 0.15</td>
</tr>
<tr>
<td>Liver</td>
<td>0.48 0.14</td>
<td>0.26 0.11</td>
<td>0.35 0.16</td>
<td>0.38 0.17</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.49 0.43</td>
<td>0.60 0.26</td>
<td>1.30 0.60</td>
<td>3.00 1.31</td>
</tr>
<tr>
<td>Bone</td>
<td>0.43 0.12</td>
<td>0.27 0.11</td>
<td>0.38 0.18</td>
<td>0.44 0.17</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.07 0.02</td>
<td>0.05 0.02</td>
<td>0.08 0.04</td>
<td>0.11 0.05</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.66 0.19</td>
<td>0.53 0.23</td>
<td>0.40 0.19</td>
<td>0.73 0.32</td>
</tr>
<tr>
<td>Ovary</td>
<td>1.44 0.42</td>
<td>0.79 0.34</td>
<td>0.60 0.28</td>
<td>0.09 0.04</td>
</tr>
</tbody>
</table>

\[ \text{%gm} = \frac{\text{counts per gram of tissue}}{\text{injected counts}} \]

\[ \text{T/B} = \frac{\text{% per gram tissue}}{\text{% per gram blood}} \]
PLATE II

PAPER ELECTROPHORESIS AND RADIOAUTOGRAPHY OF Cr\(^{51}\) LABELED ALBUMIN

2% SOLUTION - BEEF SERUM ALBUMIN (ARMOUR)

DYED PAPER STRIP – ⬆️ + ➖⬅️ POINT OF ORIGIN

RADIOAUTOGRAPH

PAPER ELECTROPHORESIS AND RADIOAUTOGRAPHY OF Cr\(^{51}\) LABELED PROLACTIN

2% SOLUTION - PROLACTIN (SQUIBB #71713)

DYED PAPER STRIP – ⬆️ + ➖⬅️ POINT OF ORIGIN

RADIOAUTOGRAPH
DISCUSSION

If one merely examines the results obtained in these experiments without considering other factors, it would appear that radioactive chromium is as good, if not a better, tagging agent for prolactin than radioactive iodine. Using the criteria of Sonenberg (1952), the appearance of radioactivity concentrations higher than blood in target endocrine tissue could be interpreted as localization. The high uptake in the adrenal and ovary is as striking as that reported by Sonenberg et al. (1951a) or that obtained by Kraintz (Master's Thesis, 1952) using I 131 prolactin. It is thought, however, that the results simply show that significant localization of radioactivity in a particular tissue can result from the rapid removal of the radioactive material from the circulation, as the mere retention of radioactivity in a tissue during this time results in increasing tissue/blood ratios. If the radioactivity in the tissue represented a turnover of the tagged protein hormone, these results would be more meaningful. The fact that trivalent chromium will combine with proteins in vivo (Gray and Sterling, 1950; and Kraintz and Talmage, 1952a) would imply that any radioactivity appearing in a tissue might represent chromium that has been split from the tagged preparation metabolically and subsequently became bound to another protein in that tissue.

Despite the finding that the major portion of radioactivity was not associated with the main component of the prolactin preparation, uptake in target tissue still occurs. This uptake may represent the localization of only the biologically active component of the prolactin preparation, but it is more reasonable to assume that this uptake, particularly in the adrenals, might be due to the contaminants.

(34)
It would be tempting to assume that the high uptake in the adrenal is due to the presence of ACTH as the tagged contaminant in the prolactin preparation, however, no evidence has been presented to verify such an assumption. The very high uptake of the chromium 51 tagged prolactin in the liver, kidney, and spleen indicates that the animal is removing this material from the circulation as it would other foreign proteins. The fact that the uptake of radioactivity can be demonstrated in the ovary and adrenal with chromium tagged prolactin, as well as with I 131 tagged prolactin, could be interpreted as evidence that the intact protein molecules actually do localize in these sites.

SUMMARY

A prolactin preparation was tagged with chromium 51 and distribution studies were done in female rats after intravenous injection. Chromium tagged albumin and chromium 51 chloride were used as control substances. Paper electrophoresis studies revealed that the tagging of the prolactin preparation was not homogenous. Evidence has been presented to indicate that this preparation of prolactin localizes radioactivity in the adrenals and ovaries, substantiating other studies done with I 131 tagged prolactin preparations. This indicates that the intact protein molecules probably localize in these tissues, but no evidence has been presented to demonstrate that the biologically active component is responsible for the localization.
GENERAL DISCUSSION OF STUDIES WITH TAGGED RADIOACTIVE PROTEIN HORMONES

Specific experiments with tagged protein hormones which are reported independently, without the use of other similarly tagged protein hormones as controls, can be misleading. Williams and Turner (1954) injected I 131 tagged prolactin intraductally into the mammary glands of pseudo pregnant rabbits and demonstrated that the radioactivity localized in the particulate fractions of the epithelial cells of the mammary glands. No additional studies were done to determine whether I 131 alone, I 131 tagged serum albumin, or any preparation tagged with I 131 would result in similar findings.

The whole concept of the biological activity and identity of a hormone is based on its ability to produce a specific response in the so-called target tissue that is unique for that hormone. The concept of specific localization of a protein hormone in its target tissue should meet the same criteria. A case in point is the work of Sonenberg et al. (1951, 1951a). In the experiments with I 131 ACTH, the appearance of radioactivity to the extent of 1% per gram of adrenal tissue was considered evidence of localization, in spite of the fact that blood concentrations were higher. This work on ACTH considered by itself appears highly significant, however, if we look at their publication with I 131 prolactin, the appearance of radioactivity in the adrenal was essentially ignored since the target tissue was defined as the ovary. One of the criteria for localization of radioactivity in this latter study was the tissue/blood ratio. However, if one calculates the tissue/blood ratios obtained with the ACTH preparations in the adrenal, and compares them with the tissue/blood ratios
obtained for the adrenal with the prolactin preparations, one finds that the adrenal must be target tissue for prolactin as well as for ACTH.

The position taken in this discussion must be further clarified. It is not assumed that tropic hormones do not localize in target tissue, but rather that other protein hormones or possibly tissue protein extracts of similar molecular weight range may appear to localize in the same tissue. The binding of the target tissue to the tropic hormone cannot be considered specific.

The studies reported in this present investigation demonstrated that parathyroid extract tagged with I 131 resulted in localization of radioactivity in the adrenal. In vitro studies with I 131 tagged, ACTH, prolactin and insulin demonstrated that all three preparations showed binding with adrenal tissue and that no preferential binding of ACTH could be shown. In addition, experiments with radioactive chromium tagged prolactin indicated that localization of radioactivity occurs in the ovaries and adrenals in a similar pattern as that obtained by other workers with I 131 prolactin. This is interpreted as tentative evidence that binding of target tissue occurs with the intact prolactin proteins as it is unlikely that this localization could be due to a similar metabolism of iodine and chromium.

As the result of the findings in these studies and critical evaluation of the work done by other investigators in this field, it was decided to attempt to prepare a radioactive protein hormone biosynthetically. One could be fairly certain that similar studies with a biosynthetically labeled protein would represent the metabolism of the naturally occurring protein.
THE BIOSYNTHESIS OF RADIOACTIVE Labeled PROLACTIN

The successful labeling of a protein hormone biosynthetically would open the way to numerous physiological and biochemical studies concerning the mechanisms involved in the biosynthesis of the hormone and also the metabolism of that hormone. The in vitro incorporation of radioactive amino acids into proteins with both tissue slices and tissue homogenates has been reported by numerous workers. A detailed review of the literature in this field has been presented by Borsook (1950). Recently Melchior and Halikis (1952) demonstrated in vitro incorporation of S 35 methionine into protein of bisected rat pituitaries. It was pointed out that this incorporation was greater than that obtained with liver slices. Naturally the task of isolating a specific protein hormone from even large numbers of rat pituitaries would be a difficult procedure, however, Pettinga and Rice (1952) reported obtaining milligram quantities of labeled insulin by incubating S 35 methionine with large amounts of foetal beef pancreas.

In view of these reports, the possibility of utilizing large amounts of beef pituitary slices for the biosynthesis of anterior pituitary hormones was considered. Of the anterior pituitary hormones, prolactin appeared to be ideally suited as the hormone to be studied. It contains 4-5% methionine and can be isolated in relatively pure form. A standardized commercial preparation was available in quantity for comparative study. A survey of the extraction methods indicated that a yield of 1-2 milligrams of prolactin could be obtained from 1 gram of pituitary tissue using the method of Schwenk et al. (1943). In addition, as will be pointed out, prolactin preparations can be readily characterized by filter paper
electrophoresis studies.

**Description of the Problem**

Since no other work has been reported on the use of surviving beef pituitary slices, it was first necessary to establish a practical method of harvesting this tissue so that a minimum of damage to the tissue would occur between the time of the removal from the animal and the incubation. A preliminary series of manometric experiments were done to demonstrate survival of the pituitary slices. These studies were followed by incubations of large amounts of pituitary tissue with S 35 methionine so that appreciable amounts of prolactin could be extracted for identification and assay of incorporated radioactivity. Lastly, a series of incubations were done over varying time intervals to demonstrate the relationship of time to the rate of incorporation of methionine into the prolactin.

The criteria for the identification of anterior pituitary protein hormones by any physical or chemical procedures have actually never been proposed, however, these methods can be used to characterize the biologically active material extracted. If the preparation studied is shown to be biologically active and is prepared in pure form, studies such as ultracentrifugation or electrophoresis can be used to demonstrate the homogeneity of such preparations. The filter paper electrophoresis method described by Kunkel and Tiselius (1950) is a relatively simple and inexpensive technique to characterize protein solutions. This technique and its modifications have been successfully applied to the separation and identification of serum proteins by numerous workers (Durrum, 1950; Cremer and Tiselius, 1950; Kunkel and Tiselius, 1950). Several models of the apparatus are available commercially since it has become an essential laboratory
tool for the study of proteins.
MATERIALS AND METHODS

Preparation of Tissue

Arrangements were made with a local packing house to obtain fresh pituitaries during the slaughtering of cattle. The time required to obtain the pituitary gland after killing the cattle was determined with a stop watch, and found to vary from 15 minutes to 35 minutes. The glands were dissected free of the surrounding tissue, placed in a container surrounded by cracked ice and transported to the laboratory. The maximum total time elapsing between the collection of the pituitaries and the slicing was never over two hours.

Immediately on arrival in the laboratory the glands were sliced manually with a Stadie slicer blade. The thickness of the slices was approximately 0.3 millimeters. The slices were immediately immersed in oxygenated cold Krebs-Ringer solution where they remained until the desired weight of tissue was obtained. They were then rinsed in fresh medium, blotted on filter paper, weighed and transferred to the incubation mixture.

Incubation Mixture

The incubation mixture used in all experiments was identical except for the substitution of radioactive methionine for the stable methionine in the incorporation studies. The base medium used was oxygenated Krebs-Ringer bicarbonate solution at pH 7.4 containing $8 \times 10^{-5}$ M sodium succinate and $0.25 \times 10^{-3}$ M methionine. This medium is similar to that described by Melchior and Halikis (1952). They found that the use of succinate as an energy source instead of glucose resulted in higher protein
labeling. The S \textsuperscript{35} methionine used in this work was obtained from D.L. Tabern of the Abbott Laboratories. The specific activity of the methionine was said to be 14 microcuries per milligram.

**Survival Study**

Survival of the tissue slices over the desired time period (2 hours) was determined by the oxygen consumption, using manometric techniques.

Approximately 200 milligrams (1 or 2 slices) of pituitary tissue was placed in each of 4 Warburg vessels containing 3 milliliters of the non-radioactive incubation medium. Two similar vessels were prepared with boiled slices to serve as controls. The vessels were placed in a water bath at 38 degrees Centigrade and filled with a mixture of 95% oxygen and 5% carbon dioxide. They were shaken at a constant rate and manometer readings were taken at 15 minute intervals for 2 hours. The oxygen consumption was calculated per 100 milligrams of wet tissue for the 2 hours.

**Incorporation Study**

In order to isolate a sufficient quantity of prolactin for identification, an adequate amount of pituitary tissue was sliced to obtain a wet weight of approximately 20 grams. The tissue was added to 80 milliliters of incubation mixture containing the radioactive methionine. This was incubated in a 500 milliliter Ehrlemeyer flask immersed in a water bath at 38 degrees centigrade and shaken at 2 strokes per second. A continuous supply of oxygen was provided by directing a stream of warm oxygen over the surface of the medium. At the end of 2 hours the flask was removed from the water bath, chilled to five degrees centigrade and extracted for prolactin by the method of Schwenk et al. (1943). Using this method
they reported the extraction of prolactin with an activity of 30 international units per milligram, however, no physical-chemical data concerning the purity of their preparation was presented.

The method consists briefly of homogenating the pituitary slices with the medium in a Waring Blender, adding \( \frac{3}{4} \) volume of chloroform, adjusting to pH 5.6, and then centrifuging at 2000 rpm. Three distinct layers are formed. The lower layer consists of chloroform containing most of the lipoidal substances. The layer above the chloroform is a gel consisting of the bulk of the tissue proteins together with prolactin and ACTH. The top layer is a clear aqueous solution containing the gonadotropic hormones, thyrotropin, posterior lobe hormones and other easily soluble substances. The remainder of the extraction procedure for the prolactin is confined to the chloroform gel layer. Repeated extractions with absolute methanol and subsequent salt fractionations of the extracts results in a purified prolactin preparation. The entire extraction procedures are carried out in a cold room (at 5 degrees Centigrade). The final prolactin preparation is washed with cold ether, thoroughly dried in a desiccator, and weighed.

All the preparations were dialyzed against 20 liters of distilled water at 5 degrees Centigrade to determine whether any of the radioactivity could be removed by this procedure. A portion of the labeled prolactin prepared as a 2% solution in distilled water, was used for the identification and the determination of radioactivity. The remainder was saved for the other studies.

A control study was done to determine the possibility of absorption or inclusion of radioactive methionine as the result of the extraction procedure. This type of control is referred to as the "zero time control"
by Melchior and Halikis (1952).

To determine the effect of time on the incorporation of radioactivity into the prolactin, 4 flasks containing similar amounts of pituitary tissue and radioactivity were prepared. The incubation was halted at 30 minutes, 1 hour, 2 hours, and 3 hours respectively by the initiation of the extraction procedure as outlined above.

**Biological Assay**

Due to the limited amount of labeled prolactin obtained in these studies it was only possible to attempt a qualitative bioassay. For this purpose the minimal stimulation method of McShan and Turner (193c) was used. This method is based on the observation that prolactin will produce a definitive thickening of the mucosa of the crop gland of pigeons and is considered a *subjective* assay. The solution tested is injected into the pectoral muscles daily for 5 days.

Both the standard preparation and the labeled preparations were tested at two different dose levels (0.1 milligrams per day and 0.25 milligrams per day). A total of 15 White Carneau pigeons weighing approximately 450 grams each were used for the assay. Three birds were injected with the standard preparation at each dose level, three with the labeled preparation at each dose level, and three birds were left uninjected as controls. The pigeons were killed 24 hours after the last injection. The crop sacs were removed, dissected free of surrounding tissue and examined visually by transmitted light for thickening of the mucosa in the crop gland area.

Arrangements were made with Dr. Robert Bates of the National Institutes of Health to have quantitative bioassay determinations made on
three samples of the labeled prolactin.

Identification of Prolactin

1. Electrophoresis

The electrophoresis paper strip method of Kunkel and Tiselius (1950) was used to characterize the preparations. A standard preparation of purified sheep prolactin from the Squibb Laboratories (lot #71713), said to contain 25-30 international units per milligram of protein, was used as a reference control for all of the electrophoresis studies. A 2% solution of this material was prepared in distilled water. It was found that a 0.1 M veronal buffer of pH 8.8 was the ideal electrolyte to use in the electrophoretic studies in order to obtain the greatest separation between the various components present. Bromphenol blue dye was used to stain the paper strips.

The electrophoretic method on filter paper utilizes a wide strip of Whatman #1 filter paper (14 cm. x 55 cm.) held between two glass plates with the two ends of the paper dipping into dishes containing the buffer solution and electrodes. The filter paper is soaked in the same buffer solution and the excess buffer squeezed out by the use of a rubber coated roller. From 5 to 20 microliters of protein solution is applied two centimeters from the center of the paper strip toward the negative electrode. By spacing the application of the solutions every 2 centimeters across the width of the paper as many as 5 determinations can be run simultaneously for comparative studies. Usually a voltage of 270 to 300 volts D.C. is applied to the paper (8 volts/cm. length of paper between the points of contact with the buffer in the electrode vessels) resulting in a cur-
rent of 6-7 milliamperes (0.5 m.a./cm. width of filter paper). At the end of a given time period (14-20 hours), the current is turned off and the filter paper strip removed and dried in an oven at 100 degrees Centigrade. The strip is then stained for 5 minutes in 0.05% bromphenol blue dye containing 1% mercurous chloride and 2% acetic acid. The excess dye is removed by rinsing in acid water and the strip is dried again in an oven. Since the bromphenol blue dye is yellow at an acid pH, ammonia vapor is introduced into the oven during the drying to change the color to a deep blue.

Direct densitometer readings of the developed filter paper strips were made with the aid of a modified Klett Summerson photometer. Readings were made for every 0.5 cm. of the strip. The density of the dye was plotted on a graph to further characterize the preparation.

2. Determination of Radioactivity

All radioactive determinations were made with a Windowless Geiger Flow Counter. Twenty microliter samples of the diluted standards and the dissolved prolactin were plated on stainless steel planchets. Self absorption corrections were made when necessary. All samples were counted for 5000 counts, giving an error of plus or minus 2.25%. Background corrections were made on all determinations. The radioactivity of the prolactin preparation is given in counts per minute per milligram. In addition, the values are calculated to represent the number of micromoles of methionine incorporated into 1 micromole of prolactin (30 mgms.), assuming an average molecular weight of approximately 30,000 for the prolactin (White, 1949).

Radioautography was also used to demonstrate the position of the ra-
radioactive prolactin on the electrophoresis strip. No screen X-ray film was placed in intimate contact with the undyed strip, usually for a period of two weeks. Later the film was developed and the paper strip dyed to determine the position of the protein.

In addition, strips were cut up into 1 centimeter squares after densitometer readings were made and placed in planchets. These samples were counted so that the readings indicating the position of the protein could be directly correlated with the radioactivity on the paper. Considerable self absorption of the S 35 would occur with this method, but it was only used to demonstrate the relation of radioactivity to stained protein on the strip. These results were also graphed.

3. Amino Acid Analysis - Chromatography

In order to show that the radioactivity present in the prolactin was incorporated into the protein, acid hydrolysis of the labeled preparation was done. This hydrolysis was identical to that previously described for the parathyroid extract except that the hydrolysis reagent was 12% formic acid in concentrated hydrochloric acid. This method, described by Miller and duVigneaud (1937), is recommended for studies on sulfur amino acid composition of protein. The hydrolysate was subjected to one-dimensional ascending chromatography in water-saturated phenol to identify the radioactive methionine. A control with radioactive methionine alone was done to determine the position of the methionine.

Two identical samples of the hydrolysate, plus the control, were run simultaneously on the same paper. Instead of spraying the whole sheet with ninhydrin to determine the positions of the amino acids, the paper was cut horizontally into 3 strips so that each strip could be treated in
a different manner. Of the 2 strips containing the hydrolysate of the prolactin, one strip was sprayed with ninhydrin. Attempts were made to determine the radioactivity of the second strip by cutting it into equal segments for direct counting. This proved impractical because of the large amount of self absorption encountered. However, elution of these same segments of paper with boiling water, and subsequent plating and drying of the solutions, provided a better method of counting. The third strip was sprayed with ninhydrin for comparison.

An additional sample of hydrolysate was subjected to two-dimensional chromatography to determine the presence of amino acids.

RESULTS

Survival Study

The data obtained with the manometric studies on the oxygen consumption of the fresh and boiled pituitary slices are shown in Table 1a. As can be seen from the data, there was a continuous utilization of oxygen over the 2 hours by the fresh slices while no respiration was shown by the boiled tissue.

Incorporation Study

Table 2a shows the results obtained with the "zero time" control and two separate two hour incubations (#1 and #2). As can be seen from these data, the surviving pituitary tissue incorporated approximately 10 times more radioactivity than is obtained with the "zero time control".

The incorporation of the S 35 methionine into the protein increased with time, as can be seen in the same table. However, it should be noted that the total amount of prolactin extracted in these experiments decreased
Table 1a

RESPIRATION OF FRESH AND BOILED BEEF PITUITARY SLICES IN KREBS RINGER BICARBONATE SOLUTION, pH 7.4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fresh Tissue</th>
<th>Boiled Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet Weight-mgs.</td>
<td>A 211</td>
<td>D 199</td>
</tr>
<tr>
<td></td>
<td>B 273</td>
<td>E 252</td>
</tr>
<tr>
<td></td>
<td>C 237</td>
<td>F 213</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Manometer Readings</th>
<th>Control Manometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A 282</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 246</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 259</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D 298</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E 264</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 242</td>
<td></td>
</tr>
<tr>
<td>15 min.</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>30 min.</td>
<td></td>
<td>159</td>
</tr>
<tr>
<td>45 min.</td>
<td></td>
<td>156</td>
</tr>
<tr>
<td>60 min.</td>
<td></td>
<td>161</td>
</tr>
<tr>
<td>75 min.</td>
<td></td>
<td>161</td>
</tr>
<tr>
<td>90 min.</td>
<td></td>
<td>161</td>
</tr>
<tr>
<td>105 min.</td>
<td></td>
<td>161</td>
</tr>
<tr>
<td>120 min.</td>
<td></td>
<td>161</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML.02</td>
<td>174 215 195 153</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Corrected

| ML.02 | 209 255 234 180 |
|       |               |

ML.02/100 mgs.

Wet tissue/2hrs. 99 93 99 91
Table 2a

METHOD OF CALCULATION

Specific Activity of methionine = 14 Mc/mg.

One $\mu$M of methionine = 0.149 mg.

Specific activity $\mu$M methionine = 2.08 $\mu$c/$\mu$M.

Counting rate of methionine standard = 16,000 cts/min for .001 mg.

Calculated cts/min/$\mu$M methionine = 2.38 x 103

One uM of prolactin = 30 mg.
### Table 2a

RADIOACTIVITY OF ISOLATED PROLACTIN PREPARATIONS

<table>
<thead>
<tr>
<th>Preparation and Time</th>
<th>Mgs. Protein Isolated</th>
<th>Mgs. Protein per sample</th>
<th>Cts./min. per sample</th>
<th>Cts./min. MM. methionine per mg. prolactin</th>
<th>MM. methionine per mole prolactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>21</td>
<td>0.4</td>
<td>52.8</td>
<td>132</td>
<td>0.0016</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>49.6</td>
<td>124</td>
<td>0.0016</td>
</tr>
<tr>
<td>A-2hour</td>
<td>18</td>
<td>&quot;</td>
<td>512.0</td>
<td>1280</td>
<td>0.0162</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>508.4</td>
<td>1271</td>
<td>0.0161</td>
</tr>
<tr>
<td>B-2hour</td>
<td>26</td>
<td>&quot;</td>
<td>568.0</td>
<td>1420</td>
<td>0.0179</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>579.2</td>
<td>1448</td>
<td>0.0182</td>
</tr>
</tbody>
</table>

EFFECT OF DIFFERENT INCUBATION TIMES

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Mgs. Protein Isolated</th>
<th>Mgs. Protein per sample</th>
<th>Cts./min. per sample</th>
<th>Cts./min. MM. methionine per mg. prolactin</th>
<th>MM. methionine per mole prolactin</th>
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</thead>
<tbody>
<tr>
<td>C-30 min.</td>
<td>20</td>
<td>0.4</td>
<td>187</td>
<td>468</td>
<td>.0059</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>181</td>
<td>453</td>
<td>.0057</td>
</tr>
<tr>
<td>D-1 hour</td>
<td>19</td>
<td>&quot;</td>
<td>284</td>
<td>711</td>
<td>.0089</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>290</td>
<td>739</td>
<td>.0091</td>
</tr>
<tr>
<td>E-2 hours</td>
<td>11</td>
<td>&quot;</td>
<td>520</td>
<td>1230</td>
<td>.0155</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>500</td>
<td>1251</td>
<td>.0162</td>
</tr>
<tr>
<td>F-3 hours</td>
<td>6</td>
<td>&quot;</td>
<td>1830</td>
<td>4568</td>
<td>.0575</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>1835</td>
<td>4583</td>
<td>.0580</td>
</tr>
</tbody>
</table>
with time. It is difficult to determine whether this decrease in yield was the result of losses occurring during the fractionation.

**Bioassay of Labeled Preparation**

At a dose of 0.25 milligrams a day, a striking increase in the thickness of the crop gland mucosa, compared to the controls, was seen in all of the birds receiving the standard and in 2 of the 3 birds receiving the labeled preparation. No response was obtained in the crop sacs of the pigeons injected intramuscularly with the .01 milligrams a day of the standard preparation or with the isolated labeled preparation.

The quantitative bioassay of the labeled preparation was not completed at the time of this writing, however, an addendum will be made to the thesis when these assays are reported.

**Identification of Prolactin**

1. Electrophoresis

The prolactin preparations proved particularly well suited for studies with filter paper electrophoresis. Both the commercial and the isolated labeled preparations had a migration rate toward the positive electrode of about 7 millimeters per hour under the conditions described. Rather than using precise mobility determinations to characterize the proteins, each preparation was run simultaneously with a known standard preparation of Squibb prolactin (lot #71713). By using this technique, factors such as changes in temperature and buffer concentration, due to evaporation, would affect the standard as well as the unknown.

The top half of Plate Ia is a reproduction of a filter paper strip. The preparation at position 1 is 5 microliters of a 2% solution of the
PLATE Ia

PAPER ELECTROPHORESIS OF PROLACTIN PREPARATIONS AND RADIOAUTOGRAph
COMPARISON OF BROMPHENOL BLUE DENSITY OF FILTER PAPER ELECTROPHORESIS STRIP OF STANDARD PROLACTIN AND LABELED PROLACTIN

The graph shows the results of paper electrophoresis analysis of standard prolactin (Squibb #71713) and labeled prolactin (preparation A). The bromphenol blue density was determined every 0.5 centimeter.

Experimental Conditions:
Quantity of protein - 5 µl. of a 2% solution (0.1 mg.)
Buffer - 0.1M veronal, pH 8.8
Voltage - 3 volts/centimeter length
Current - 0.5 milliamps/centimeter width
Duration - 14 hours
PLATE IIa

X---X DENSITY OF SQUIBB PROLACTIN (# 71713)
●---● DENSITY OF LABELED PROLACTIN (preparation A)

DENSITY OF BROMPHENOL BLUE DYE

350
300
250
200
150
100
50

DISTANCE TRAVELLED IN CENTIMETERS

POINT OF ORIGIN
standard Squibb prolactin. Three separate components could be seen on the original strip. Positions 2, 2, and 4 represent three different amounts, 5, 10, and 20 microliters respectively, of a 2% solution of the preparation of labeled prolactin. Different amounts of labeled protein were used in order to obtain sufficient radioactivity for a radioautograph, and also to try to reveal the presence of the same components that occur in the standard preparation. The 5 microliter sample resulted in a single component showing slight tailing toward the positive electrode and is also a small amount of contaminant protein evident at the point of origin. With increasing amounts of the labeled preparation, the presence of the contaminant becomes more evident, and it is possible to visualize at least 2 components in the major portion of the protein that moved. It should be emphasized that, in addition to the small amount of contaminant present at the point of origin, only one component is evident in the labeled prolactin when small amounts (5 microliters) are tested. Attempts to use large amounts of either the standard prolactin or the labeled preparation in these studies were impractical because of the suitable densitometer readings could not be made of these strips when the staining was too dark.

Plate IIa shows the result of densitometer readings for the standard preparation and the radioactive preparation. This illustrates in a more graphic way the similarity of the two preparations in respect to their mobility. In addition, disregarding the contaminant in the labeled preparation, it appears that the labeled prolactin is a relatively more homogeneous protein than the standard prolactin. All labeled preparations were shown to be identical when subjected to electrophoresis, including those extracted at different time intervals. (Plate IIIa).
PLATE IIIa

COMPARISON OF FOUR LABELED PROLACTIN PREPARATIONS WITH SQUIBB PROLACTIN

- 1. 0.5 M HCl, 0.5/200
- 0.5 M urea, 0.5 ml/cm
- 30 minutes

5 solutions

- 2. 1 hour
- SQUIBB PROLACTIN

- 3. 2 hours

- 4. 0.5 hours

POINT OF ORIGIN
The bottom half of Plate Ia is a reproduction of an X-ray film exposed to the above filter paper strip. As expected, the standard preparation produced no darkening of the film, since it was not radioactive. One can see that the preparations at positions 2, 3, and 4 produced discernible darkening at the same location as that of the major protein component.

Plate IVa is a graphic representation of similar findings. Sufficient radioactivity was present on the filter paper strip so that direct counting could be done.

2. Amino Acid Analysis - Chromatography

Plate Va is a photograph of a one-dimensional chromatogram showing the separation of the amino acids obtained in the radioactive prolactin hydrolysate. The position of methionine can be assumed from numerous studies reported by other workers (Jones, 1952). However, control studies with both stable and radioactive methionine designated the precise area for comparison. Since no other sulfur amino acid contained in protein hydrolysates would appear in the same position, it is a valid assumption that any radioactivity at this location must represent methionine. The counts per minute, obtained by elution of segments of the strip, are shown along the strip. It can be seen that the majority of the detectable radioactivity is associated with the methionine. An appreciable amount of radioactivity is present in an area probably occupied by cystine or some of its oxidation products. Studies of a more quantitative nature concerning the exact amount of radioactivity incorporated as methionine in the prolactin preparation were not undertaken.

The amino acids in the labeled prolactin preparation were identified
qualitatively by their color and position on a two-dimensional chromatogram sprayed with ninhydrin. The following amino acids were identified: arginine, aspartic, cysteic?, glutamic, glycine, isoleucine?, leucine?, lysine, methionine, phenylalanine?, proline, serine, threonine, tyrosine, valine, and alanine. Except for the presence of alanine and the absence of histidine and tryptophane, these results would agree with other reports (White, 1949). The absence of tryptophane can be explained by its destruction in acid hydrolysis. The presence of alanine and the absence of histidine in the isolated labeled prolactin does not agree with the reported amino acids in purified prolactin. Purified preparations do not contain any alanine, but do contain histidine.

It is possible that the alanine, along with other amino acids, comes from the small amount of contaminant protein seen in the labeled preparation. If histidine were present, it is possible that it may not have been detected by this method.

The preceding studies were repeated on all of the prolactin preparations isolated and the results were in agreement with those presented.
PLATE IVa

COMPARISON OF RADIOACTIVITY TO BROMPHENOL BLUE DENSITY OF FILTER PAPER ELECTROPHORESIS STRIP OF ISOLATED PROLACTIN

The graph shows the results of paper electrophoresis analysis of labeled prolactin (preparation A). The bromphenol blue density was determined every 0.5 centimeters, and radioactivity determinations were made with 2 centimeter segments of the strip.

Experimental Conditions:
Quantity of protein - 5 µl. of a 2% solution (0.1 mg.)
Buffer - 0.1 M veronal, pH 8.8
Voltage - 3 volts/centimeter length
Current - 0.5 milliamps/centimeter width
Duration - 14 hours
PLATE IVa

> DENSITY OF LABELED PROLACTIN (preparation A)

> RADIOACTIVITY

DENSITY OF BIOPHOREL BLUE DYE

RADIOACTIVITY IN COUNTS/60 MINUS BACKGROUND

POINT OF ORIGIN

DISTANCE TRAVELLED IN CENTIMETERS

POSITIVE
PLATE Va

FILTER PAPER CHROMATOGRAM OF PROLACTIN HYDROLYSATE

Point of application →
DISCUSSION

It can be stated that strong evidence has been presented to show that surviving beef pituitary slices can incorporate radioactive methionine into a protein which possesses several of the properties of known prolactin preparations. One can question whether, under the conditions of these experiments, actual biosynthesis of prolactin occurred. Several alternatives must be considered as possible mechanisms of incorporation before stating categorically that biosynthesis of radioactive prolactin has been achieved.

It is generally recognized that methionine can be converted indirectly to cysteine metabolically (du Vigneaud, 1952). Whether pituitary slices are capable of producing this transformation has not been established. The sulfhydryl groups of the cysteine can be oxidized to give disulfide bonds. The coupling of two cysteine molecules in this manner to form cystine occurs very readily. The cysteine can also react with any free sulfhydryl groups of a protein to form a similar disulfide linkage between the cysteine and the protein. This type of reaction could be responsible for the appearance of radioactivity in a protein obtained from tissue slices incubated with S 35 methionine, but would not represent true metabolic incorporation. Prolactin does not contain any free sulfhydryl groups but does contain cystine (White, 1949). It is unlikely that the incorporation of radioactivity into this preparation was due to the formation of disulfide linkages as most of the radioactivity in the prolactin could be shown to be present as methionine.

It is of course possible that this radioactive prolactin preparation
is contaminated with other protein components showing a similar electrophoretic pattern, since any methionine containing protein in the pituitary probably incorporates the S 35 methionine. The fact that biological activity could be demonstrated in this preparation indicates that some if not all of the protein in this preparation represents biologically active prolactin. Although no studies have been reported on the production of pituitary hormones by surviving tissue slices of this organ, it is assumed that the pituitary is the site of synthesis of these hormones. Proof that this synthesis actually occurs in the pituitary is of an indirect nature. Observations that anterior pituitary homo-transplants in hypophysectomized animals in regions remote from the central nervous system can act as partial replacement therapy in hypophysectomized animals, have been interpreted as evidence that the pituitary continues to manufacture hormones in spite of the absence of a direct connection with the brain (Cheng et al., 1949). On the other hand, studies on the production of hormones by explants of pituitary tissue in tissue culture, reported by Anderson and Haymaker, (1935) did not reveal any new secretion of gonadotrophic, thyrotropic, or adrenocorticotropic hormone. No studies have been reported on the elaboration of prolactin by tissue culture of the pituitary. It would be difficult to determine whether any new production of hormones occurred in tissue cultures. If one assumes that there is an equilibrium between the synthesis and breakdown of a protein, then one could only detect an increase or decrease in the rate of protein synthesis rather than any new synthesis by any method used to measure the amount of hormone present in tissue cultures. The
use of a radioactive amino acid should enable one to follow the synthesis of a protein in tissue cultures and presumably tissue slices.

A certain amount of enzymatic hydrolysis of protein is bound to occur with tissue slice studies. The mechanical damage to the cells of the cut surface of the slice may result in the cytolysis of these cells with the subsequent release of proteolytic enzymes. If the hydrolysis of protein does indeed occur under these conditions, incorporation of an amino acid could result from a simple equilibrium established between the protein and its amino acids since enzymatic reactions are theoretically reversible. Bates (personal communication) has expressed doubt as to the possibility that the prolactin initially present in the pituitary would withstand the incubation period of two hours since prolactin like many other pituitary protein hormones is destroyed enzymatic hydrolysis in pituitary homogenates at room temperatures. It should be emphasized, however, that these studies were done with pituitary slices rather than tissue homogenates. If destruction of prolactin occurred as the result of enzymatic hydrolysis, incorporation could be explained on the basis of the equilibrium discussed. The demonstration that the incorporation of radioactivity into the prolactin preparation increased with time is evidence that the incorporation was associated with some active biochemical process. Regardless of the mechanism or mechanisms of incorporation these studies do demonstrate that a radioactive labeled prolactin preparation can be prepared biosynthetically.
**ADDENDUM**

(See page 49)

Bioassay of Prolactin reported by R.W. Bates. May 28, 1954

<table>
<thead>
<tr>
<th>Total Dose</th>
<th>C. G. wt.</th>
<th>Equiv. Units</th>
<th>u/mg*</th>
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</thead>
<tbody>
<tr>
<td>3-S-35 (#1)</td>
<td>1.7 mg.</td>
<td>2.96±0.36</td>
<td>12</td>
</tr>
<tr>
<td>4-S-35 (#2)</td>
<td>3.0 mg.</td>
<td>3.25±0.27</td>
<td>24.4</td>
</tr>
<tr>
<td>71713 Std</td>
<td>1.0 mg.</td>
<td>2.94±0.14</td>
<td>13</td>
</tr>
</tbody>
</table>

*71713 was taken as 30 u/mg. and the other fractions corrected by multiplying by 30.*
SUMMARY

1. On the basis of the studies presented here, it would appear that a radioactive protein preparation can be obtained from surviving beef pituitary slices incubated with S 35 methionine by using an extraction method that is presumably specific for prolactin.

2. The incorporation of the S 35 methionine into this protein increased with time indicating that this incorporation was an active process.

3. Tentative evidence has been presented that this preparation has some biological activity known to be present in prolactin.

4. Filter paper electrophoresis revealed that this preparation consisted of a major protein component which was similar to that of a standard purified preparation of prolactin.

5. Most of the radioactivity present in the protein was shown to be in the form of methionine while the remainder was presumably in cystine or its oxidation products.
GENERAL SUMMARY OF THESIS

STUDIES WITH CHEMICALLY TAGGED RADIOACTIVE PROTEIN HORMONES

Parathyroid extract was tagged with I 131 without apparent loss of biological activity. Distribution studies in normal and parathyroidectomized rats, after intravenous administration, were done at time intervals up to 3 hours. No difference between the two groups was revealed.

ACTH, prolactin, insulin and bovine serum albumin, all tagged with I 131, were incubated with rat adrenal slices in an attempt to determine whether selective "binding" of the ACTH with the adrenal occurred. No preferential binding of the I 131 tagged ACTH could be determined.

A preparation of prolactin was tagged with trivalent chromium 51. Distribution studies in female rats showed that the adrenal and ovary apparently localized radioactivity after intravenous injection of the chromium 51 prolactin. Paper electrophoresis revealed that the radioactivity of the preparation was not associated with the major protein component. Interpretations of these studies, in view of other studies reported with I 131 prolactin, are discussed.

Distribution studies in animals with both I 131 parathyroid extract and chromium 51 prolactin indicated that the uptake of radioactivity in the adrenal, in studies reported with I 131 ACTH, cannot be considered specific by merely measuring the radioactivity in that tissue.

THE BIOSYNTHESIS OF RADIOACTIVE LABELED PROLACTIN

Because of the difficulties encountered in interpreting the results
obtained with radioactive chemically tagged protein hormones, studies were undertaken to demonstrate that a pituitary protein hormone preparation, prolactin, could be labeled biosynthetically by surviving beef pituitary slices incubated with a medium containing S 35 methionine. A quantitative bioassay of the isolated radioactive prolactin has not been completed, however, paper electrophoresis studies indicate that it is similar to a commercial preparation. The various interpretations of these studies are discussed.
BIBLIOGRAPHY


