1960

Studies on Human and Rat Plasma Parathyroid Hormone Activity

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STUDIES ON HUMAN AND RAT PLASMA PARATHYROID HORMONE ACTIVITY

by

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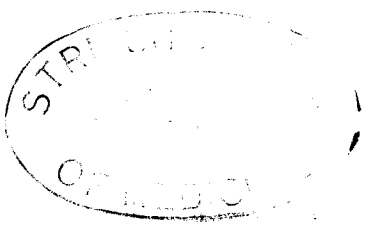
A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy.

February, 1960



LIFE

Leo Edmund Reichert, Jr., was born in New York City, New York, on January 9, 1932. He received his high school diploma from De La Salle Institute, New York City, New York, in June, 1949.

From August, 1950, to March, 1952, he served on active duty with the United States Marine Corps. He graduated from Manhattan College, New York City, New York, in June, 1955, with the degree of Bachelor of Science in Chemistry. During his senior year at Manhattan College, he received the Sinclair, Valentine and Company award in chemistry.

In September, 1955, he began graduate work in the Department of Biochemistry of Loyola University at the Stritch School of Medicine. From that time until June, 1957, he held the position of Teaching Assistant in the Department of Biochemistry. In July, 1957, he was awarded a Standard Oil Foundation Fellowship for one year, and in February of 1958 he received the degree of Master of Science in Biochemistry. In June, 1958, he was appointed a Postgraduate Fellow in the Department of Biochemistry and was elected an associate member of the Loyola University Chapter of the Society of the Sigma Xi. He has completed his graduate program under the terms of a National Institute of Health terminal year pre-doctoral research fellowship which was awarded in December, 1958.

He is co-author of the paper "In Vitro Effect of Parathyroid Gland Extract Upon the Ultra-Violet Absorption of Reduced Coenzyme II" which he presented before the Chicago meeting of the American Chemical Society in September, 1958.

He is also co-author of the following publication: "In Vitro Effect of Parathyroid Hormone on TPNH" J. of Endocrinology, in press.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. Maurice V. L'Heureux for his patience and encouragement throughout the course of this investigation.

He also wishes to thank Dr. Hugh J. McDonald for his generous cooperation in making available funds for the purchase of the necessary experimental materials, without which this dissertation could not have been undertaken.

Further, the author wishes to express his appreciation to Dr. Martin B. Williamson for making available to him the lyophilizer utilized so often in this work, and to Dr. Norten C. Melchior for giving so freely of his time, help and advice whenever it was requested.

Thanks are due to Dr. John R. Tobin, Director, Cook County Blood Bank, Cook County Hospital, Chicago, Illinois, for making available some of the human plasma utilized in this study.

Indebtedness is also acknowledged to Mr. J. E. Brackett and Dr. Glenn W. Irwin of Eli Lilly and Company, Indianapolis, Indiana, for the generous supply of some of the Injection Parathyroid, USP, used in this work.

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CHAPTER I

INTRODUCTION AND STATEMENT OF THE PROBLEM

"A Hormone is not something that occurs in the urine or something that is knocked up by a chemist in his laboratory; a hormone is something that goes round in the blood to act in another part of the body" Dr. A. S. Parkes, Chairman, Ciba Foundation Colloquia on Endocrinology, "Hormones in Blood" February 12, 1957.

This statement and the existence of the Colloquia cited above, reflect the growing awareness of medical scientists in the fundamental importance of studies which are concerned with elucidating the nature of hormones as they exist in their true physiological environment, the blood. Dr. Parkes' theme "Back to the Blood" has indeed been one of the inspirations of this work.

The difficulties encountered in investigations dealing with hormones in the blood are rather formidable. They have been amply described in two reviews (Wolstenholme, 1953) (Wolstenholme and Millar, 1957), and their relationship to this dissertation will be discussed in detail in a subsequent chapter. Nevertheless, the stimulating and exciting observations recorded by those who were willing to enter this field have proven well worth the time and effort required to overcome whatever obstacles that may have rise in their path. Information has

been obtained concerning the status of almost all of the protein peptide, amino acid and steroid hormones in the blood with one notable and regretable exception. At the time this investigation was initiated there was, to the author's knowledge, absolutely no information available concerning the characteristics of the parathyroid hormone as it existed in the circulation.

It was the purpose of this dissertation, then, to bridge this gap in our knowledge of plasma hormone activity.

CHAPTER II

THE PARATHYROID HORMONE

Description of the Parathyroid Glands. The parathyroid glands, which are the source of the parathyroid hormone, develop from the entoderm of the branchial clefts (the primordial gills) and are found as small paired bodies in the region of the thyroid gland. The number and position of these glands varies widely even between individuals of the same species. From one to four pairs (usually two) are seen. One pair, the superior or internal parathyroids may be imbedded entirely in the thyroid gland. Others, the inferiors or external glands, are usually found near the dorsolateral surface of the thyroid, but accessory tissue may develop in widely scattered positions in the neck and upper thoracic region. (Hopkins and Chandler, 1925) The total weight of the parathyroid tissue in man is about 0.1 gram and in the rat about 0.0003 grams. In histological appearance, the parathyroid tissue somewhat resembles hyperplastic thyroid tissue without colloid, a fact which led early investigators to consider the parathyroid to be accessory or embryonic thyroid tissue.

Historical Basis of Current Knowledge. The historical evolution of our current knowledge of parathyroid function may be considered to rest upon four major developments. 1) The discovery by Gley (1897) that the tetanic spasms and convulsions which sometimes followed thyroidectomy were observed to

occur only when the parathyroid glands were also removed; 2) The findings of MacCallum and Voegtlin (1909) that the symptoms of parathyroid insufficiency can be prevented and alleviated by the administration of calcium salts, as well as by the injection of crude extracts of parathyroid glands; 3) The work of Collip (1925) who succeeded in preparing a sterile, reasonably stable, physiologically active extract of the parathyroid glands which could be used for experimental purposes, and whose significant report definitely established the endocrine nature of parathyroid function; 4) The report of Rose and Wood (1942) which conclusively established the active principle of parathyroid gland extracts to be protein in nature.

Clinical and Experimental Basis of Current Knowledge. Fundamentally, all our knowledge concerning the physiological role of the parathyroid hormone can be considered to have developed from a relatively few carefully documented observations. The first of these is parathyroidectomy, experimental or inadvertently produced by surgery in man. Another is spontaneous hyperparathyroidism, either adenoma or hyperplasia, the two being indistinguishable clinically (Howard, 1956). In accepting data derived from spontaneous hyperparathyroidism for use in defining normal parathyroid function, one must, of course, make the assumption that the parathyroid adenoma secretes an excess of hormone produced by the normal glands. It would seem likely

that this is so, since the biochemical changes in hyperparathyroidism are strikingly the converse of those found after parathyroidectomy. The final basic source of information stems from experiments involving use of glandular extracts. Although recently there have been reports of the final attainment of a pure parathyroid hormone preparation from bovine glands (Rasmussen, 1957), practically all work reported to date has involved use of cruder hydrochloric acid or acetic acid extracts of glandular tissue. Despite this fact however, there is good reason to believe that such crude extracts contain one or more principles possessing the same physiological activities as does the secretion of normal parathyroid glands.

It has been well documented that in man and in the rat doses of parathyroid extract produce the same symptoms and chemical changes in the serum as those found in spontaneous hyperparathyroidism. Also, in experimental animals administration of large amounts of parathyroid extract result in production of osteitis fibrosa indistinguishable from that which accompanies parathyroid adenoma. Further, the biochemical changes of hypoparathyroidism can be restored to normalcy by appropriate doses of parathyroid extract (Howard, 1956). It seems logical, then, to assume that the parathyroid extracts currently available contain the active principle of the parathyroid gland.

Physiology of the Parathyroid Hormone. Much has been

reported upon the nature, effects, and sites of action of the parathyroid secretions although the physiological mode of action of the active principle(s) is still a point of controversy. Dominating thought for many years was the concept of Albright et. al. (1948) that the parathyroid hormone caused increases in urinary excretion of phosphate as its primary effect. Secondarily, this phosphaturia was believed to cause a hypophosphatemia, which induced dissolution of bone salt, thereby causing increased serum calcium levels. This historic concept has now given way to the idea of a dual action of the parathyroid secretions. Although there is still disagreement, the modern theory holds that the principle and primary effect of the parathyroid hormone is on bone itself, resulting in a transfer of calcium and phosphate from bone to serum. Secondly and independently, there is a decreased reabsorption of phosphate in the kidney tubule leading to phosphaturia and hypophosphatemia. At least three authoritative review have appeared summarizing the evidence for these conclusions. (Bartter, 1954) (Howard, Wolstenholme and O'Connor, 1956) (McLean and Bourne, 1956) The mechanism of the primary effect of the parathyroid hormone on bone is little understood at present, and again, the source of much controversy. The most recent theory in this regard has been forwarded by Neuman and his associates in a series of reports (Neuman et. al., 1956) (Neuman, 1958) (Firschein et. al., 1958) (Martin et. al., 1958)

and summarized in two excellent review (Neuman and Neuman, 1958) (Firschein et. al., 1959). This worker and his associates have suggested that the parathyroid hormone exerts an inhibitory effect upon TPNH-linked reactions, in vivo, with a resulting shunt of glucose metabolism to the production of citrate ion, which is the moeity he feels is responsible for the dissolution of bone salts and the resulting increase in levels of serum calcium observed in hyperparathyroidism and subsequent to administration of parathyroid extracts. This proposed effect of parathyroid hormone upon TPNH linked reactions in vivo, is based upon a reported observation (Neuman, 1956) that commercial parathyroid extract "destroys the chromophoric group of reduced coenzyme II, in vitro." Reichert (1958) and Reichert and L'Heureux (1958) (1959), have shown, however, that this effect of parathyroid extract upon TPNH, in vitro, is not exclusively associated with the hormonal potency of the extract. Neuman's concept, therefore, although having received rather wide acceptance has yet to be experimentally confirmed.

Chemistry of the Parathyroid Hormone. The parathyroid hormone has historically been considered to be protein in nature. Ross and Wood (1942) have amply demonstrated this with their parathyroid hormone preparation on the basis of its inactivation following pepsin digestion and its typical protein ultraviolet absorption spectrum. The voluminous amount of work reported on

the purification of the parathyroid hormone have culminated in the reports of Rasmussen (1957) and Munson (1959) in which they describe the preparation of the most potent parathyroid gland extracts yet realized. Rasmussen (1957) utilized zonal electrophoresis of acetic acid extracts of bovine parathyroid glands powder on polyvinyl chloride to obtain a preparation which sedimented in a single peak upon ultracentrifugation, and migrated as a single zone during electrophoresis. This seemingly homogenous preparation possessed a potency of approximately 200 units/mg. protein when assayed by two different methods, had a calculated molecular weight of about 10,000 and contains 16.4% Nitrogen. Munson (1959) has reported preparation of an even more potent preparation but makes no claims as to homogeneity. This worker's technique consists of extraction of ground frozen bovine parathyroid glands with hot hydrochloric acid, precipitation of the extract with ammonium sulfate according to the method of Friedman and Munson (1958) and column chromatography of the resulting material on carboxymethycellulose followed by gradient elution with sodium chloride. The resulting preparation possessed activities of 380 units/mg. protein, and is the most potent preparation yet reported.

Rasmussen (1958) has demonstrated that the purified parathyroid hormone obtained by Rasmussen (1956) does not contain free sulfhydryl groups. This worker has also shown purified

parathyroid hormone to be inactive after treatment with hydrogen peroxide, and re-activated by subsequent reduction with cysteine. The extent and reversibility of the inactivation is dependent upon pH.(Rasmussen, 1958).

CHAPTER III

HORMONES IN THE BLOOD

A. DIFFICULTIES ENCOUNTERED AND TECHNIQUES UTILIZED IN STUDYING HORMONES IN THE BLOOD.

The methodological problems which arise when one attempts to study plasma hormone activity have been amply described in a recent review (Wolstenholme and Millar, 1957). Let us consider here the more fundamental problems which arise in this work. Gaddum (1953) in referring to the problem of detecting hormonal activity in plasma, has commented "The task is difficult because the amount (of hormone) present is very small, and because accuracy can be achieved only with much labour and at vast expense." Obviously, in order to study plasma hormone activity, we must be able to obtain plasma samples possessing sufficient hormonal potency to be detected by a bioassay system, and we must have an assay system of sufficient sensitivity and reliability for use in the analysis of plasma and plasma concentrates. The former question will be considered at this time. The problem of suitable assay systems will be dealt with in a separate chapter.

Although there are instances where untreated plasma has been found to possess sufficient hormonal potency to elicit responses from biological assay systems, such occurrences are the exception rather than the rule. In almost all cases it has been found necessary to concentrate plasma hormone activity in

one way or another before its characteristics could be studied. There are three basic techniques which are most frequently employed for this purpose.

The first of these involves removal of hormonal potency from plasma by a specific absorbant, as for example, the removal of Insulin activity from plasma by absorbance upon Dowex-50 cationic exhcnage resin (Antoniades et. al., 1958) (Beigelman and Antoniades, 1958) and the concentration of plasma ACTH activity by absorption on oxycellulose (Payne, 1950). The Biological activity of various parathyroid hormone preparations has been shown to be absorbed in varying degrees by oxycellulose (Munson, 1959) (Auerbach, 1959), permutit (Ross and Wood, 1942), and Dowex-50 resin (Rasmussen and Westfall, 1957), and to be extractable to a considerable extent with phenol (Auerbach, 1959) (Rasmussen and Craig, 1959) and benzoic acid (Ross and Wood, 1942), as well as with ammonium sulfate, (Friedman and Munson, 1958). None of these techniques, however, have been applied to the study of plasma parathyroid hormone activity.

The second technique utilized in the preparation of plasma possessing sufficient hormonal potency for detection in assay animals employs intravenous or intraperitoneal administration of exogenous hormone. This technique has yielded much valuable information about plasma hormone activity and has been frequently utilized. An example of this is the work of Bethune et. al. (1958)

in which ACTH was administered intravenously to human subjects and the resulting plasma subjected to chemical fractionation and biological analysis. Significantly, the activity of plasma collected from the human patients who had been injected with exogenous ACTH intravenously was found localized in the same plasma protein fraction as the endogenous ACTH activity obtained from plasma of normal patients. Roberts (1957) has also utilized this technique in studying distribution of rat plasma ACTH activity among plasma protein fractions with considerable success.

The last technique which will be mentioned and the one which has been utilized most frequently and with the greatest success, is chemical fractionation of plasma which permits concentration of hormone activity in plasma protein fractions. A number of fractionation techniques are available for this purpose, such as salt fractionation, fractionation on various types of ion-exchange columns, electrophoretic separations, and others. The technique which has proved most suitable for use in the study of plasma hormone activity, however, has been the cold ethanol fractionation method originally developed by Cohn et. al. (1946) specifically for human plasma, and since modified by a number of workers (Li and Tarver, 1954) (Roberts and Kelley, 1956) for use with rat plasma. This method has the advantage of being designed for low temperatures, thereby reducing the chances of denaturation and inactivation of the biologically active principles being in-

investigated. It also permits use of relatively large quantities of plasma, an important advantage.

Let us now review the successes attained through use of chemical fractionation techniques in detecting and studying the characteristics of a number of hormones in the plasma.

B. APPLICATION OF FRACTIONATION TECHNIQUES TO THE STUDY OF HORMONES IN THE BLOOD.

Since the reports in this area have been so voluminous, it seems most appropriate to examine the information available on the plasma characteristics of the various hormones individually.

Insulin. Most of the work involving plasma insulin activity has been concerned with the problem of detection, localization and estimation of this hormone in normal human plasma. A number of workers have utilized untreated plasma in attempts to detect plasma insulin activity, with widely varied results. (Willebrands and Groen, 1956) (Vallance-Owen and Hurlock, 1954) (Randle, 1954). Weitz and Hagedorn (1954) extracted plasma from normal human subjects with acid-alcohol and assayed the resulting preparation for hormonal activity with some success. Goetz et. al. (1954) demonstrated a marked insulin like activity in a "protein-globulin precipitate," which was a globulin rich fraction precipitated from plasma with zinc. Beigelman et. al. (1956) successfully employed the cold ethanol fractionation techniques of Cohn et. al. (1946) to localize endogenous insulin activity of normal human

plasma in a beta-globulin, gamma-globulin, lipoprotein fraction (Fraction II-III). Antoniades et. al. (1958) and Beigelman and Antoniades (1958) then developed a method for concentration of plasma insulin activity by absorption on Dowex-50 cationic exchange resin. Randle and Taylor (1958) studied the insulin activity of protein fractions of normal human serum prepared by zone electrophoresis on columns of treated cellulose. These preparations were found to contain insulin activity mostly in a beta-gamma globulin fraction. Bolinger et. al. (1959) subjected normal human serum to starch column fractionation and detected insulin activity in the beta-gamma globulin fractions. Randle (1957) studied the distribution of insulin activity in protein fractions prepared from plasma of hypophysectomized rats utilizing the cold ethanol fractionation techniques of Ulrich, Li and Tarver (1954). Activity was found in Fraction II, which contains beta and gamma globulins, and also in Fraction III, which contains alpha globulins.

Growth Hormone. The sustained interest in the role of the pituitary growth hormone in human physiology has resulted in much work being reported on the problem of estimating and localizing growth hormone activity in human and rat plasma protein fractions. Kinsell et. al. (1948) were able to detect growth hormone-like activity by injecting 3-10 mls. of lyophilized plasma from a patient with gigantism into hypophysectomized female rats and

observing the growth of the tibial epiphysis. Greenspan (1950) was unable to show any growth hormone activity in pooled, lyophilized plasma from 54 growing children. Greenspan (1950) then attempted to concentrate growth hormone in plasma through use of chemical fractionation techniques. By adding purified growth hormone to plasma and treating it with methanol at 3°C., he was able to recover hormonal activity in one fraction. Cotes and Young (1951) precipitated human plasma to which growth hormone had been added with 50% ammonium sulfate and recovered the hormone in the lyophilized supernatant liquid. Application of the same technique to human plasma failed to produce signs of activity. Following this, Gemzell et. al. (1955) employing cold ethanol fractionation techniques, added purified growth hormone to human plasma in vitro and succeeded in recovering 25% of the added hormone in 15% of the total plasma protein. The fraction in which activity was found corresponded to Cohn Fractions IV-I and IV-4, which consist primarily of alpha-globulins and beta-globulins respectively. Although Gemzell, utilizing the same technique, was unable to detect hormonal activity in fractions of normal human plasma, he was able to demonstrate activity in fractionated plasma from young pigs and calfs, as well as in fractionated plasma of human retroplacental blood, in plasma from umbilical cord, and in plasma from patients suffering from acromegaly and gigantism.

Thyrotrophic Hormone. Querido and Lameyer (1956) utilized the cold ethanol fractionation techniques of Cohn et. al. (1946) to study localization of thyrotrophic hormone activity in human plasma protein fractions. They reported the interesting observation that in males, endogenous thyrotrophic hormone activity was localized in Cohn Fraction II-III, which consists mostly of gamma and beta globulins, while in females hormonal activity was associated with Cohn Fraction IV-I, which consists mostly of alpha globulins. Roberts (1957), utilizing his modification of the Cohn method, studied the distribution of thyrotrophic hormone activity in plasma protein fractions of the rat, and found hormonal activity to be associated with both the beta globulin and albumin fractions of rat plasma.

Gonadotrophic Hormone. Although a great deal of research has been concerned with the biological determination and clinical significance of pituitary and chorionic gonadotrophins, there have been, until recently, relatively few attempts made to determine the distribution of these hormones in plasma protein fractions.

Reisfeld et. al. (1959) studied the plasma distribution pattern of gonadotrophic hormone in normal pregnant patients and patients suffering from trophoblastic tumors. Zonal electrophoresis and chromatography on anion exchange cellulose columns followed by paper electrophoresis were employed to obtain the plasma

fractions for biological assay. The sera of tumor patients showed hormonal activity in the beta and alpha$_2$ globulin fractions, while the sera from normal pregnant women exhibited gonadotrophic activity in the alpha and beta globulin fractions. These findings suggested to the authors the very interesting possibility that the hormone with gonadotrophic activity in the serum from pregnant women may be structurally different from that found in women from the trophoblastic tumors.

Perhaps a classical example of what can be attained through application of cold ethanol fractionation procedures to the study of plasma hormone characteristics may be seen in the work of McArthur et. al. (1956) and Antoniades et. al. (1957B). McArthur et. al. (1956) first reported a study on the distribution pattern of pituitary gonadotrophic hormone activity in fractions of human postmenopausal plasma. Such plasma was fractionated by the cold ethanol method of Cohn et. al. (1946), and then assayed for hormonal activity. Although traces of activity were found in all fractions, the bulk of hormonal activity was observed associated with Cohn Fraction II-III, which consists mostly of beta and gamma globulins and beta-lipoproteins. Following this report, Antoniades et. al. (1957) prepared large quantities of Fraction II-III protein and then subfractionated this fraction by two different methods. In each case they obtained a highly potent preparation of endogenous plasma pituitary gonadotrophic hormone,

the actual chemical species of the hormone as it appears in its normal physiological environment, the blood.

Plasma Thyroxin. Various chemical fractionation procedures have also been applied to the study of plasma thyroxin and of thyroxin binding protein. Schmid (1953) found the highest content of thyroxin in his Fraction IV of normal human plasma, which is a mixture of alpha globulins. Freinkel et. al. (1955) applied the cold ethanol fractionation method of Cohn et. al. (1946) to human plasma and found the greatest enrichment of thyroxin binding protein to be present in Cohn Fraction IV-4, which consists primarily of alpha and beta globulins.

Adrenocorticotrophic Hormone (ACTH). The techniques employed in the study of plasma ACTH activity illustrate very well some basic approaches to problems of this type.

Human and rat plasma has been injected without processing into assay animals in the hope of detecting endogenous ACTH activity with varying success. Plasma ACTH activity has been concentrated from plasma by the oxyellulose absorption technique of Payne, Raben and Astwood (1950) as modified by Bartholomew (1953). This is an extremely potent and sensitive method for estimating blood ACTH levels. Finally, as has been the case time and time again, cold ethanol fractionation has been utilized to concentrate plasma fractions prior to administration and analysis for hormonal potency.

Bethune et. al. (1958) applied the cold ethanol fractionation technique of Cohn et. al. (1946) to human plasma in an attempt to localize ACTH activity in specific plasma protein fractions. These workers studied the distribution of ACTH activity in plasma collected from normal human donors, plasma collected from normal human donors to which had been added ACTH, in vitro, and plasma collected from patients following intravenous infusion of ACTH. It was found that in each case hormonal activity was primarily associated with protein Fraction II-III, the same fraction in which plasma insulin, plasma thyrotrophic hormone, and plasma gonadotrophic hormone activities had been detected.

Roberts (1957) has utilized cold ethanol fractionation (Roberts and Kelley, 1957) to study the localization of ACTH activity in rat plasma protein fractions. Two types of plasma were fractionated and assayed for activity, a) normal rat plasma to which had been added ACTH in vitro, and b) plasma obtained from rats which had been intravenously infused with an ACTH preparation. In the latter case ACTH activity was localized primarily in the albumin fraction (Fraction V), while in the former case activity was found associated with the albumin fraction (Fraction V) as well as with other protein fractions. The author concluded that "Endogenous circulating ACTH in the rat is transported mainly or exclusively in the albumin fraction of the plasma proteins."

Steroid Hormones. Finally, cold ethanol fractionation has been successfully applied to the problem of studying the plasma characteristcs of various steroid hormones.

Sandberg et. al. (1957) has reported that Cortisol bound more per unit protein to Cohn Fraction IV-I, which contains mostly alpha globulins, than to other plasma protein fractions. Antoniades et. al. (1957) utilized Cohn method 6 to study the binding of C^{14} labeled steroids and metabolites in plasma. He observed most of the protein bound radioactivity in Fraction IV-I and Fraction V. The author concludes that this indicates a binding of steroid hormones to these particular fractions. Roberts and Szego (1946) reported estrogen activity to be associated with a subfraction of Cohn Fraction II-III. Gardner (1954) utilized the Cohn fractionation method to study distribution of 17-keto steroids in various plasma protein fractions. He found indications of hormonal activity in Cohn Fraction IV-4, which contains mostly alpha and beta globulins. Antoniades et. al. (1956) (1957) demonstrated that 2 hours after intravenous injection of estrone, hormonal activity was localized in Cohn Fractions IV-I and V.

Summary. In summary, then, it appears that human plasma hormone activity can be associated with specific plasma protein fractions by chemical fractionation techniques, especially cold ethanol fractionation. Also, this plasma hormone activity is

usually not associated with the plasma albumin fractions, but seems to be primarily co-precipitated with, bound to, or transported in specific plasma globulin fractions.

C. EXPERIMENTAL APPROACH TO BE UTILIZED IN THIS DISSERTATION.

As stated earlier, the purpose of this dissertation is to obtain initial information characterizing plasma parathyroid hormone activity. It is only logical that the experimental approach to this problem be influenced by those techniques found most suitable in the study of plasma characteristics of various other plasma hormone activities. In view of the previous summary, it is felt that there can be little doubt that application of the cold ethanol fractionation method 6 of Cohn and his collaborators (1946) is the method of choice, and has been the method most commonly and most successfully used in investigations of this sort. Consequently, this type of chemical fractionation will be utilized to concentrate and localize parathyroid hormone activity in human and rat plasma protein fractions.

CHAPTER IV

BIOLOGICAL ASSAY OF PARATHYROID HORMONE ACTIVITY

Methods Available For Assay Of Parathyroid Hormone Activity. A reliable biological assay system is, of course, of basic importance in any attempt to study plasma hormone characteristics. Lack of easy, cheap, sensitive, specific and precise analytical systems for detection of hormonal activity have been a serious drawback to hormone research in general, and to parathyroid hormone research in particular. The problems associated with the bioassay of hormonal potency in the presence of plasma proteins have been discussed in detail in two reviews. (Wolstenholme, 1953) (Wolstenholme and Millar, 1957)

In the case of the parathyroid hormone there have been a number of methods introduced for assaying the hormonal activity of glandular extracts. In general, all such assays are based upon one or more of the four basic changes observed in the chemical composition of body fluids following administration of parathyroid extract. These changes are 1) increases in serum calcium concentrations, 2) increase in urinary calcium excretion, 3) a decrease in serum phosphorus concentration and 4) an increase in urinary phosphorus excretion.

Let us review here briefly some of the techniques available for bioassay of parathyroid hormone potency.

Kenny et. al. (1954) and Kenny and Munson (1959) developed a method of parathyroid hormone assay based upon the total urinary excretion of inorganic phosphorus by young male rats during the 6 hours immediately following parathyroidectomy and injection of the hormonal preparation. Rubin and Dorfman (1953) reported a method based on the measurement of P^{32} excreted in the urine after simultaneous intraperitoneal injection of P^{32} labeled Na_2HPO_4 and parathyroid preparation in rats which had been parathyroidectomized at least 24 hours previously. Intact mice are the tools of a phosphaturic assay method developed by Davies, Gordon and Musett (1955) in which the urinary P^{32} excretion of saline loaded mice are measured about 3 hours subsequent to administration of the hormonal preparation. As can be seen, each of these methods is dependent upon urinary phosphorus excretion following administration of parathyroid hormone. Aside from the difficulties inherent in the obtaining of complete urine samples, there is one other critical fact to be considered. Although the changes in urinary phosphorus following administration of parathyroid extract is a well documented phenomenon, there is still much controversy over whether or not the hyper-phosphaturic and hypo-phosphotemic effects of such extracts are due to an active hormonal principle of the parathyroid gland, or merely to the presence of some pharmacologically active artifact. Stewart and Bowen (1952) have reported that extracts of thymus and spleen are

capable of eliciting phosphaturic effects in assay animals similar to that produced by parathyroid extract. Although this report could not be confirmed by Kenny and Munson (1959), it would seem that at present the most reliable measure of parathyroid hormone activity involves consideration of the hormones effect on serum calcium levels.

Beiring (1950) developed an assay method for parathyroid hormone activity which was relatively simple, and involved measuring the increase in serum calcium of 150 gram intact rats 18 hours following injection of a test preparation. This method, however, has a very low sensitivity, requiring from 150 to 1000 USP units of activity per rat to elicit a significant response. Davies et. al. (1954) have described a method in which assay rats are parathyroidectomized 10 days prior to the date of assay, and then maintained on a stock low calcium diet. Serum calcium is determined before and 21 hours after injection of the extract. Rasmussen (1956), Rasmussen and Westfall (1956), and Rasmussen (1959), however, have exhaustively investigated the question of what time interval is required for assay animals to respond maximally to injections of parathyroid hormone preparations, as measured by increases in serum calcium levels. These workers found that 6 hours after administration of the hormone, the maximum response has usually been attained. After this time the response may decrease sharply or plateau off gently depending

upon the injection medium (Rasmussen, 1959). As a result, analytical data based upon increments of serum calcium at times other than 6 hours after injection of the test material, are rendered suspect.

Finally, Munson (1953) has described a method which is based on the maintenance of serum calcium levels in calcium depleted, parathyroidectomized rats in the 6 hours immediately following operation and injection of the hormonal preparation. If large numbers of animals are utilized, however, as is usually necessary, this method becomes technically rather cumbersome.

Biological Assay Procedure Utilized In This Dissertation. The basis of the biological assay procedure to be utilized in this dissertation, is the effect of parathyroid hormone upon the serum calcium levels of parathyroidectomized female rats of the Sprague-Dawley strain, six hours subsequent to intraperitoneal administration of the test material.

Parathyroidectomy is accomplished by cauterization with a hot wire. Details of this procedure are given in the Appendix. Cauterization was found superior to surgical removal of the thyroparathyroid apparatus, a technique utilized by many workers, because it 1) permitted destruction of the parathyroid glands with a minimum of damage to the thyroid gland, 2) practically eliminated bleeding as a source of trauma to the operated animal and 3) in general, reduced the overall shock to the animal

during the course of the parathyroidectomy, with a resultant increased percentage of successful operations.

Following parathyroidectomy the animals are allowed to recover for a period of at least 3 days before being prepared for assay. Throughout this work female rats of the Sprague-Dawley strain are used, and these are maintained on a Purina Dog Chow diet, with tap water supplied ad libitum.

Preparation for assay consists of fasting the animals for two nights and one day prior to use. During the fasting period animals are allowed tap water ad libitum.

The actual assay procedure usually requires one day for completion. Blood is drawn in the morning, following the previously described fasting period, by means of cardiac puncture or venous tail bleeding. Immediately after removal of the initial blood sample, aliquots of the material to be assayed are injected intraperitoneally into the assay animals. It was found that removal of 0.75 to 1.0 cc. of blood provided ample serum for analysis and did not adversely effect the assay animal. The final blood sample is collected 6 hours subsequent to administration of the test dose.

Serum is obtained by centrifugation of the blood for five minutes in the clinical centrifuge. Samples taken prior to and subsequent to the six hour test period were analyzed simultaneously for calcium content.

Chemical Analysis of Serum for Calcium and Protein. Chemical analysis of the serum for calcium was performed according to the method of Natelson and Penniall (1956), with some slight modification. In this procedure, calcium in triethanolamine solution reacts with alizarin (1,2 dihydroxy-anthraquinone) in octanol solution, to give a purple colored complex which is soluble in the octanol. After shaking to insure complete extraction, the calcium alizarinate-octanol mixture (upper layer) is transferred to a colorimeter and intensity of color is measured. In this work a Klett-Summerson photoelectric colorimeter fitted with a number 56 filter (560 mu.) was routinely employed.

The alizarin solution and the triethanolamine solution used were of the same concentration as outlined in the original method. The modification consisted in using 6 ml. of the alizarin-octanol reagent instead of 3 ml. and in pipetting a 0.050 ml. serum sample for analysis instead of the 0.020 ml. serum sample suggested by Natelson and Penniall (1956). For extraction of the calcium alizarinate complex, it was found convenient to use 12 ml. glass stoppered centrifuge cones. Pipetting of serum samples was accomplished through use of 50 lamda micro-pipettes. The procedure for calcium analysis is given in detail in the Appendix.

The calcium standard curve is presented in Figure I, and the per cent recovery of calcium from freshly collected rat

plasma which is afforded by this method is shown in Table I.

The protein concentration of the various plasma fractions assayed for biological activity was determined by the Biuret reaction (Natelson, 1956). Crystallized Bovine Plasma Albumin (Armour Lab. Lot #29633) is utilized as a standard. Two stock solutions are prepared: a) 25% NaOH and b) 2% $CuSO_4.5H_2O$. Prior to analysis the Biuret reagent is prepared by mixing 50 ml. of a) and 10 ml. of b) followed by dilution to 100 ml. with distilled water. To a 0.1 ml. aliquot of the protein solution to be analyzed is added 6 ml. of the Biuret reagent. This mixture is then shaken immediately, and again after 20 minutes. The resulting color is read on a Klett-Summerson colorimeter fitted with a # 54 (540 mu.) filter. The protein standard curve obtained is indicated in Figure 2.

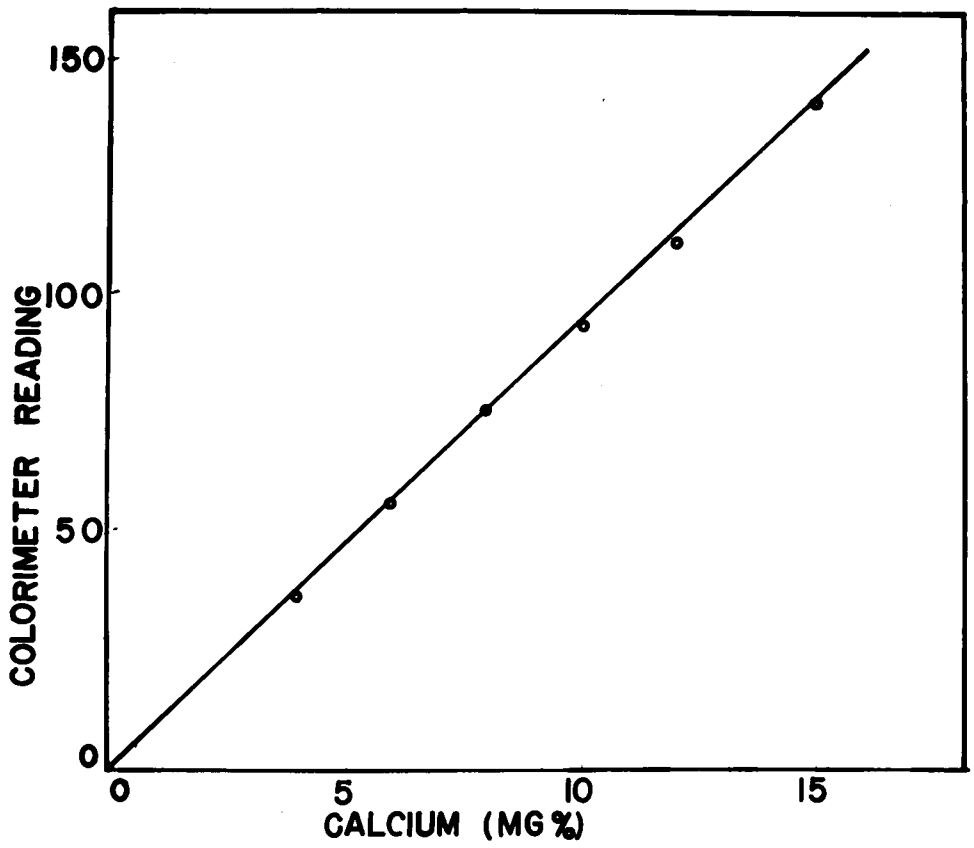

FIGURE I

STANDARD CURVE FOR CALCIUM ANALYSIS

TABLE I

RECOVERY OF CALCIUM FROM FRESHLY COLLECTED RAT PLASMA

*Expt.	Calcium Originally Present (mg.)	Calcium Added As $CaCO_3$ (mg.)	Total Calcium Present (mg.)	Total Calcium Observed	% Recovery
1	0.0015	0.0025	0.0040	0.0037	93.0
2	0.0015	0.0050	0.0065	0.0062	95.5
3	0.0015	0.0100	0.0115	0.0116	101.0
4	0.0030	0.0025	0.0055	0.0057	103.2
5	0.0030	0.0050	0.0080	0.0078	97.5
6	0.0030	0.0100	0.0130	0.0129	99.1
				Mean	98.2

* Each experiment indicates mean value of 6 individual determinations.

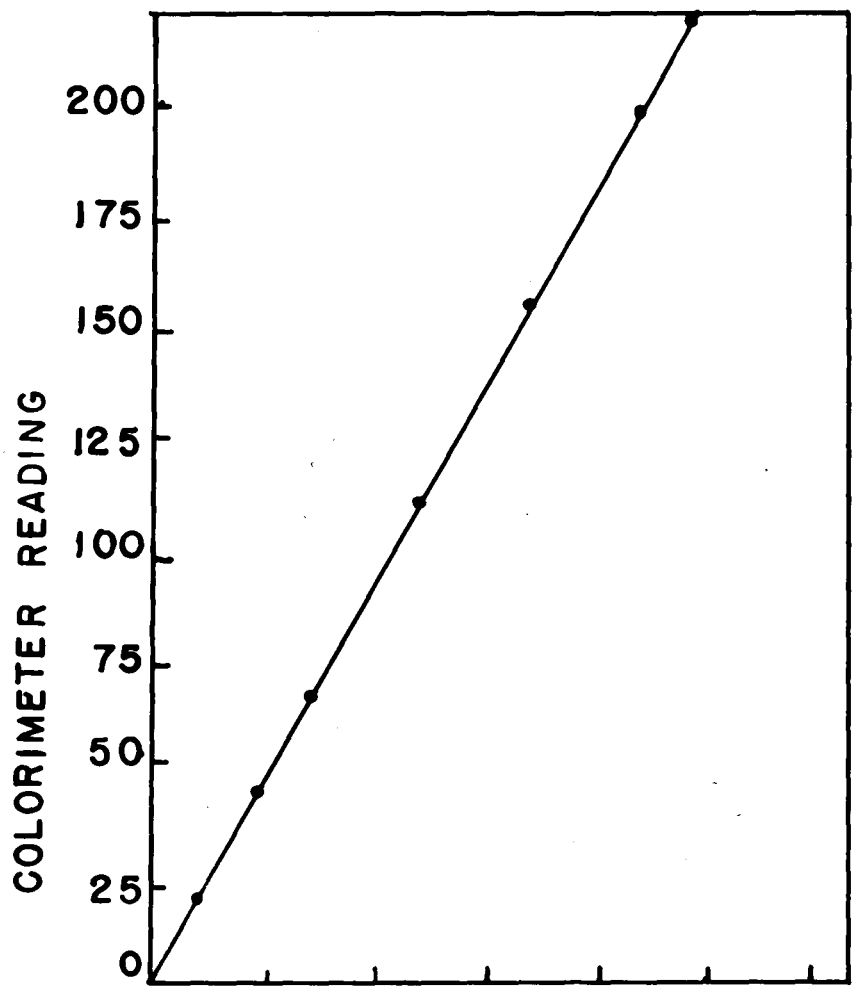

FIGURE 2

STANDARD CURVE FOR PROTEIN ANALYSIS

CHAPTER V

EVALUATION OF BIOLOGICAL ASSAY SYSTEM

The ultimate significance of any attempt to quantitively estimate or qualitatively localize plasma hormone activity depends, of course, upon the reliability of the biological assay system in use. Usually, however, a number of practical and methodological problems arise with which one must deal before further investigations are warrented and before valid interpretations can be drawn from the analytical data compiled. The more commonly used types of biological assay procedure available for estimation of parathyroid hormone potency have been reviewed in a previous section, as has been a general description of the procedure to be employed in this work. Let us now critically examine the biological assay system from the points of view of a) practical aspects involved in its use, and b) its reliability when subjected to experimental evaluation.

A. PRACTICAL CONSIDERATIONS WHICH BEAR UPON THE CHOICE OF A BIOLOGICAL ASSAY SYSTEM.

Cost of Materials. In considering use of any particular biological assay system one must bear in mind the necessary expenses involved in the attainment and preparation of the assay animals. Bearing directly upon this problem are the questions of how many assay animals will be required for a suitable assay group, the number of fatalities to be expected from any preparatory

operational procedures (such as parathyroidectomy), and the possible loss of animals during preparation for and use in the assay procedure itself.

In the case of the assay system involved in this report, a rather large number of animals are required. In addition, rats of only one sex are utilized to avoid the possibility of a species difference in the response of the assay animals to hormonal preparations. The parathyroidectomy to which the animals are initially subjected usually results in the loss of about 20% of the starting group. As described previously, the animals are permitted to recover from the operation and are then prepared for assay by a fast of two nights and a day. This additional stress upon the already sensitive parathyroidectomized animals results in additional losses. Usually it requires an original group of 10 rats in order to have available for assay a group of 6 suitably prepared animals. Use of rats, however, is considerably cheaper than use of dogs or rabbits.

Time Required For Assay. This is a major drawback common to all biological assay procedures. As can be seen from the reports summarized in Chapter III, part B, research involving biological assay of plasma protein fractions are usually undertaken by teams of investigators and technicians. In this work it routinely required a total of one week to parathyroidectomize a group of animals, permit their recovery, prepare the surviving animals for

assay, and then assay a preparation for hormonal activity. When one considers, for example, the time required for the collection of rat plasma subsequent to cold ethanol fractionation of this material, and final biological assay of the resulting five plasma protein fractions on a minimum of a 5 animal assay group per fraction, requiring at least 25 animals which have survived the preparatory procedures, one can get an idea of the significance of the time element in research of this type.

Ease of Performance of the Biological Assay Procedures. This again is a serious drawback to most biological assay procedures, which, for the most part, require experienced technicians for their adequate performance. In the assay system outlined here, the technician must be skilled in the critical technique of parathyroidectomy, as well as proper method of cardiac puncture and familiarity with the calcium analysis, upon which everything actually depends.

Despite the obvious drawbacks inherent in biological assay procedures in general, and the assay procedures to be utilized in this dissertation in particular, such methods are the only ones available to scientists for the study of hormonal activities. As stated previously, however, the results obtained are usually worth the difficulties one must overcome.

B. EXPERIMENTAL EVALUATION OF THE BIOLOGICAL ASSAY SYSTEM

Sensitivity of the Biological Assay System to Various Levels of Parathyroid Hormone Activity. Perhaps the most important characteristic of any biological assay system is its sensitivity to the hormone in question. In this experiment the sensitivity of the assay system employed in this dissertation to various levels of parathyroid hormone activity was determined.

Let us consider briefly the source of this hormonal activity, which will be utilized here and throughout this investigation whenever exogenous parathyroid hormone is required.

The parathyroid preparation to be employed is "Injection Parathyroid, USP," manufactured by Eli Lilly and Company, Indianapolis, Indiana. (formerly called "Solution Parathyroid Extract") Almost all of the information presently available concerning the physiological mechanism of action of the parathyroid hormone has been obtained through use of this particular preparation.

"Injection Parathyroid, USP" is prepared by a commercial process which is based upon the classical hot hydrochloric acid extraction of fat free, excised bovine parathyroid glands. The marketed extract has been assigned a potency by the manufacturer of not less than 100 USP units of activity per milliliter of solution. One USP parathyroid unit of activity is defined as

one one-hundredth the amount required to raise the serum calcium content of 100 cc. of blood serum of normal dogs 1 mg. within 6 to 18 hours after its administration. Mature male dogs free from gross evidence of disease and weighing between 10 and 12 Kg. are utilized. The rules of assay of the United States Pharmacopoeia are followed.

This commercial extract is used clinically in the treatment of acute hypoparathyroidism with tetany. Ordinarly, it has been found that in humans, serum calcium will begin to rise about four hours after injection. Patients soon become refractory to parathyroid extract, and its prolonged use is not recommended. Injection parathyroid is also used in the Ellsworth-Howard test (Ellsworth and Howard, 1934) which is useful in establishing the diagnosis of idiopathic and post-operative hypoparathyroidism.

"Injection Parathyroid, USP" has been utilized extensively as a reference against which are compared other parathyroid preparations for evaluations of their hormone potency.

Unless otherwise noted, the source of parathyroid hormone activity employed in this work is "Injection Parathyroid, USP" (Eli Lilly) Lot # 4064-720452, Expiration date - April 1, 1960.

The sensitivity of the biological assay system was investigated by administering 25, 50 and 100 USP units of parathyroid extract intraperitoneally to assay animals prepared as previously described, and observing the effect of these levels

of parathyroid hormone activity upon the serum calcium level of each individual member of the respective assay groups. The results obtained are indicated in Table 2.

Effect Of Serum Proteins Upon The Sensitivity of the Biological Assay System To Various Dose Levels of Parathyroid Hormone Activity. In fulfilling the objectives of this dissertation it was necessary to be able to detect parathyroid hormone activity in the presence of large amounts of plasma and serum proteins. This experiment was designed to examine the effect of excess serum proteins upon the sensitivity of the biological assay system.

A 15 gm.% solution of bovine serum proteins (Bovine Serum, Lyophilized, Pentex, Inc. Lot # 1507) was prepared, and to 5 cc. aliquots of this preparation were added 25, 50 and 100 USP units of parathyroid extract. The resulting mixtures were immediately injected intraperitoneally into the assay animals. The analytical data recorded is shown in Table 3.

A comparison of the sensitivity of the biological assay system to various dose levels of parathyroid hormone activity with the sensitivity of this assay system to the same dose levels of hormone activity when administered in a medium of concentrated serum proteins, is shown in Figure 3.

TABLE 2

SENSITIVITY OF BIOLOGICAL ASSAY SYSTEM TO VARIOUS LEVELS OF PARATHYROID HORMONE ACTIVITY

a) Dose Level: 25 USP Units

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	11.2	12.3	1.1
2	8.2	9.4	1.2
3	9.0	9.0	0.0
4	7.2	8.6	1.4
5	7.0	8.8	1.8
6	7.8	9.9	2.1
7	7.9	8.5	0.6
8	8.7	10.5	1.8
9	9.6	9.6	0.0
		Mean and Standard Error	1.1±0.3

b) Dose Level: 50 USP Units

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	9.9	10.1	0.2
2	7.8	9.2	1.4
3	8.9	11.1	2.2
4	7.6	9.7	2.1
5	7.7	9.5	1.8
6	6.6	9.2	2.6
7	6.6	9.0	2.4
8	7.4	7.6	0.2
9	7.2	10.2	3.0
10	8.1	10.8	2.7
		Mean and Standard Error	1.9±0.3

TABLE 2 (Cont'd.)

c) Dose Level: 100 USP Units

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.2	9.5	3.3
2	7.1	10.1	3.0
3	8.0	12.0	4.0
4	8.6	10.8	2.2
5	5.6	6.2	0.6
6	7.8	11.8	4.0
7	6.7	10.8	4.1
8	8.2	8.2	0.0
9	5.7	9.2	4.1
10	8.0	9.2	1.2
		Mean and Standard Error	2.7±0.5

TABLE 3

EFFECT OF SERUM PROTEINS UPON THE SENSITIVITY OF ASSAY SYSTEM TO VARIOUS LEVELS OF PARATHYROID HORMONE ACTIVITY.

a) Dose Level: 25 USP units in 5 mls. of a 15 gm.% Serum Protein Solution

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	12.2	12.9	0.7
2	8.5	9.3	0.8
3	7.5	9.3	1.8
4	8.0	9.6	1.6
5	8.5	9.1	0.6
6	6.8	7.6	0.8
7	9.0	10.3	1.3
8	7.3	9.0	1.7
9	10.3	11.0	0.7
10	7.2	8.2	1.0
		Mean and Standard Error	1.1 ± 0.2

b) Dose Level: 50 USP units in 5 mls. of a 15 gm.% Serum Protein Solution

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.8	8.5	1.7
2	10.9	11.1	0.2
3	11.4	11.5	0.1
4	7.6	9.0	2.4
5	10.7	13.0	2.3
6	10.8	12.7	1.9
7	9.2	11.9	2.7
8	6.7	8.3	1.6
9	7.0	8.9	1.9
10	7.9	9.9	2.0
		Mean and Standard Error	1.7 ± 0.3

TABLE 3 (Cont'd.)

c) Dose Level: 100 USP units in 5 mls. of a 15 gm.% Serum Protein Solution

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.5	10.5	3.0
2	9.2	13.2	4.0
3	7.7	7.7	0.0
4	8.3	8.8	0.5
5	7.6	11.0	3.4
6	8.1	12.0	3.9
7	6.9	9.6	2.7
8	7.3	8.1	0.8
9	7.6	10.4	2.8
10	6.7	10.2	3.5
		Mean and Standard Error	2.5±0.5

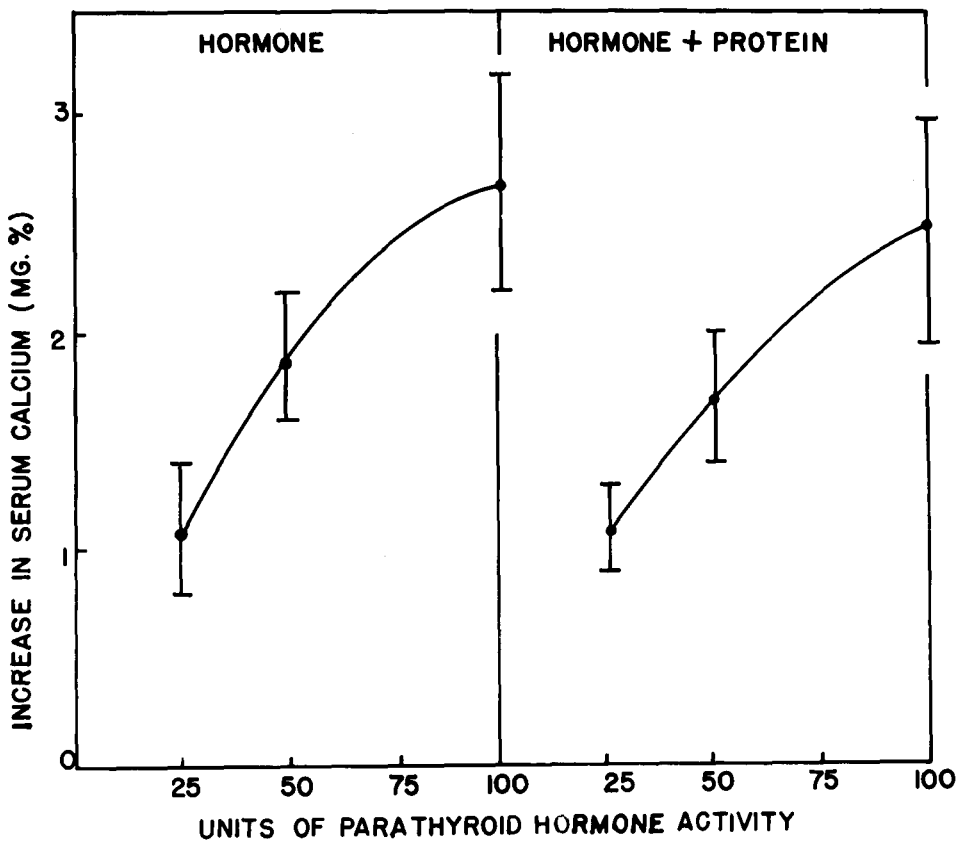

FIGURE 3

EFFECT OF SERUM PROTEINS UPON THE SENSITIVITY OF THE BIOLOGICAL ASSAY SYSTEM

(The Vertical Bar Represents The Standard Error of the Mean)

It would seem that a number of observations may be made at this time in light of these results.

The biological assay system employed has been shown to be sensitive to at least 25 USP units of parathyroid hormone activity. Excess serum proteins do not seem to effect the sensitivity of this analytical system to any significant degree. The data conclusively demonstrate that the particular lot of commercial extract to be utilized in this dissertation is hormonally potent. In this connection Reichert (1958) has shown that sterile vials of commercial extract may retain full activity for up to 10 years <u>past</u> the listed expiration date.

Another significant point is that volumes of injection fluid up to 5 ml. do not alter the response of the assay system. No volume greater than 5 cc. was employed for injection purposes during the course of this investigation. Also, since the plasma does not affect the response of the assay animals to the various dose-levels of parathyroid extract administered, we may consider the experiments outlined in Table 2 and Table 3 to be duplicate experiments. The results tend to confirm the reproducibility of the analytical data obtained through use of this bioassay system.

Finally, the data provides at least a semi-quantitative dose-response curve from which approximations of the parathyroid hormone potency of various preparations can be made.

Effect Of Serum Proteins Upon The Time-Response Of The Biological Assay System To Parathyroid Hormone Activity. The biological assay system under consideration is dependent upon the effect of intraperitoneally injected hormonal preparations upon the serum calcium level of parathyroidectomized female rats, SIX hours subsequent to its injection. The use of the six hour time interval is justified, since it has been well documented (Rasmussen, 1956) (Rasmussen and Westfall, 1956) (Rasmussen, 1959) that the response of parathyroidectomized rats to injected parathyroid hormone preparations occurs maximally 6 hours subsequent to administration. Rasmussen (1959) and Melius (1956) have confirmed this fact in the specific case of "Injection Parathyroid, USP." Rasmussen (1959) has also shown that the response of the assay rat to injected parathyroid hormone preparations either peaks and falls, or peaks and plateaus, at this six hour time interval regardless of the injection medium employed. In this investigation, however, we are attempting to detect hormonal activity in the presence of vast excesses of plasma proteins and in relatively large volumes of injection fluid. It seemed necessary, therefore, to examine the effect of these parameters upon the response of the assay system to parathyroid hormone potency.

To do this a 15 gm.% solution of Bovine serum proteins (Bovine Serum, Lyophilized, Pentes, Inc. Lot # 1507) was

prepared. To 5 cc. aliquots of this preparation were added 50 USP units of parathyroid extract with slow stirring over a magnetic stirrer, and the resulting mixture was injected immediately into the assay animals. The response of each animal was recorded 3, 6 and 9 hours subsequent to administration of the test dose. The individual response of the assay animals at each time interval can be found in Table 4, and the results are summarized in Figure 4.

These results confirm that the maximum response of the assay animal to administration of parathyroid hormone activity in the presence of excess amounts of serum proteins occurs at the 6 hour interval subsequent to the intraperitoneal injection of the preparation.

TABLE 4

EFFECT OF SERUM PROTEINS UPON TIME RESPONSE OF THE BIOLOGICAL ASSAY SYSTEM TO PARATHYROID HORMONE ACTIVITY

Animal Number	Initial Serum Calcium, mg.%	Serum Calcium After 3 hours mg.%	Serum Calcium After 6 hours mg.%	Serum Calcium After 9 hours mg.%
1	7.0	7.8 (0.8)*	9.0 (2.0)	9.1 (2.1)
2	6.3	7.1 (0.8)	7.5 (1.2)	7.6 (1.3)
3	7.2	7.9 (0.7)	8.8 (1.6)	8.7 (1.5)
4	7.8	9.8 (2.0)	10.8 (3.0)	10.6 (2.8)
5	7.2	7.5 (0.3)	7.9 (0.7)	8.1 (0.9)
6	7.6	8.0 (0.4)	9.5 (1.9)	9.3 (1.7)
7	8.3	9.4 (1.1)	10.0 (1.7)	9.9 (1.6)
Mean and Standard Error		0.9±0.2	1.7±0.3	1.7±0.2

*() indicates increase in serum calcium level (mg.%) over initial value.

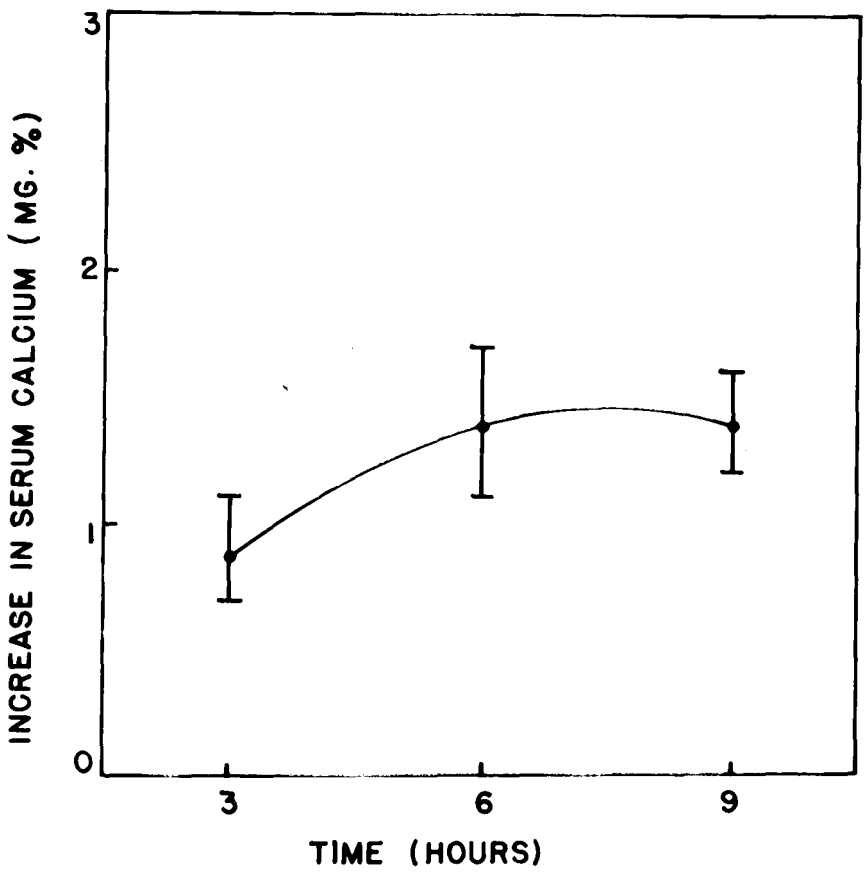

FIGURE 4

TIME RESPONSE RELATIONSHIP OF BIOLOGICAL ASSAY SYSTEM TO PARATHYROID HORMONE ACTIVITY

(The Vertical Bar Represents The Standard Error of the Mean)

Effect of Incubation With Freshly Collected Rat Plasma Upon The Hormone Potency Of Parathyroid Extract. The nature of this investigation made it necessary to obtain information concerning the effect that prolonged incubation with freshly collected rat plasma would have upon the hormonal potency of various parathyroid preparations. When, for example, we added commercial parathyroid extract to freshly collected rat plasma we had to consider the possible existence of some parathyroid hormone inactivating system in the plasma. The existence of such plasma hormone inactivating systems in vitro, has been demonstrated to occur in the case of plasma ACTH (Reiss et. al., 1951) (Pincus et. al., 1952) (White and Gross, 1957) (Mirsky et. al., 1959) as well as with plasma glucogon and plasma somatotrophin (Mirsky, 1959).

It also seemed probable that situations would arise where it would be necessary to store hormonally active plasma preparations for various periods prior to use for assay. Information upon the stability of hormonal potency under such conditions was, therefore, critical.

In order to examine these questions, normal female rats of the Sprague-Dawley strain, weighing about 200 grams each, were anesthetized with ether and exanguinated via heart puncture. To 31.5 cc. of plasma collected in this manner was added 350 USP units of parathyroid extract. The solution was permitted to mix over a magnetic stirrer for 5 minutes, allowed to incubate at

room temperature (20° C.) for two hours, and then placed under refrigeration for 18 hours at 5° C. Following this, 5 cc. aliquots of the hormone-plasma preparation, containing 50 USP units of activity per aliquot, were warmed to room temperature and administered intraperitoneally to each of the 7 animals in the assay group. The results obtained are shown in Table 5.

The high initial serum calcium values obtained in this instance are most likely due to incomplete parathyroidectomy, although Like and Orbison (1958) have reported serum calcium levels for parathyroidectomized rats in the same range as those observed here. The results indicated that the administered preparations were biologically active, and were capable of eliciting a response even in those animals with relatively high serum calcium levels. The results further indicate that a parathyroid hormone-inactivating system either is not present in rat plasma, or if present, is not active under the incubation conditions outlined. It would seem, therefore, that in this work we need not be seriously concerned about any inactivation of parathyroid hormone activity by rat plasma, in vitro, and that we may store biologically active plasma-hormone preparations for considerable periods in the cold without significant loss of potency.

TABLE 5

EFFECT OF INCUBATION WITH FRESHLY COLLECTED RAT PLASMA, UPON HORMONAL POTENCY OF PARATHYROID EXTRACT.

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	10.9	10.9	0.0
2	10.3	12.4	2.1
3	10.7	12.1	1.4
4	9.0	10.8	1.8
5	8.3	11.7	3.4
6	11.1	13.3	2.2
7	12.3	14.6	2.3
		Mean and Standard Error	1.9±0.4

Parathyroid Hormone Potency of Freshly Collected Rat Plasma. The possibility that normal rat plasma contained sufficiently high levels of parathyroid hormone activity to elicit measurable responses in our bio-assay system remained to be considered. The occurrence in untreated plasma of sufficient hormonal potency to be detected by biological assay systems is a situation to be hoped for but seldom realized.

In order to experimentally define this contingency in the case of the parathyroid hormone, 40 cc. of fresh rat plasma were collected, via cardiac puncture, from normal female rats of the Sprague-Dawley strain, weighing approximately 200 grams each. Since the total permissible volume of injection dose has been limited to 5 cc., this volume of fresh rat plasma was injected into each animal of the assay group. The results are outlined in Table 6.

Quite obviously, no detectable parathyroid hormone activity is present in 5 ml. aliquots of freshly collected, untreated rat plasma. This experiment would seem to confirm the necessity of employing a concentration technique in order to study characteristics of rat plasma parathyroid hormone activity.

TABLE 6

PARATHYROID HORMONE ACTIVITY OF FRESHLY COLLECTED RAT PLASMA

Dose: 5 ml. Freshly Collected Rat Plasma per Animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.1	8.2	0.1
2	10.8	10.6	-0.2
3	7.2	7.3	0.1
4	7.6	7.5	-0.1
5	7.6	8.2	0.6
6	8.3	8.4	0.1
7	8.4	8.4	0.0
8	7.2	7.4	0.2
		Mean and Standard Error	0.1±0.1

Effect Of Nembutal (Sodium Pentobarbital) Upon The Sensitivity of the Biological Assay System. As has been described earlier, blood is taken from the assay animal for calcium analysis before and after a six hour time period through use of either cardiac puncture technique or by venous tail bleeding. The former case requires anesthetizing the assay animal prior to removal of the serum sample in order to obtain the highest percentage of successful cardiac punctures. Although ether proved to be the anesthetic of choice, Nembutal (Sodium Pentobarbital) was also considered for use during the early stages of this work. In order to assess the effect of Nembutal anesthesia upon the biological response of our assay system, assay animals were anesthetized by nembutal injection prior to administration of 50 USP units of parathyroid extract. Sufficient nembutal (40 mg./Kg. body weight) was given to render the animals unconscious for 4 of the 6 hours in the test period. The interesting results obtained are indicated in Table 7.

TABLE 7

EFFECT OF NEMBUTAL (SODIUM PENTOBARBITAL) UPON SENSITIVITY OF THE BIOLOGICAL ASSAY SYSTEM

Experiment 1

Dose: 50 USP units of parathyroid extract/assay animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.8	7.6	0.8
2	7.9	8.2	0.3
3	9.9	9.0	-0.9
4	12.1	13.8	1.7
5	13.6	14.3	0.7
		Mean and Standard Error	0.4±0.4

Experiment 2

Dose: 50 USP units of parathyroid extract/assay animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.8	7.7	0.9
2	7.6	7.9	0.3
3	8.5	8.5	0.0
4	5.7	6.9	1.2
5	7.8	8.5	0.7
6	6.7	7.2	0.5
		Mean and Standard Error	0.6±0.2

The experimental results indicate that Nembutal anesthesia markedly decrease the response normally elicited by 50 USP units of parathyroid extract.

Somewhat analagous findings have been recently indicated by Royce and Sayers (1958). These workers reported a reduction of blood ACTH levels as a result of the action of Nembutal. Their supposed reduction in ACTH levels, however, could conceivably be due to a phenomenon somewhat similar to that observed here. Perhaps sodium pentobarbital in some manner interfered with the sensitivity of the ACTH assay system utilized. If this were so, it would be erroneous to conclude that this drug decreased the plasma ACTH levels.

In any event, the use of Nembutal as an anesthetic in this biological assay system appears precluded.

Further, use of Nembutal as an anesthetic in any biological assay system for parathyroid hormone activity would seem to require experimental inquiries similar to those reported here.

C. COMMENTS ON THE RELIABILITY OF THE BIOLOGICAL ASSAY SYSTEM.

It seems appropriate at this time to evaluate the biological assay system in light of the preceding experimental data.

Laraine (1957) has proposed five main problems associated with bioassay of hormones in the blood. Let us list these problems and examine them as they apply to our analytical system.

Problem of Sensitivity. The problems associated with the sensitivity of biological assay systems have been adequately discussed in a previous section. We have demonstrated that the assay system under consideration possesses sufficient sensitivity to parathyroid hormone activity to warrant its use in this investigation.

Problem of Specificity. Bioassay procedures are often complicated by the problem that positive tests may be given by moeities present in the plasma other than the hormone in question. Li (1953) found that the commonly used assay for growth hormone activity may be interfered with by the thyroid hormone, the thyrotrophic hormone and ACTH. The Insulin-like activity of plasma has been attributed to the action of Insulin itself (Groen et. al., 1952) (Vallence-Owen and Hurlock, 1954) but also to non-specific plasma proteins (Park and Bornstein, 1953). As has been pointed out previously, the hyperphosphaturic and hypophosphatemic responses of assay systems to various parathyroid hormone preparations has been rendered suspect by the work of

Stewart and Bowen (1952), who obtained similar response with non-hormonal glandular extracts.

To the author's knowledge, however, there has never been any report which attributes calcium mobilizing ability to any source other than the parathyroid hormone. It is felt that we may be quite sure of the specificity of our biological assay system for parathyroid hormone activity.

Problem of Precision. Our evaluation work has amply demonstrated that we are working with an analytical system capable of providing reproducible results. As in the case of most bioassay procedures, this system cannot give exact quantitative information. It can, however, be expected to provide definite qualitative indications of parathyroid hormone activity as well as a good semi-quantitative estimation of such potency as may be present in a particular test preparation. If, for example, an assay group of five animals responded to a particular plasma fraction with a mean increase in serum calcium of 1.9 mg.%, it is felt that we may conclude with reasonable certainty that the animals are responding to about 50 USP units of parathyroid activity per assay rat.

Problem in Relation to Existing International Standards. In order to estimate levels of parathyroid hormone activity, a suitable reference standard must be available. The preparation employed for that purpose in this investigation was "Injection

Parathyroid, USP," which has been described in detail previously.

The USP unit of activity associated with this extract has been defined earlier, and is the unit of activity which is employed almost exclusively by workers in the field for estimations of parathyroid hormone activity.

Problem Associated With The Injection of Serum or Plasma into the Test Animals. This critical parameter has been exhaustively evaluated with regard to its effects upon the sensitivity of the biological assay system. It may be stated rather categorically that plasma and serum proteins do not significantly effect the sensitivity of the assay system utilized in this investigation.

CHAPTER VI

FRACTIONATION OF PLASMA PARATHYROID HORMONE ACTIVITY

A. PREPARATION OF HORMONALLY ACTIVE RAT PLASMA

In considering the problem of localizing and characterizing rat plasma parathyroid hormone activity it was, of course, necessary to be able to obtain rat plasma possessing sufficient hormonal potency to be detectable by biological assay. Since normal rat plasma, in doses suitable for use in the assay method adopted, lacked this amount of parathyroid hormone activity, it became necessary to develop a technique which would increase rat plasma hormonal potency to measurable levels.

A first possibility was the direct injection of parathyroid extract into the circulatory system. In the case of a number of protein hormones, however, this usually results in a rapid inactivation of the exogenous hormonal activity. This general problem has recently been reviewed by Mirsky et. al. (1959), who have advance a possible explanation for this phenomena based upon a suggested hydrolytic inactivation of protein hormones in the circulation by the enzyme, plasmin. Also, a massive "flooding" of plasma with large quantities of injected hormone could result in a binding of the hormone molecule to protein fractions with which it is not normally associated. Such a situation has been suggested by a number of reports, including those of Robbins and Rall (1955) and Roberts (1957).

It appeared that some method of introduction of exogenous hormone other than intravenous injection would be more appropriate and suitable for use in this work. Consequently it was decided to administer the exogenous hormone via intraperitoneal injection, collect the plasma via cardiac puncture after a pre-determined time interval and assay the plasma obtained in this manner for hormonal activity. It was felt that the intraperitoneally injected hormone would diffuse slowly but steadily into the circulation, where it would equilibrate with the various plasma protein fractions and have the opportunity to become associated with specific plasma protein fractions in a physiological manner.

In the initial attempt to prepare hormonally active plasma, 400 USP units of parathyroid extract were administered intraperitoneally into each of nine normal female rats of the Sprague-Dawley strain, weighing about 200 grams each. The animals were exanguinated by cardiac puncture <u>one hour</u> subsequent to the administration of the extract, with heparin being employed as the anticoagulant. A total of 23 cc. of pooled plasma were obtained in this manner, and immediately refrigerated until the following morning. At that time, the refrigerated pooled plasma was warmed to room temperature in a luke-warm water bath, and 4 cc. aliquots of this preparation were then injected into each member of the five animal assay groups.

The results obtained, presented in Table 8, Experiment 1, suggest the presence of parathyroid hormone activity in the plasma samples assayed.

Next, the experiment previously described was essentially repeated with some slight modifications. In this attempt, 500 USP units of parathyroid extract were administered to each of eleven 200 gram female rats of the Sprague-Dawley strain. The animals were exanguinated after one hour, the plasma collected and stored in the cold overnight. Thirty cc. of rat plasma were collected, and 5 cc. aliquots of this preparation were administered to the individual members of a six animal assay group. Each test dose contained 385 mg. of plasma protein. The results outlined in Table 8, Experiment 2, are similar to those observed in the previous experiment, and again indicate the presence of parathyroid hormone activity.

The one hour time interval before exanguination was an arbitrarily chosen condition. The possibility existed that a greater level of plasma hormone activity might be obtained at time intervals other than one hour. To evaluate this contingency 500 USP units of parathyroid extract were administered to each of twelve 200 gram female rats of the sprague-Dawley strain, with the plasma being obtained and prepared as in the previous experiments except that exanguination was accomplished three hours subsequent to the administration of the parathyroid extract.

Thirty-six cc. of rat plasma were collected and five cc. aliquots administered to each of six animals in the assay group. Each test dose contained 350 mg. of plasma protein. The results are given in Table 8, Experiment 3.

The data recorded in these experiments indicate that rat plasma collected one and three hours subsequent to intraperitoneal administration of parathyroid extract possesses sufficient hormone-like activity to be detected by our biological assay system. This hormone-like potency, however, must be due to the hormonal principle of the parathyroid extract employed since it has previously been shown (Table 6) that 5 cc. aliquots of freshly collected plasma from normal untreated rats does not possess similar biological activity.

At this time the author would like to point out some factors which bear upon this technique. The experiments just completed required the sacrifice of 32 "bleeding" animals, and use of about 150cc. (30 vials) of parathyroid extract, the latter item being a rather expensive commodity. Because of the expense involved in the preparation of hormonally active plasma it is necessary to use the minimum amount of parathyroid extract and bleeding animals required to yield sufficient plasma volumes for analytical evaluation. Consequently, preparations of active plasma of sufficient volume to be assayed in assay groups of more than five or six animals are precluded from practical

consideration. Assay groups of this size, however, are amply sufficient for the type of information desire here. The reliability of the qualitative and semi-quantitative date realized is heightened by the fact that the response of each individual assay rat is measured. Often investigators (Melius, 1955) (Friedman and Munson, 1957) base their estimation of hormonal potency upon the difference in calcium levels between pooled plasma samples taken from assay rats before and after the test period.

The variability of response elicited by doses of equal hormonal potency from members of an animal assay group, however, is well known and, indeed, is a major parameter associated with the interpretation of biological assay data (Wolstenholme, 1953). Following the response of individual members of an assay group therefore, would seem to be the method of choice and one capable of providing reliable information of a qualitative and a semi-quantitive nature.

TABLE 8

BIOLOGICAL ASSAY OF PLASMA FROM RATS TREATED WITH PARATHYROID EXTRACT

Experiment 1

23 cc. plasma collected one hour after intraperitoneal injection of 400 USP units of parathyroid extract per "bleeding" animal. 4 cc. aliquots administered per assay animal.

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	10.3	13.2	2.9
2	11.5	15.5	4.0
3	10.2	9.8	-0.4
4	10.0	10.0	0.0
5	9.4	11.5	2.1
		Mean and Standard Error	1.7±0.8

Experiment 2

30 cc. plasma collected one hour after intraperitoneal injection of 500 USP units of parathyroid extract per "bleeding" animal. 5 cc. aliquots administered per assay animal.

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change in Serum Calcium, mg.%
1	9.3	11.6	2.3
2	8.3	9.6	1.3
3	7.9	8.9	1.0
4	8.5	10.6	2.1
5	7.3	7.2	-0.1
6	6.7	8.5	1.8
		Mean and Standard Error	1.4±0.4

TABLE 8 (Cont'd.)

Experiment 3

36 cc. plasma collected three hours after intraperitoneal injection of 500 USP units of parathyroid extract per "bleeding" animal. 5 cc. aliquots administered per assay animal.

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.0	9.4	1.4
2	8.2	9.7	1.5
3	7.3	6.4	-0.9
4	6.9	8.6	1.7
5	6.7	8.7	2.0
6	7.8	10.0	2.2
		Mean and Standard Error	1.3±0.5

B. EFFECT OF DIALYSIS AND OXIDATION UPON PLASMA PARATHYROID HORMONE ACTIVITY

The feasibility of applying chemical fractionation procedures to hormonally active rat plasma was now investigated.

In order to ascertain whether or not even a crude partition of plasma parathyroid hormone activity could be realized, it was decided to apply the classical ammonium sulfate fractionation procedures to hormonally active rat plasma and assay the resulting fractions for biological activity.

Before such analytical data could be correctly interpreted, however, there remained a critical parameter, inherent in the salt fractionation procedure, to be considered. Plasma protein fractions obtained as a result of salting out with ammonium sulfate would have to be dialyzed free of the salt prior to biological analysis. This presented the problem of determining what effect dialysis would have upon such parathyroid hormone potency as may be present in a particular plasma protein preparation.

There have been reports in the literature which suggest the active principle of the parathyroid gland to be capable of passing through membrane barriers subsequent to dialysis and ultrafiltration, (Kenny et. al., 1956) (Davies and Gordon, 1954) (Rasmussen and Westfall, 1957). These investigations, however, were all performed on parathyroid gland extracts other then the commercial extract employed here and not on normal

plasma or on plasma-parathyroid hormone preparations. Even, therefore, if the parathyroid principle of the commercial extract employed in this work was found to be dialyzable and ultrafilterable, such an observation could not be extended to include plasma parathyroid hormone activity because the possibility existed that the plasma proteins present could, in some manner, perhaps by binding, prevent the passage of the hormonal molecules through the semipermeable membrane barrier. Situations such as this have been indicated to occur in the case of the hormonal principles of the posterior pituitary (Heller, 1957), cortisone (Daughaday, 1956), thyroxine (Pitt-Rivers, 1957), and others.

The effect of dialysis upon the biological activity of commercial parathyroid extract was studied in the following series of experiments.

400 USP units of parathyroid extract were dialyzed against two liters of distilled water in an Oxford Model B Revolving Multiple Dialyzer, in the cold (2-4° C.) for 25 hours, with changes of the dialysate at 3, 6 and 9 hours. Cellulose Dialyzing Tubing (A. H. Thomas, No. 4465-A2) was employed as the dialyzing membrane. After dialysis five ml. of dialysand were recovered, and equal aliquots of this material were injected into each of eight rats. Each animal received an aliquot of the dialysand equivalent to 50 USP units of activity before dialysis.

The second experiment, 350 USP units of parathyroid extract were added to 26.5 cc. of distilled water. This preparation was subjected to dialysis as before, after which a total of 35 cc. of dialysand were recovered. Five cc. aliquots of the solution were administered to each of 7 assay animals of the assay group. Each animal, then, received the equivalent of 50 USP units of parathyroid activity before dialysis.

In the final experiment of this sequence, 500 USP units of parathyroid extract were added to 15 cc. of distilled water and the resulting solution dialyzed in the usual manner. 25 cc. of dialysand were recovered and 5 cc. aliquots of this administered to each of five animals of the assay group. In this case each animal received a test dose containing 100 USP units of activity before dialysis.

The results of these experiments are given in Table 9, and indicate a definite and pronounced decrease in biological activity of the parathyroid gland extract subsequent to dialysis. The possible nature of this phenominon will be considered in detail in the "Discussion" section.

TABLE 9

EFFECT OF DIALYSIS UPON THE HORMONAL POTENCY OF PARATHYROID EXTRACT.

Experiment 1

Dose: 0.5 ml. (50 USP units) dialyzed extract/assay animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.2	8.4	0.2
2	8.6	8.3	-0.3
3	8.3	9.1	0.8
4	8.3	7.4	-0.9
5	9.4	9.3	-0.1
6	9.6	8.7	-0.9
7	9.1	10.3	1.2
8	8.3	7.9	-0.4
		Mean and Standard Error	-0.4±0.3

Experiment 2

Dose: 0.5 ml. (50 USP units) dialyzed extract in 5 ml. distilled water/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	5.4	7.0	1.6
2	6.7	6.3	-0.4
3	5.3	5.5	0.2
4	6.6	6.9	0.3
5	6.8	6.7	-0.1
6	5.7	6.9	1.2
7	5.8	5.8	0.0
		Mean and Standard Error	0.4±0.3

TABLE 9 (Cont'd.)

Experiment 3

Dose: 1.0 cc. (100 USP units) dialyzed extract in 5 ml. distilled water/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.8	7.3	0.5
2	6.4	6.5	0.1
3	12.5	11.8	-0.7
4	9.5	9.5	0.0
5	7.8	7.8	0.0
		Mean and Standard Error	0.0±0.2

The effect of dialysis upon plasma parathyroid hormone activity was studied in the following set of experiments.

300 USP units of parathyroid extract were added to 22 cc. of freshly collected rat plasma. The resulting mixture was allowed to incubate for one hour at room temperature (18° C.) with continuous stirring over a magnetic stirrer. The preparation was then subjected to dialysis as in the previous experiments. Thirty cc. of dialysand were recovered, and five cc. aliquots were administered to each of six animals of the assay group. Before dialysis, then, each aliquot of test material contained 50 USP units of activity.

In the second experiment of this sequence 500 USP units of parathyroid extract were freeze-dried and added to 19 cc. of freshly collected rat plasma. After incubation for one hour as before, the preparation was dialyzed in the usual manner. 26 cc. of dialysand were recovered and aliquots of this were administered to each of five animals of the assay group. Each animal received a test dose containing 100 USP units of activity before dialysis.

The final experiment of this sequence dealt with the effect of dialysis on "active" plasma. 500 USP units of parathyroid extract were administered to each of seven animals. After one hour the animals were exanguinated by cardiac puncture, the procedure yielding 21 cc. of hormonally active plasma. This

material was then subjected to dialysis in the usual manner. 25 cc. of dialysand were recovered and equal aliquots of this preparation administered to each of five animals in the assay group.

The results obtained from this series of experiments (Shown in Table 10) demonstrate that the hormonal activity of a parathyroid extract-plasma protein mixture is not lost upon dialysis as is the active principle of the parathyroid extract when the extract is dialyzed by itself. Also, the potency of the hormonally active rat plasma appears to be uneffected by the dialysis procedure.

In addition to the effect of dialysis upon plasma parathyroid hormone activity, there remained one other parameter to examine. Rasmussen (1959) has reported that his acetic acid extracts of bovine parathyroid glands were oxidatively inactivated by hydrogen peroxide, and reactivated by cysteine to varying degrees depending upon pH. There was no report on the effect of this hydrogen peroxide treatment upon the commercial parathyroid extract utilized in this work, nor was any information presented concerning the effect of oxidation upon plasma parathyroid hormone activity. The following experiments were designed to study this problem.

250 USP units of parathyroid extract were diluted to a total volume of 25 cc. with distilled water (final pH. 4.1),

TABLE 10

EFFECT OF DIALYSIS UPON PLASMA PARATHYROID HORMONE ACTIVITY

Experiment 1

Dose: Dialyzed solution of 50 USP units of parathyroid extract in 5 cc. rat plasma/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.9	8.7	1.8
2	7.3	9.2	1.9
3	5.8	7.8	2.0
4	8.9	10.6	1.7
5	6.7	7.7	1.0
6	5.9	7.2	1.3
		Mean and Standard Error	1.6±0.2

Experiment 2

Dose: Dialyzed solution of 100 USP units of parathyroid extract in 5 cc. rat plasma/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	10.7	12.6	1.9
2	6.3	8.8	2.5
3	6.0	9.5	3.5
4	9.3	12.0	2.7
5	7.0	8.7	1.7
		Mean and Standard Error	2.4±0.6

TABLE 10 (Cont'd.)

Experiment 3

Dose: 4 cc. of dialyzed, hormonally active rat plasma in 5 cc. soltuion of plasma and distilled water/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.9	9.6	1.7
2	6.8	8.0	1.2
3	9.4	10.3	0.9
4	5.7	7.9	2.2
5	7.7	8.7	1.0
		Mean and Standard Error	1.4±0.3

and to this solution was added enough 30% hydrogen peroxide to make the solution 0.1 M. The oxidation was allowed to continue 30 minutes at room temperature (25 to 28° C.) after which the reaction was terminated by addition of 1.0 mg. of catalase. The procedure is essentially that outlined by Rasmussen (1958). Five cc. aliquots of this oxidized preparation, each of which contained 50 USP units of parathyroid hormone activity prior to treatment with hydrogen peroxide, were administered to each of five animals of an assay group. This experiment was repeated again in exactly the same manner and the results obtained in these duplicate experiments are presented in Table 11.

In the first two experiments of this sequence, 300 USP units of parathyroid extract were added to 27 cc. of freshly collected rat plasma, and the mixture allowed to incubate at room temperature (25° C.) for one hour with stirring over a slow magnetic stirrer. Following this, the preparation was treated with hydrogen peroxide as previously described. Five cc. aliquots of this treated plasma-hormone mixture, containing 50 USP units of activity before oxidation, were administered to each of six animals of the assay group. The analytical data recorded for these duplicate experiments are included in Table 12 (Experiment 1 and 2).

The final experiment of this set was performed to study the effect of this oxidative treatment upon biological potency of hormonally active rat plasma.

TABLE 11

EFFECT OF OXIDATION UPON THE HORMONAL POTENCY OF PARATHYROID EXTRACT

Experiment 1

Dose: Oxidized solution of 50 USP units of parathyroid extract in 5 cc. distilled water/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.2	8.5	0.3
2	8.6	8.4	-0.2
3	7.2	8.2	1.0
4	6.4	6.5	0.1
5	7.2	7.5	0.3
		Mean and Standard Error	0.3±0.2

Experiment 2

Dose: Oxidized solution of 50 USP units of parathyroid extract in 5 cc. distilled water/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.8	6.7	-0.1
2	7.3	7.7	0.4
3	5.5	5.8	0.3
4	8.7	8.2	-0.5
5	8.0	8.6	0.6
		Mean and Standard Error	0.1±0.2

500 USP units of parathyroid extract were administered to each of nine 200 gram female rats of the Sprague-Dawley strain. After one hour the animals were exanguinated via cardiac puncture. 25 cc. of plasma were obtained in this manner, and this preparation was subjected to treatment with peroxide in the usual manner. Five cc. aliquots of this oxidized hormonally active plasma preparation were administered to each of five animals in the assay group. The analytical data obtained is presented in Table 12 (Experiment 3).

The data recorded in Tables 9, 10, 11 and 12 demonstrates quite conclusively that dialysis and peroxide oxidation result in a loss of hormonal potency of parathyroid extract, but have no effect upon the biological activity of parathyroid extract after incubation with freshly collected rat plasma. Neither dialysis nor peroxide oxidation affects the potency of hormonally active rat plasma obtained from animals treated with parathyroid extract.

As suggested earlier, it is possible to extend the experimental information presented here to imply additional properties of plasma parathyroid hormone activity in order to explain the seeming "protective effect" afforded such hormonal activity by the plasma proteins. This subject will be discussed more fully in the Discussion, but it may be mentioned, at this time, that a number of workers (Heller, 1957) (Daughaday, 1956) (Pitt-Rivers, 1957) and others, have interpreted data similar to that presented

TABLE 12

EFFECT OF OXIDATION UPON PLASMA

PARATHYROID HORMONE ACTIVITY

Experiment 1

Dose: Oxidized solution of 50 USP units of parathyroid extract in 5 cc. plasma/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	11.4	12.8	1.4
2	7.3	8.6	1.3
3	6.9	9.0	2.1
4	7.3	9.1	1.8
5	8.2	9.9	1.7
6	5.9	7.9	2.0
		Mean and Standard Error	1.7±0.1

Experiment 2

Dose: Oxidized solution of 50 USP units of parathyroid extract in 5 cc. plasma/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.9	8.9	2.0
2	7.3	8.5	1.2
3	8.1	9.8	1.7
4	10.2	11.1	0.9
5	6.6	8.6	2.0
		Mean and Standard Error	1.6±0.2

TABLE 12 (Cont'd.)

Experiment 3

Dose: 5 cc. aliquots of oxidized hormonally active plasma preparation/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	9.3	11.1	1.8
2	7.6	8.5	0.9
3	8.7	10.1	1.4
4	6.9	7.9	1.0
5	7.9	9.2	1.3
		Mean and Standard Error	1.3±0.1

here as being indicative of a binding of hormonal moeities to plasma proteins.

Of immediate importance to this investigation, however, is that the work just outlined permits application of ammonium sulfate fractionation procedures to the study of localization of plasma parathyroid hormone activity.

C. AMMONIUM SULFATE FRACTIONATION OF HORMONALLY ACTIVE RAT PLASMA

The traditional method for separation of plasma proteins is by fractional salting out with ammonium sulfate. Approximately 0.2 to 0.25 saturation is used to precipitate the fibrinogen, 0.33 saturation to separate the euglobulin fraction, and 0.5 saturation to remove the pseudoglobulin fraction. The albumin remaining in solution after removal of the globulins is usually precipitated by full saturation with ammonium sulfate (Gutman, 1948). A number of workers, among them Tiselius (1937) and Svensson (1941) have shown that the fractions obtained in this manner are heterogeneous and susceptible to further separation. Cohn et. al. (1940) in a classical report, have summarized the results of a detailed study of serum fractions obtained by precipitation with increasing concentrations of ammonium sulfate. Their findings may be summarized in the following tabular form.

Saturation with Ammonium Sulfate	Percent of Total Protein in Precipitate	Composition of Precipitate
0.34	20	Mostly gamma globulins
0.40	15	alpha, beta, gamma globulins
0.50	14	alpha, beta globulins
0.68	16	chiefly crystalline albumins

Although salt fractionation of this type is no longer the method of choice for separation of plasma proteins, having been replaced by such techniques as cold ethanol fractionation (Cohn et. al., 1946) (Roberts and Kelley, 1956), electrophoresis (Bier, 1959) and chromatography (Moore and Stein, 1956) (Peterson and Sober, 1956) (Sober et. al., 1956) (Porath, 1957) (Sober and Peterson, 1958), it is still frequently utilized where crude separations of plasma proteins are desired (Wolfson et. al., 1948) (Cotes and Young, 1951) (Walters et. al., 1957) (Friedman and Munson, 1958) (Campbell et. al., 1959).

Fractional Precipitation of Plasma By Ammonium Sulfate. Fifty ml. of hormonally active plasma were collected from female rats of the Sprague-Dawley strain by cardiac puncture one hour subsequent to treatment of each animal with 500 USP units of parathyroid extract. The pooled plasma was then placed in the freezer at -20° C. until used for fractionation. At that time the plasma was slowly warmed to room temperature, transferred to a pyrex beaker, and to it were added two drops of 10% sulfuric acid. Next, fifty cc. of saturated ammonium sulfate solution

were added dropwise from a separatory funnel, and the mixture was allowed to stir slowly over a magnetic stirrer for one hour, after which the preparation was permitted to stand for one hour. A cardboard lift was placed under the beaker containing the reaction mixture in to minimize any heat effect from the motor of the magnetic stirrer. The saturated ammonium sulfate was prepared by adding enough salt to distilled water so that a precipitate could be observed after two days standing at room temperature with frequent shaking. The plasma globulin precipitate prepared in this manner was collected at the centrifuge and the albumin-supernate was saved for further fractionation.

The globulin precipitate was then dissolved in 100 ml. of distilled water and reprecipitated as before. Following collection at the centrifuge, the preparation was dissolved in, and diluted to, 50 ml. with distilled water, after which it was dialyzed against the same solvent. The dialyzing membrane employed was cellulose dialyzing tubing (A. H. Thomas, No. 4465-A2) possessing a flat width of 1 1/16 inch and an inflated diameter of 3/4 inch. Dialysis was carried out in an Oxford Dialyzer, Model B, in the cold with frequent changes of dialysate, until all of the excess salt had been removed as determined by the absence of a barium sulfate precipitate upon treatment of an aliquot of the dialysate with 10% barium chloride solution.

The contents of the dialysis bag were then washed out with dilute (0.1%) NaCl solution. Since the volumes of reconstituted material was too large to be used in the biological assay system, the preparation was freezedried and stored in the cold until used for assay, at which time it was reconstituted to 25 ml. with 0.1% NaCl.

The albumin fraction from the initial 50% ammonium sulfate supernate was salted out by the slow addition of an excess of finely ground solid ammonium sulfate. After this, the mixture was allowed to stand for one hour, and the albumin fraction then collected on a Whatman #2 filter paper by filtration. The fraction thus obtained was redissolved in distilled water, and reprecipitated in the same manner. The final albumin preparation was dialyzed, lyophilized and reconstituted to a volume of 25 ml. with distilled water prior to biological analysis.

Five ml. aliquots of the reconstituted fractions were administered to each of five assay animals. The percent of total protein contained in each fractions, the amount of protein administered per assay animal and the biological response elicited by each protein fraction are shown in Table 13, which represents the analytical data from duplicate experiments. The data presented are summarized in Figure 5.

TABLE 13

ASSAY OF AMMONIUM SULFATE FRACTIONS OF HORMONALLY ACTIVE RAT PLASMA

Experiment 1

Protein Analysis:

Plasma Total Protein	5.70 gm.%
Albumin Fraction	3.36 gm.%
Globulin Fraction	2.18 gm.%
% Recovery	97

Dose per Animal:

Albumin Fraction	336 mg.
Globulin Fraction	218 mg.

Assay of Globulin Fraction

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.3	8.6	2.3
2	7.0	8.5	1.5
3	10.9	10.4	-0.5
4	6.7	7.7	1.0
5	6.6	8.6	2.0
		Mean and Standard Error	1.3 ± 0.5

Assay of Albumin Fraction

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	10.9	10.7	-0.2
2	8.6	7.7	-0.9
3	7.9	7.5	-0.4
4	8.2	6.2	-2.0
5	6.3	7.8	1.5
		Mean and Standard Error	-0.4 ± 0.6

TABLE 13 (Cont'd.)

Experiment 2

Protein Analysis:		Dose per Animal:	
Plasma Total Proteins	6.38 gm.%	Globulin Fraction	228 mg.
Albumin Fraction	3.52 gm.%	Albumin Fraction	352 mg.
Globulin Fraction	2.28 gm.%		
% Recovery	91		

Assay of Globulin Fraction

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.6	8.6	2.0
2	7.0	8.5	1.5
3	6.7	7.7	1.0
4	7.0	8.5	1.5
5	6.7	7.7	1.0
		Mean and Standard Error	1.4±0.2

Assay of Albumin Fraction

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	5.7	5.0	-0.7
2	8.4	7.9	-0.5
3	5.0	4.8	-0.2
4	6.0	6.9	0.9
5	8.0	7.5	-0.5
		Mean and Standard Error	-0.2±0.2

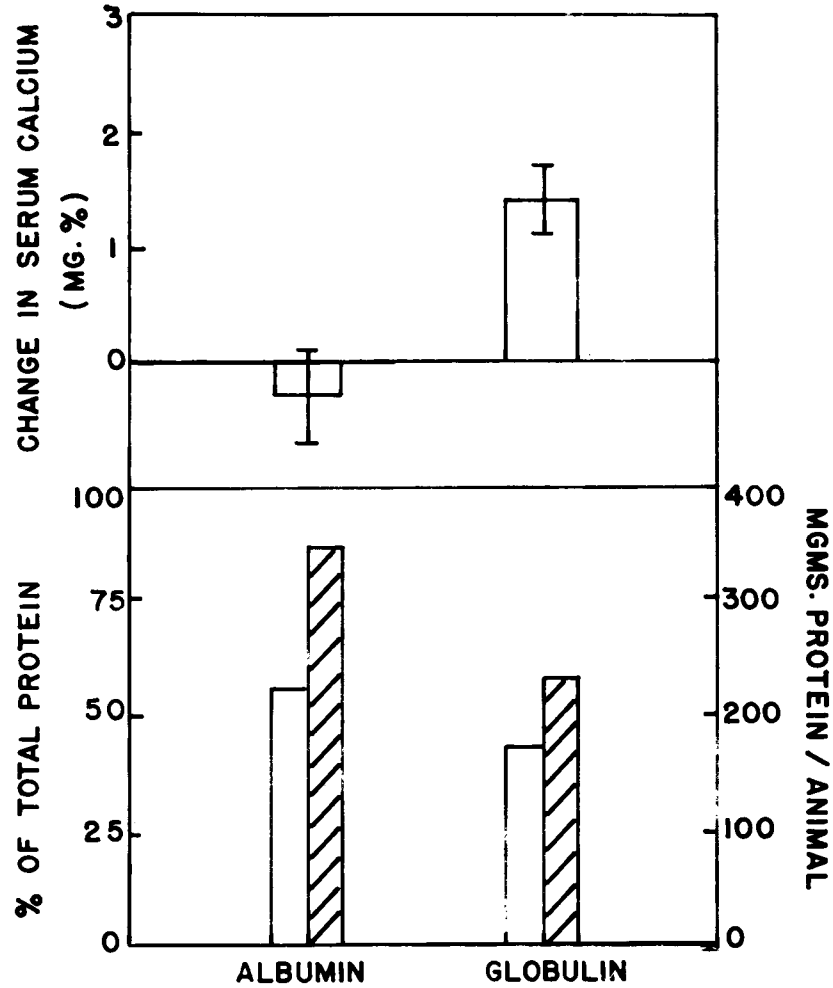

FIGURE 5

DISTRIBUTION OF PARATHYROID HORMONE ACTIVITY IN PLASMA PROTEIN FRACTIONS OBTAINED BY AMMONIUM SULFATE FRACTIONATION

(The Open Columns Are Read From The Left Hand Legend, And The Shaded Columns Are Read From The Right Hand Legend)

The recorded data indicates that the parathyroid hormone potency of hormonally active rat plasma can be localized in plasma fractions by chemical fractionation techniques. One may conclude from this information that parathyroid hormone activity is associated with the globulin fraction in this instance, that is, it is precipitated from plasma with the globulin fraction at 50% saturation of ammonium sulfate.

Additional observations may be made at this time. The fact that activity could be localized in a particular plasma protein fractions confirms, once again, the efficacy and reliability of the technique which has been described for the preparation of hormonally active rat plasma. Also, Reiss et. al. (1951) have reported a rapid inactivation of ACTH activity during incubation with heparinized plasma from rats, rabbits and humans. It will be recalled that heparinized plasma was also used in the work just concluded. It would appear, then, that unlike the situation which exists in the case of ACTH, plasma parathyroid hormone activity is not adversly affected by contact with heparin.

It is felt that a firm foundation has now been set for the application of more refined chemical fractionation techniques to the study of plasma parathyroid hormone activity.

D. COLD ETHANOL FRACTIONATION OF RAT PLASMA

The technique of cold ethanol fractionation was now employed to study the distribution of parathyroid hormone activity in protein fractions of rat plasma. For this purpose the method of Roberts and Kelley (1956), a modification of the method of Ulrich, Li and Tarver (1954) and of Cohn and his associates (1946), was chosen, since it had been specifically developed for use in preparing rat plasma protein fractions. The following are the details of the cold ethanol fractionation procedure employed in this work.

Cold Ethanol Fractionation Method of Roberts and Kelley (1956)

Adult female rats of the Sprague-Dawley strain weighing approximately 200 grams and maintaind ad libitum on Purina Laboratory Chow were utilized as the source of plasma for subsequent fractionation. (The Roberts and Kelley method was developed for use specifically on plasma from animals of this strain.) Citrated plasma (1 ml. of 4% sodium citrate per 10 ml. of blood collected) was prepared by centrifugation of freshly drawn blood for thirty minutes at 0° C. and 3000 rpm. and stored in the cold (2° C.) until utilized for fractionation, at which time it was placed in a cooling bath kept at -5° C. Initially, a rock salt-crushed ice mixture was used for cooling, but subsequently an ammonium chloride-crushed ice mixture was found to be suitable for this purpose. Addition of ethanol was begun when plasma had reached

a temperature of about 1° C., after which no freezing occurred at the temperature of bath. All additions were made while the plasma was stirring slowly over a magnetic stirrer and were accomplished in a dropwise fashion from either a pipette or a separatory funnel. All buffers utilized were prepared fresh the day before use and refrigerated until needed, at which time they were again checked for proper pH. pH measurements of plasma protein fractions were made by pipetting off 0.4 ml. of protein solution and adding to this 1.6 ml. of 0.02 M. NaCl which, after mixing, was measured for acidity in a Beckman pH Meter utilizing micro-glass electrodes. The instrument was checked continually against suitable buffer standards to insure proper function. All reagents were refrigerated prior to use.

Initially, fifty percent ethanol was added to the plasma to a concentration of eight percent, after which the solution was brought to pH 6.9±0.02 with 0.125 M. acetate buffer, pH 3.6. The ethanol concentration was then readjusted to 8%, and the precipitate removed immediately by centrifugation at -5° C. and 3000 rpm. for 40 minutes. The material collected (Fraction I) was mostly fibrinogen and was immediately placed in the deep freeze until utilized for biological assay, at which time it was warmed to room temperature in a warm water bath and then re-constituted to an appropriate volume, usually 25 ml. This was the routine procedure employed for handling the various plasma

protein precipitates. Fraction II was obtained by adding 50% ethanol to the supernate from Fraction I, to a final concentration of 20% and readjusting the pH to 6.9±0.02 with 0.09 M. acetate buffer, pH 3.5. Fraction II contains 65% beta-globulin and 35% gamma-globulin. 95% ethanol was then added to the supernate fluid from Fraction II to a final concentration of 40%, and the pH was lowered to 4.8±0.02 with 0.09 M. acetate buffer, pH 2.3. The precipitate thus obtained was the "crude albumin" precipitate, and the supernate of this precipitate was designated as Fraction SCA. This supernate was allowed to remain overnight in the Deepfreeze (-20° C.) where a precipitate formed which was recovered by centrifugation. Fraction SCA consists mainly of beta-globulin. The reconstituted crude albumin solution was then brought to a final ethanol concentration of 14% by addition of 95% ethanol, and the pH adjusted to 4.30 with 0.09 M. acetate buffer, pH 2.3. The resulting precipitate, Fraction IV, was removed by centrifugation in the cold, and consists mostly of alpha globulins. Finally, 95% ethanol was added to the supernate from Fraction IV to a final concentration of 40% and the pH was raised to 5.40 with 0.01 M. carbonate buffer, pH 8.6. The resulting fraction, Fraction V, was removed by centrifugation and consists of the plasma albumins.

An outline of this procedure is shown in Figure 6.

Biologically Active Rat Blood

Centrifuged for 30 min., 3000 rpm., 0°C.

Biologically Active Rat Plasma

50% EtOH to final conc. of 8% pH. to 6.90 with 0.125 M. Acetate Buffer, pH. 3.6

Fraction I
Fibrinogen

Supernate From Fraction I

50% EtOH to final conc. of 20%. pH. to 6.90 with 0.09 M. Acetate Buffer, pH. 3.5

Fraction II
65% Beta-Globulin
35% Gamma-Globulin

Supernate From Fraction II

95% EtOH to final conc. of 40%. pH. to 4.80 with 0.09 M. Acetate Buffer, pH. 2.3

Crude Albumin Precipitate

Dissolved in H_2O
95% EtOH to final conc. of 14%. pH. to 4.30 with 0.09 M. Acetate Buffer, pH. 2.3

Supernate of Crude Albumin Ppt.

Stored overnight in freezer

Fraction SCA
Beta-Globulin

Fraction IV
Alpha-Globulins

Supernate of Fraction IV

95% EtOH to final conc. of 40%. pH. to 5.40 with 0.01 M. Carbonate Buffer pH. 8.6

Fraction V
Albumins

FIGURE 6

OUTLINE OF COLD ETHANOL FRACTIONATION

TECHNIQUE OF ROBERTS AND KELLEY

Cold Ethanol Fractionation Of Rat Plasma To Which Has Been Added Parathyroid Extract In Vitro. The cold ethanol fractionation technique just described was now employed to study the distribution of parathyroid hormone activity in rat plasma protein fractions.

In the initial approach, parathyroid extract was added to freshly collected rat plasma, in vitro, and the distribution of hormonal activity among the various protein fractions noted. This technique has been utilized successfully by a number of workers for similar studies. Gemzell et. al. (1955) added growth hormone to normal human plasma and subjected the mixture to cold ethanol fractionation. He observed that the growth hormone activity of this plasma preparation was associated with the same fractions as the endogenous growth hormone activity of young pig and calf plasma, and of retroplacental human plasma. Bethune (1958) added ACTH to normal human plasma prior to cold ethanol fractionation and observed the distribution pattern of hormonal activity to be the same as that observed in fractions of normal human plasma, and in fractions of plasma obtained from normal patients who had been previously infused with ACTH. Roberts (1957) added ACTH to freshly collected rat plasma, in vitro, and utilized the resulting information as an integral part of his discussion of the distribution of endogenous ACTH in rat plasma.

Studies involving addition of hormonal preparations to plasma in vitro, therefore, has usually provided a basis for interesting observations on plasma hormone characteristics. It was hoped that the same situation would manifest itself in the case of parathyroid hormone.

Experimental. 600 USP units of parathyroid extract were lyophilized and the resulting solid dissolved in 50 ml. of rat plasma which had been collected from adult female rats of the Sprague-Dawley strain, by means of the cardiac puncture technique. Nineteen rats were required to supply this volume of plasma. The resulting preparations was fractionated according to the method of Roberts and Kelley, previously described. All fractions were collected in the refrigerated centrifuge at -5° C. and then stored immediately in the freezer at -20° C. Prior to assay, each fraction was slowly warmed to room temperature in a warm water bath, and then reconstituted to a volume of 25 ml. with warm distilled water. This warming procedure was necessary so that the preparation would be suitable for injection into the assay animals. After this, five cc. aliquots of the various fractions were administered to each animal of five animal assay groups.

The distribution of protein in the plasma fractions of Sprague-Dawley strain rats which had been previously treated with parathyroid extract, is shown in Table 14. The biological assay

TABLE 14

PROTEIN ANALYSIS OF RAT PLASMA PROTEIN FRACTIONS PREPARED BY THE COLD ETHANOL FRACTIONATION METHOD OF ROBERTS AND KELLEY

Fraction	Contents	Protein gm.%	mg. Protein In 50 ml. Plasma	% of Total Protein	Dose/animal mg. protein
I	Fibrinogen	0.7	350	11.3	70
II	Beta,Gamma-Globulin	1.2	600	19.4	120
SCA	Beta-Globulin	0.3	150	4.8	30
IV	Alpha-Globulin	1.1	550	17.7	110
V	Albumins	2.9	1,450	46.8	290
Total		6.2			
Whole Plasma		6.1			
Recovery		102%			

of plasma protein fractions prepared in duplicate experiments is presented in Table 15 and the experimental results are summarized in Figure 7.

The data indicates a definite partition of parathyroid hormone activity among rat plasma protein fractions subsequent to cold ethanol fractionation. The exogenous parathyroid activity appears primarily associated with Fraction IV and Fraction V, with no detectable activity being observed in Fraction I, II, or SCA.

These experiments also afforded an opportunity to study the percent recovery of plasma parathyroid activity obtainable through use of the biological assay system.

It will be noted that the biological responses elicited by Fraction IV and Fraction V are of the same order as that observed when 50 USP units of parathyroid hormone activity are administered to individual members of an assay group (Table 2, Table 3). On this basis, it would seem reasonable to assume that each animal of the assay group treated with Fraction IV protein and Fraction V protein were responding to approximately 50 USP units of hormonal activity. We can, therefore, account for 500 of the 600 USP units of parathyroid activity which were added to the freshly collected rat plasma prior to fractionation. This, then, represents a recovery of about 80% of the hormonal activity known to be present.

TABLE 15

BIOLOGICAL ASSAY OF PROTEIN FRACTIONS OBTAINED FROM RAT PLASMA TO WHICH HAD BEEN ADDED PARATHYROID EXTRACT, IN VITRO

Experiment 1

Fraction I (Fibrinogen)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.5	8.0	0.5
2	6.7	6.8	0.1
3	6.9	7.3	0.4
4	7.5	8.3	0.8
5	6.2	6.4	0.2
		Mean and Standard Error	0.4±0.1

Fraction II (65% Beta-Globulin, 35% Gamma-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.2	6.8	0.6
2	7.5	7.9	0.4
3	6.7	6.9	0.2
4	6.2	6.2	0.0
5	7.4	7.7	0.3
		Mean and Standard Error	0.3±0.1

Fraction SCA (Beta-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.5	6.7	-0.8
2	9.6	9.6	0.0
3	7.8	7.7	-0.1
4	7.3	7.5	0.2
5	6.2	6.4	0.2
		Mean and Standard Error	-0.1±0.2

TABLE 15 (Cont'd.)

Experiment 1 (Cont'd.)

Fraction IV (Alpha-Globulins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.6	9.4	1.8
2	7.7	8.8	1.1
3	8.9	11.7	2.8
4	6.5	7.6	1.1
5	6.9	9.8	2.9

Mean and Standard Error 1.9 ± 0.4

Fraction V (Albumins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.8	10.5	1.7
2	7.1	9.0	1.9
3	7.9	10.5	2.6
4	8.4	8.7	0.3
5	8.5	8.4	-0.1

Mean and Standard Error 1.3 ± 0.5

Experiment 2

Fraction I (Fibrinogen)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.4	7.9	0.5
2	8.7	9.0	0.3
3	5.9	5.9	0.0
4	7.2	7.2	0.0
5	7.1	7.5	0.4

Mean and Standard Error 0.2 ± 0.1

TABLE 15 (Cont'd.)

Experiment 2 (Cont'd.)

Fraction II (65% Beta-Globulin, 35% Gamma-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.9	7.0	0.1
2	6.6	6.6	0.0
3	5.4	5.5	0.1
4	7.5	7.9	0.4
5	5.9	6.0	0.1
		Mean and Standard Error	0.1±0.1

Fraction SCA (Beta-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.0	6.3	0.3
2	7.0	7.4	0.4
3	8.1	8.5	0.4
4	6.6	6.5	-0.1
5	7.5	7.5	0.0
		Mean and Standard Error	0.2±0.2

Fraction IV (Alpha-Globulins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.8	9.5	1.7
2	6.0	8.6	2.6
3	7.7	8.8	1.1
4	7.4	8.7	1.3
5	6.8	8.6	1.8
		Mean and Standard Error	1.7±0.3

TABLE 15 (Cont'd.)

Experiment 2 (Cont'd.)

Fraction V (Albumins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.9	7.9	0.0
2	6.5	9.3	2.8
3	6.4	8.2	1.8
4	6.6	9.1	2.5
5	5.4	7.1	1.7
		Mean and Standard Error	1.8±0.5

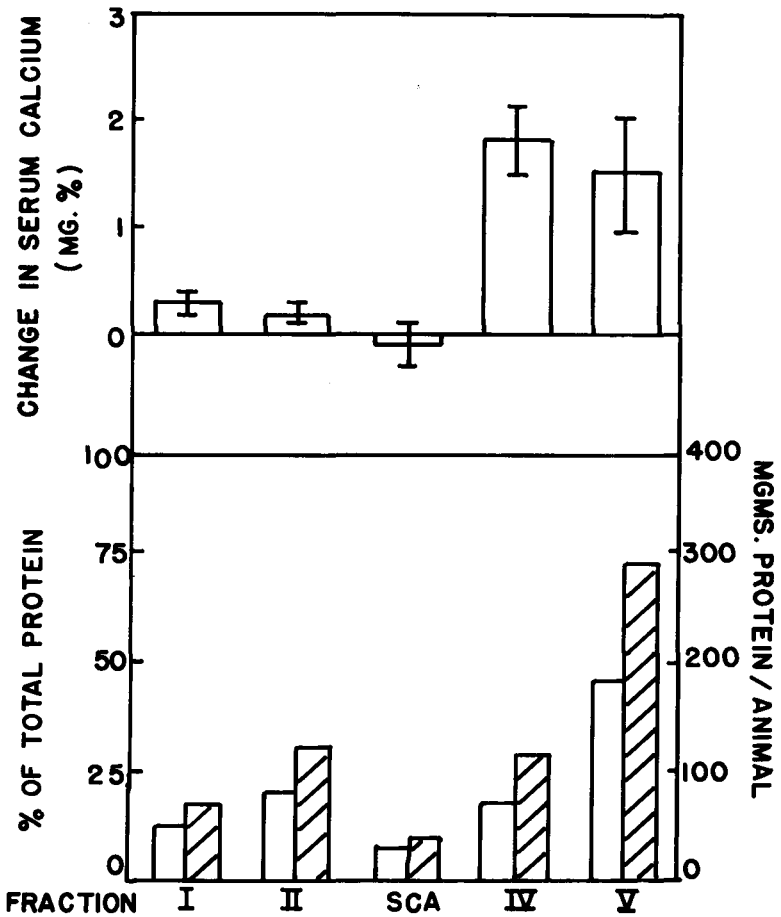

FIGURE 7

DISTRIBUTION OF PARATHYROID HORMONE ACTIVITY IN PLASMA PROTEIN FRACTIONS OBTAINED FROM FRESHLY COLLECTED RAT PLASMA TO WHICH HAD BEEN ADDED PARATHYROID EXTRACT, IN VITRO

Three main explanations may be forwarded to explain the lack of complete recovery of added hormone. 1) The semi-quantitative nature of these estimations of parathyroid activity could not be expected to account for all hormonal activity present. 2) The unaccounted-for activity could well have been distributed in other plasma fractions but at levels too small to be detected by our assay system. 3) It is possible that some of the parathyroid activity was destroyed as a result of the fractionation procedures. Probably all three of these factors are involved to some extend in our failure to obtain complete recovery of the added hormone. The 80% recovery reported here, however, is quite acceptable for this type of analysis. Gemzell et. al. (1955) for example, was able to recover only 25% of his added growth hormone activity from fractionated human plasma.

It is felt that the significance of the association of activity with Fraction IV (alpha-globulin) and Fraction V (albumin) is best discussed in light of subsequent experimentation and will, therefore, be considered at a later point in this dissertation.

Cold Ethanol Fractionation Of Hormonally Active Rat Plasma. In view of the success in obtaining a partition of hormonal activity among protein fractions of rat plasma to which had been added parathyroid extract in vitro, it was now decided to study the partition of hormonal activity among protein fractions of

hormonally active rat plasma. The preparation and securing of this material have been described in detail in Part A. of this chapter.

The administration of exogenous hormone to experimental animals and humans has been a technique utilized profitably by many workers to obtain a wide variety of information concerning plasma hormone characteristics. Bolinger (1959) used such an approach to confirm that the insulin-like activity of plasma was due to hormonal insulin and not to an artifact. Antoniades et. al. (1957) studied the binding and distribution of intravenously injected C-^{14} steroids and metabolites in human plasma and human plasma protein fractions obtained through cold ethanol fractionation. Greenspan et. al. (1950) employed intravenous injections of ACTH to study half-life of that hormone in the human circulation. Daughaday (1958) studied the binding of Cortisol-4-C^{14} with various plasma protein fractions after intravenous administration of that material.

In a report very pertinent to this work, Bethune et. al. (1957) studied the distribution of hormonal activity in plasma protein fractions of normal human subjects by means of the cold ethanol fractionation procedure, following intravenous infusion of ACTH. Activity was observed in the same fraction with which the endogenous ACTH activity of untreated normal human plasma subsequently was identified. Roberts (1957) also utilized this

approach to study the corticotrophic activity of rat plasma.

The technique of administrating exogenous hormone to experimental animals and humans, then, has permitted accumulation of important information concerning characteristics of a number of hormones as they exist in the plasma, and its use in this investigation, therefore, seemed justified.

Experimental. 500 USP units of parathyroid extract were administered via intraperitoneal injection to individual female rats of the Sprague-Dawley strain, weighing about 200 grams each. After one hour the animals were exanguinated to as great a degree as possible through use of the cardiac puncture technique. The plasma was collected at the centrifuge and immediately placed in the freezer (-20° C.) until ready for fractionation. In each experiment, a sufficient number of animals were sacrificed to provide 50 ml. of plasma. This usually required from 15 to 20 rats per experiment.

The day following its collection, the hormonally active rat plasma was subjected to cold ethanol fractionation according to the method of Roberts and Kelley (1956), described on page 88. the plasma protein fractions prepared in this manner were collected at the refrigerated centrifuge (-5° C.) and immediately placed in the freezer (-20° C.) until assayed. At that time the preparations were slowly warmed to room temperature in a warm water bath and reconstituted to a total volume of 25 ml. with

warm distilled water. Five ml. aliquots of the reconstituted fractions were administered intraperitoneally to each member of a five animal assay group.

Protein analysis of the various plasma fractions is shown in Table 16. The biological assay data from four individual experiments is presented in Table 17, and Figure 8 summarizes the results of these experiments.

The following observations may be made on the basis of the analytical data presented in Table 17.

a) Fraction I (fibrinogen) was prepared in experiments 2, 3 and 4, and was shown to lack detectable hormonal potency in each case.

b) Fraction II (beta and gamma globulins) was prepared in experiments 1, 2, 3 and 4, and was shown to be devoid of measurable hormonal activity in every instance.

c) Experiment 1 indicated the possible presence of hormonal activity in the combined protein of Fraction SCA (beta globulins), Fraction IV (alpha globulins) and Fraction V (albumins).

d) Fraction SCA, however, was found to be biologically inactive in experiment 3, and again in experiment 4.

e) Also, Fraction V was shown to lack hormonal potency in experiments 2, 3 and 4.

f) Experiment 2 indicated parathyroid activity to be present in Fraction IV, and this was confirmed in experiments 3 and 4.

TABLE 16

PROTEIN ANALYSIS OF PLASMA PROTEIN FRACTIONS OBTAINED FROM HORMONALLY ACTIVE RAT PLASMA

Fraction	Contents	Protein gm.%	mg. Protein In 50 ml. Plasma	% of Total Protein	Dose/animal mg. protein
I	Fibrinogen	0.6	300	10.2	60
II	Beta,Gamma-Globulins	1.1	550	18.8	110
SCA	Beta-Globulins	0.3	150	5.1	30
IV	Alpha-Globulins	1.0	500	17.0	100
V	Albumins	2.6	1,300	44.0	260
Total		5.6			
Whole Plasma		5.9			
Recovery		95%			

TABLE 17

BIOLOGICAL ASSAY OF PROTEIN FRACTIONS OBTAINED FROM HORMONALLY ACTIVE PLASMA

Experiment 1

Fraction II (Beta and Gamma-Globulins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	12.0	12.0	0.0
2	11.5	11.1	-0.4
3	8.2	8.2	0.0
4	5.4	7.6	2.2
5	12.5	11.5	-1.0

Mean and Standard Error 0.2 ± 0.5

Fraction SCA-IV-V (combined) (Beta-Globulin, Alpha-Globulin, Albumins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	9.2	10.2	1.0
2	8.6	12.0	3.4
3	6.4	7.4	1.0
4	9.3	7.4	-1.9
5	5.6	8.0	2.4

Mean and Standard Error 1.2 ± 0.9

TABLE 17 (Cont'd.)

Experiment 2

Fraction I (Fibrinogen)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.3	6.8	-0.5
2	6.6	6.6	0.0
3	6.6	7.0	0.4
4	10.0	10.4	0.4
5	6.2	6.7	0.5

Mean and Standard Error 0.2 ± 0.2

Fraction II (Alpha-Globulins, Beta-Globulins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.5	6.6	-0.9
2	7.4	6.6	-0.8
3	10.0	9.0	-1.0
4	6.7	7.0	0.3
5	8.4	8.8	0.4

Mean and Standard Error -0.4 ± 0.3

Fraction IV (Alpha-Globulins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.0	6.8	-0.2
2	6.8	8.0	1.2
3	8.5	10.1	1.6
4	6.4	8.4	2.0
5	7.3	7.1	-0.2

Mean and Standard Error 0.9 ± 0.5

TABLE 17 (Cont'd.)

Experiment 2 (Cont'd.)

Fraction V (Albumins)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.0	7.4	0.4
2	11.3	11.6	0.3
3	6.2	8.2	2.0
4	5.9	6.2	0.3
5	10.4	10.1	-0.3
		Mean and Standard Error	0.5±0.3

TABLE 17 (Cont'd.)

Experiment 3

Fraction I (Fibrinogen)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.6	6.9	0.3
2	7.5	7.7	0.2
3	8.5	8.5	0.0
4	7.1	7.6	0.5
5	7.0	7.2	0.2
		Mean and Standard Error	0.2 ± 0.1

Fraction II (Beta-Globulin, Gamma-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.5	7.8	0.3
2	6.5	7.0	0.5
3	6.7	6.4	-0.3
4	6.4	6.5	0.1
5	8.9	9.3	0.4
		Mean and Standard Error	0.2 ± 0.1

Fraction SCA (Beta-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.4	8.6	0.2
2	6.0	6.4	0.4
3	6.6	6.9	0.3
4	6.1	5.4	-0.7
5	6.3	6.3	0.0
		Mean and Standard Error	0.0 ± 0.2

TABLE 17 (Cont'd.)

Experiment 3 (Cont'd.)

Fraction IV (Alpha-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.9	8.6	-0.3
2	6.5	7.7	1.2
3	7.5	8.5	1.0
4	7.2	8.5	1.3
5	6.6	8.6	2.0

Mean and Standard Error 1.0 ± 0.4

Fraction V (Albumin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.5	6.9	0.4
2	6.2	6.1	-0.1
3	7.4	7.8	0.4
4	6.2	5.5	-0.7
5	6.6	6.9	0.3

Mean and Standard Error 0.1 ± 0.2

TABLE 17 (Cont'd.)

Experiment 4

Fraction I (Fibrinogen)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.3	7.3	0.0
2	8.0	8.3	0.3
3	7.0	6.8	-0.2
4	5.4	5.6	0.2
5	7.9	8.7	0.8

Mean and Standard Error 0.2 ± 0.2

Fraction II (Beta-Globulin, Gamma-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.6	7.7	0.1
2	7.8	8.5	0.7
3	5.5	5.5	0.0
4	7.0	6.7	-0.3
5	5.2	5.7	0.5

Mean and Standard Error 0.2 ± 0.2

Fraction SCA (Beta-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	4.6	4.7	0.1
2	5.2	5.0	-0.2
3	8.4	8.7	0.3
4	5.9	6.4	0.5
5	7.1	7.0	-0.1

Mean and Standard Error 0.1 ± 0.1

TABLE 17 (Cont'd.)

Experiment 4 (Cont'd.)

Fraction IV (Alpha-Globulin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.2	7.6	0.4
2	6.1	7.8	1.7
3	7.3	8.6	1.3
4	8.0	9.9	1.9
5	5.4	6.6	1.2
		Mean and Standard Error	1.3±0.3

Fraction V (Albumin)

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.3	7.4	0.1
2	6.1	5.9	-0.2
3	7.8	8.0	0.2
4	5.9	6.1	0.2
5	7.5	7.6	0.1
		Mean and Standard Error	0.1±0.1

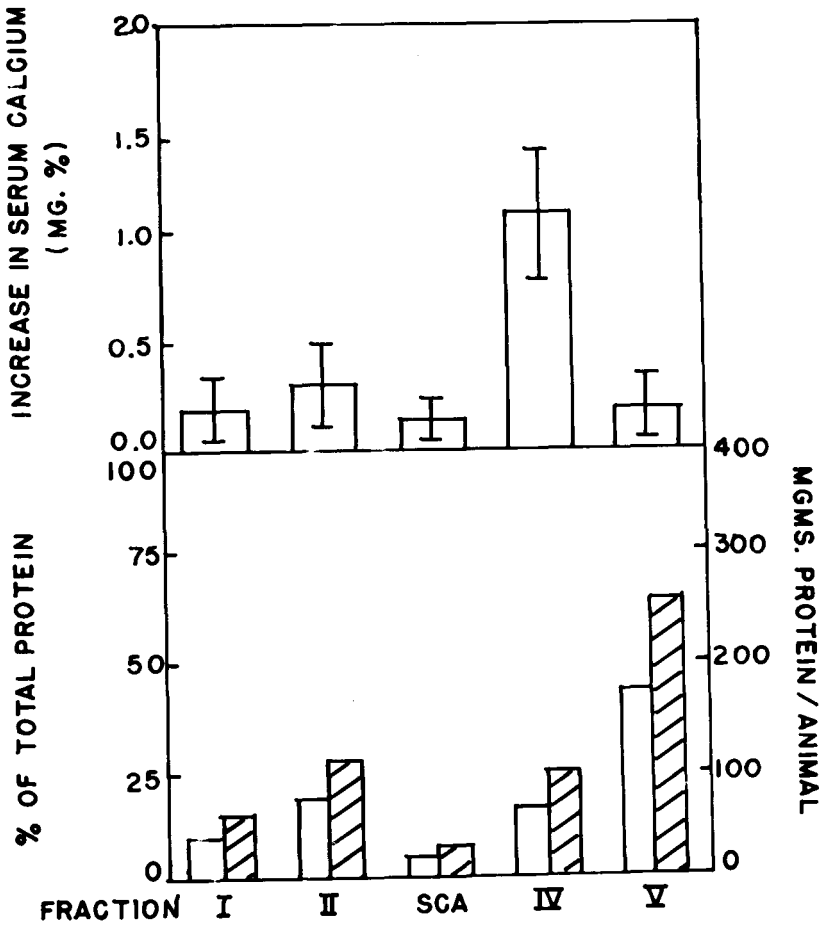

FIGURE 8

DISTRIBUTION OF PARATHYROID HORMONE ACTIVITY IN PLASMA PROTEIN FRACTIONS OBTAINED FROM HORMONALLY ACTIVE RAT PLASMA

The data presented, therefore, seem to clearly define a partition of plasma parathyroid activity among rat plasma protein fractions. This hormone activity appears to be localized primarily in Fraction IV, which contains alpha-globulins. The distribution pattern noted here is different from that observed when parathyroid extract was incubated with rat plasma in vitro, prior to fractionation. In the latter case, it will be recalled, hormonal activity was associated with Fraction V as well as with Fraction IV.

Explanations for this discrepancy and the possible significance of the variations in the distribution patterns of hormonal activity noted, will be presented in the discussion.

E. COLD ETHANOL FRACTIONATION OF NORMAL HUMAN PLASMA

Parathyroid Hormone Activity of Unfractionated Normal Human Plasma. Attempts to detect hormonal activity in untreated normal human plasma have resulted in the production of an extremely voluminous literature. A recent Ciba Foundation Colloquia on Endocrinology, "Hormones In The Blood," (1957) presents a composite report of advances made in this field. In it can be found literally hundreds of references dealing with attempts to detect various hormonal activities in whole plasma. Every known hormone has been subjected to studies of this type, with the exception (to the author's knowledge) of the parathyroid hormone.

The technique unfailingly applied in almost all of these cases has involved the administration of as large an amount as possible of whole plasma or plasma concentrated by lyophilization, to assay animals. The results obtained, as pointed out previously, are always entirely dependent upon the sensitivity of the assay system employed. One can never report, however, that there is "no activity present" in any whole plasma sample, but only that there is no detectable activity present, which is a completely different situation.

Since no information was available on the level of the parathyroid hormone in human plasma, it was decided to attempt to detect such activity in concentrated preparations of lyophilized whole plasma. Similar experiments with whole rat plasma were precluded due to the prohibitive number of rats which would have to be sacrificed in order to obtain sufficient volumes of plasma for lyophilization and analysis. In work of this nature involving rat plasma, the limiting factor would be the excessive number of rats required, while in the case of human plasma, which is readily available in relatively large volumes, the limiting factor becomes the amount of plasma protein which could be administered without adversely affecting the assay animal. These practical considerations were of considerable import in the decision to utilize normal human plasma rather than normal rat plasma for this particular investigation.

A series of studies were conducted to determine the amount of plasma protein which could be administered to the assay preparation without production of visual signs of toxicity. The maximum amount of plasma protein which could be administered in a total volume of five cc. without the production of any visible signs of toxicity was found to be 0.01 gram per gram of body weight, e.g. 1.5 grams of plasma protein per 150 gram rat. This dose limit was not exceeded in subsequent work involving human plasma protein test doses.

Plasma was obtained from a normal male donor (Negro, 36 years of age) at the Cook County Blood Bank of the Cook County Hospital, Chicago, Illinois, and was found to have a protein concentration of 6.0 gm.% 220 ml. of this plasma (13.2 grams of protein) were lyophilized and placed in the freezer until used for assay. 12.0 grams of this preparation, representing the protein present in 200 ml. of normal plasma, were then dissolved in distilled water to a total volume of 40 ml. Five ml. aliquots of this concentrated plasma protein solution were administered to each of eight assay animal. Each animal, then, received 1.5 grams of plasma protein, or the protein present in 25 ml. of normal human plasma. The results of the biological assay are shown in Table 18.

The data indicate that the level of parathyroid hormone activity in 25 ml. of normal human plasma is too small to be

TABLE 18

BIOLOGICAL ASSAY OF LYOPHILIZED, NORMAL HUMAN PLASMA

Dose: Protein equivalent of 25 cc. normal human plasma (1.5 grams protein) per animal.

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.6	7.3	-0.3
2	9.2	9.4	0.2
3	7.3	8.0	0.7
4	8.3	8.2	-0.1
5	7.9	6.7	-1.2
6	7.8	8.0	0.2
7	7.3	7.2	-0.1
8	6.9	7.0	0.1

Mean and Standard Error -0.1 ± 0.2

detected by our analytical system.

It has been shown, however, that the bioassay system employed is sensitive to at least 25 USP units of parathyroid hormone activity. On this basis, then, we may estimate that human plasma contains a parathyroid hormone level of less than 100 USP units of activity per 100 ml. of plasma.

Cold Ethanol Fractionation Method of Cohn et. al. (Method 6)

Since it was not possible to detect plasma parathyroid activity in lyophilized concentrates of whole human plasma, attention was now focused upon an approach similar to that used successfully in the investigations concerning localization of hormone activity in rat plasma protein fractions. The initial approach would be the study of the distribution of parathyroid hormone activity among protein fractions of normal human plasma, to which had been added parathyroid extract, in vitro. Plasma protein fractions would be obtained through application of the cold ethanol fractionation techniques of Cohn et. al. (1946) designed specifically for fractionation of human plasma.

During and shortly after World War II, Dr. E. J. Cohn and his associates at Harvard University developed methods for the large scale separation of blood plasma into protein fractions valuable to the medical services of the Armed Forces. (Edsall, 1947) (Cohn et. al., 1940) (Cohn et. al., 1944) (Cohn et. al., 1946) The procedure consists in the precipitation of plasma

proteins by different concentrations of ethyl alcohol at low temperatures, specific pH, and low ionic strength. The technique most often used is method 6 (Cohn et. al., 1946) by which the plasma is separated into five major fractions by alcohol precipitation. These fractions are designated as Fractions I, II-III, IV-I, IV-4 and V.

The details of method 6, as employed in this investigation, are presented below and anoutline of this procedure is shown in Figure 9.

"Plasma is stirred slowly over a magnetic stirrer and cooled as quickly as possible to 0° C. without permitting the formation of ice.

The stirring is continued while sufficient sodium acetate-acetic acid buffer in a 53.3 volume % (at 25° C.) ethanol water mixture is added to bring the pH to 7.2±0.2 and the final ethanol concentration to 8%. During such additions, the temperature is allowed to fall so that the system is maintained close to its freezing point and so that the final temperature is between -2.5° and -3° C. The initial step requires 0.177 liter (measured at -5° C.) of 53.3% ethanol for each liter of plasma (measured at 0° C.) and about one cc. of 0.8 molar sodium acetate, buffered at pH 4.0 with acetic acid, for each liter of plasma; this suffices for the pH adjustment.

Precipitate I consists principally of fibrinogen, which

is removed by centrifugation at a temperature between -2° and -3° C.

The supernatant is next brought to 25% ethanol and a pH of about 6.9 by the addition of cold 53.3% ethanol containing a sodium acetate-acetic acid buffer. This step requires, for each liter of supernate I, an ethanol buffer mixture made as follows; 601 cc. of 53.3% ethanol at -5° C., 0.88 cc. of 10M acetic acid at 25°C, 0.44 cc. of 4M sodium acetate at 25° C. and 2.30 cc. of 95% ethanol. The buffer used in this step has a molar ratio of sodium acetate to acetic acid of 0.2 and, if before the 53.3% ethanol is added, it is diluted eighty fold with water, it should have a pH of 4.00±0.02 at 25° C. No attempt has been made to vary the composition of the buffer so as to adjust the system to an exact pH and, indeed, the system varies by several tenths of a pH unit as a result of the change in the carbon dioxide concentration. The buffer added in the additions contributes 14 milliequivalents of acetic acid for each liter of plasma.

Precipitate II-III is removed by centrifugation at -5° C. and by electrophoretic measurement, consists principally of beta and gamma globulins. The supernatant from precipitate II-III is brought to a pH of 5.2 and 18% ethanol, by the addition of water and a sodium acetate buffer. This addition is carried out in two steps. The first step consists of the

addition of 311 cc. of water at 0°C. for each liter of supernate II-III. The second step is the addition to each liter of supernate II-III of 78 cc. of water at 0°C. containing enough of a sodium acetate-acetic acid buffer to lower the pH to 5.2±0.2. The mole ratio of sodium acetate to acetic acid in the buffer is 0.2.

Precipitate IV-1 is removed by centrifugation at a temperature of -5°C. By electrophoretic measurements it is shown to consist primarily of alpha globulins and lipid material. The supernate is next brought to a pH of 5.80±0.05, an ionic strength of 0.09 and an ethanol concentration of 40% at -5°C. The buffer is added first and after this the system is brought to 40% ethanol by the addition of 456 cc. of cold ethanol for each liter of supernate IV-1 while the temperature is maintained at -5°C.

Precipitate IV-4 is next removed by centrifugation at -5°C. and by electrophoretic measurements is shown to contain principally alpha and beta globulins, with some albumins. The supernate is lowered to pH 4.8 by the addition of a sodium acetate-acetic acid buffer, while the temperature is held at -5°C. and the ethanol concentration at 40%. For this addition the buffer is made up by taking for each liter of supernatant, 5.00 cc. of 10 N acetic acid, 2.5 cc. of 4 M sodium acetate, 10.5 cc. of 95% ethanol and enough water to make 25cc.

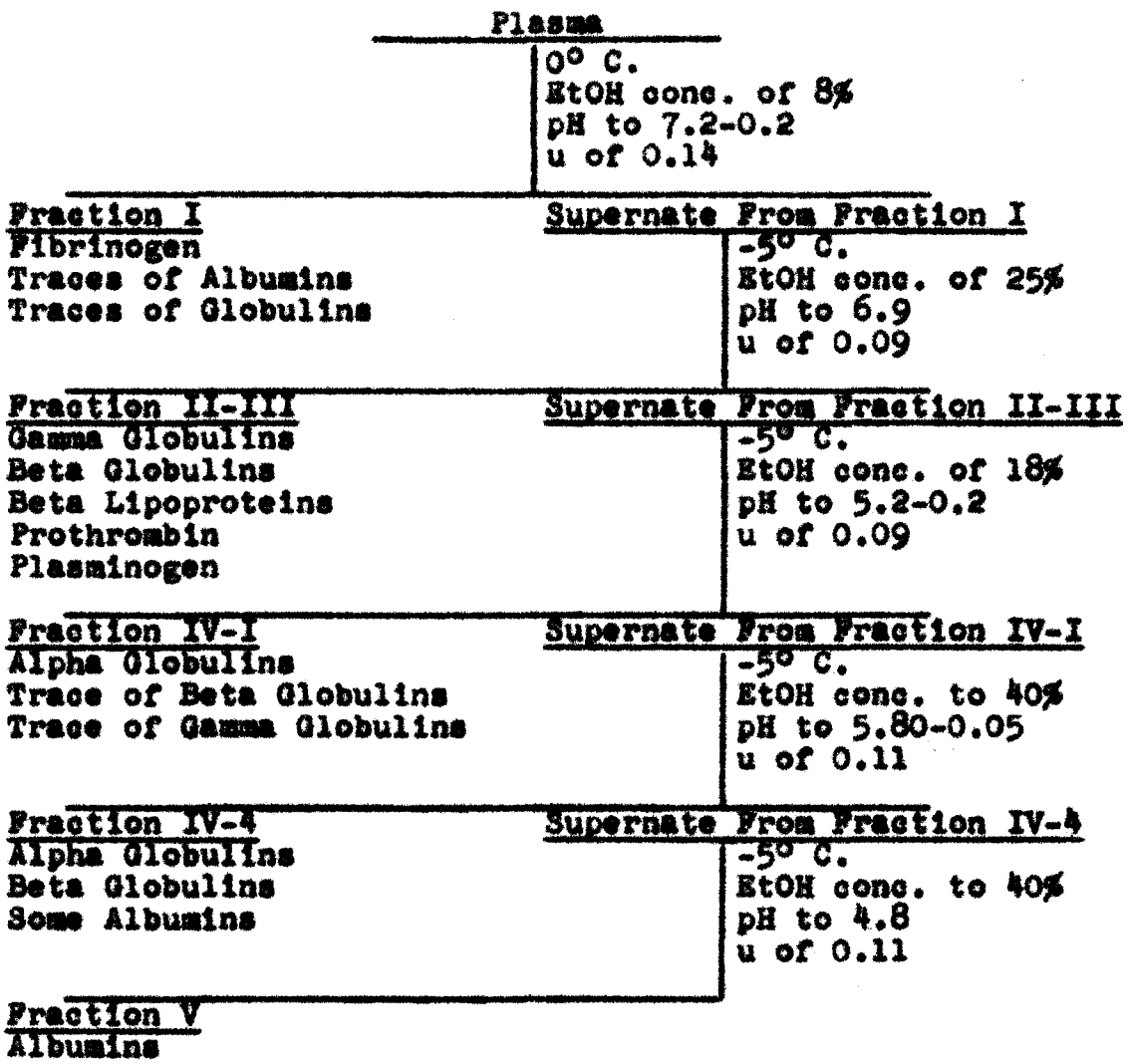

FIGURE 9

OUTLINE OF COLD ETHANOL FRACTIONATION TECHNIQUE

OF COHN ET. AL. (METHOD 6)

Precipitate 5 consisting of the plasma albumins, is collected by centrifugation at -5°C. "

Cold Ethanol Fractionation Of Normal Human Plasma To Which Had Been Added Parathyroid Extract, In Vitro. In order to study the distribution of parathyroid activity in protein fractions of human plasma, 100 ml. of plasma was collected from a normal donor (Male,Caucasian, 28 years of age) at the Cook County Blood Bank, Cook County Hospital Chicago, and in it were dissolved 500 USP units of lyophilized parathyroid extract. The mixture was allowed to stir for one hour over a slow magnetic stirrer, after which time it was subjected to cold ethanol fractionation as described above. The fractions were collected at the refrigerated centrifuge and then placed immediately in the freezer until assayed. At that time they were warmed to room temperature in a warm water bath and dissolved in warm distilled water to a total volume of 35 cc. Five cc. aliquots of this reconstituted preparation were administered to each of 7 animals of the assay group. The activity pattern observed in duplicate experiments is shown in Table 19, and a composite picture of these results is shown in Figure 10.

TABLE 19

BIOLOGICAL ASSAY OF PROTEIN FRACTIONS OBTAINED FROM NORMAL HUMAN PLASMA TO WHICH HAD BEEN ADDED PARATHYROID EXTRACT, IN VITRO

Experiment 1

Protein Analysis

Fraction	gm. % Protein	% of Total Protein	Animals per Assay Group	Dose/Animal mg. Protein
I	0.30	5.5	7	43
II-III	1.49	26.9	7	210
IV-I	0.34	6.1	7	48
IV-4	0.38	6.9	7	54
V	3.02	55.0	7	430
Total	5.54			
Whole Plasma	5.90			
% Recovery	93			

Biological Analysis

Fraction I

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.7	7.3	0.6
2	6.5	7.1	0.6
3	7.2	7.7	0.5
4	7.7	8.5	0.8
5	11.7	10.4	-1.3
6	8.4	9.1	0.7
7	6.9	7.3	0.4
		Mean and Standard Error	0.3 ± 0.3

TABLE 19 (Cont'd.)

Experiment 1 (Cont'd.)

Biological Analysis

Fraction II-III

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.5	10.3	1.8
2	6.8	8.2	1.4
3	6.3	7.5	1.2
4	8.5	11.3	2.8
5	7.2	8.7	1.5
6	7.3	11.2	3.9
7	6.0	8.0	2.0
		Mean and Standard Error	2.1±0.4

Biological Analysis

Fraction IV-I

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	9.0	8.6	-0.4
2	8.2	8.5	0.3
3	7.6	7.7	0.1
4	7.0	7.0	0.0
5	9.9	10.5	0.6
6	8.6	9.2	0.6
7	6.0	7.9	1.9
		Mean and Standard Error	0.4±0.3

TABLE 19 (Cont'd.)

Experiment 1 (Cont'd.)

Biological Analysis

Fraction IV-4

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.1	6.8	-0.3
2	9.2	9.6	0.4
3	8.2	8.7	0.5
4	6.2	6.9	0.7
5	7.2	9.0	1.8
6	7.4	7.8	0.4
7	9.3	10.0	0.7
		Mean and Standard Error	0.6±0.2

Biological Analysis

Fraction V

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.5	8.0	0.5
2	6.2	5.5	-0.7
3	8.1	8.9	0.8
4	6.9	6.5	-0.4
5	8.6	8.0	-0.6
6	9.1	8.5	-0.6
7	7.6	7.2	-0.4
		Mean and Standard Error	-0.2±0.2

TABLE 19 (Cont'd.)

Experiment 2

Protein Analysis

Fraction	gm.% Protein	% of Total Protein	Animals per Assay Group	Dose/Animal mg. Protein
I	0.30	5.5	7	43
II-III	1.49	27.1	7	214
IV-I	0.35	6.4	7	50
IV-4	0.37	7.0	7	53
V	3.03	55.0	7	420
Total	5.55			
Whole Plasma	5.89			
% Recovery	95			

Biological Analysis

Fraction I

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.9	7.1	0.2
2	7.9	8.4	0.5
3	8.4	8.9	0.5
4	7.6	7.3	-0.3
5	6.8	6.8	0.0
6	7.5	8.0	0.5
7	7.7	8.1	0.4
		Mean and Standard Error	0.3±0.1

TABLE 19 (Cont'd.)

Experiment 2 (Cont'd.)

Biological Analysis

Fraction II-III

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.7	9.6	1.9
2	8.4	10.2	1.8
3	8.5	11.3	2.8
4	8.1	10.2	2.1
5	7.8	10.5	2.7
6	7.4	8.9	1.5
7	7.7	9.9	2.2
		Mean and Standard Error	2.1±0.2

Biological Analysis

Fraction IV-I

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.1	7.2	0.2
2	7.8	8.1	0.3
3	8.5	8.3	-0.2
4	7.7	8.7	1.0
5	6.4	6.7	0.3
6	8.7	9.0	0.3
7	8.5	9.0	0.5
		Mean and Standard Error	0.3±0.1

TABLE 19 (Cont'd.)

Experiment 2 (Cont'd.)

Biological Analysis

Fraction IV-4

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.1	7.5	0.4
2	7.5	8.7	1.2
3	8.3	8.5	0.2
4	6.9	8.2	1.3
5	8.0	8.3	0.3
6	6.4	6.9	0.5
7	6.3	6.2	-0.1
		Mean and Standard Error	0.5±0.2

Biological Analysis

Fraction V

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.0	6.8	-0.2
2	7.3	7.1	-0.2
3	7.9	8.0	0.1
4	7.1	7.1	0.0
5	8.3	8.7	0.4
6	8.2	8.0	-0.2
7	7.7	8.3	0.6
		Mean and Standard Error	0.1±0.1

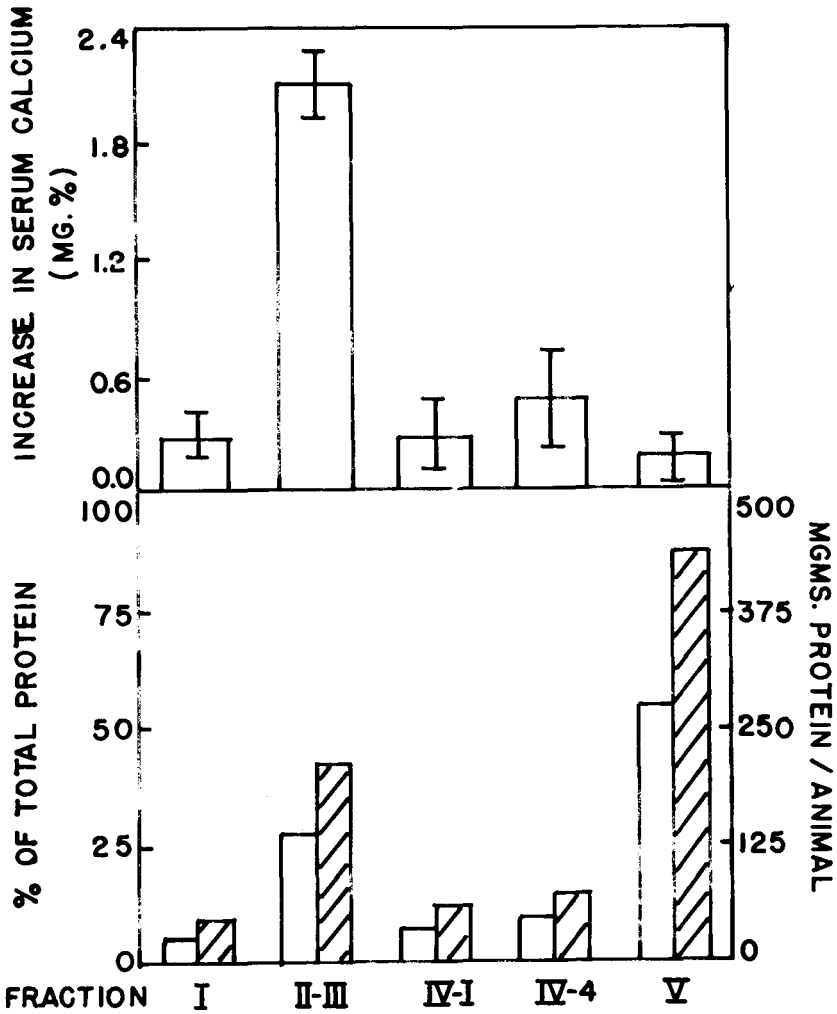

FIGURE 10

DISTRIBUTION OF PARATHYROID HORMONE ACTIVITY IN PLASMA PROTEIN FRACTIONS OBTAINED FROM NORMAL HUMAN PLASMA TO WHICH HAS BEEN ADDED PARATHYROID EXTRACT, IN VITRO

The experimental results recorded here strongly suggest a localization of exogenous parathyroid hormone activity in Fraction II-III of normal human plasma, which is composed primarily of beta-globulins, gamma-globulins and beta-lipoproteins. It is also important to note that the biological activity recovered does not seem to be distributed to any significant extent among the plasma protein fractions, but appears to be concentrated almost completely in Fraction II-III.

The mean response of the assay group utilized in the bio-assay of Fraction II-III is in the area of the response which would be elicited by at least 50 USP units of parathyroid hormone activity (Table 2 and 3). The seven animals of the assay group employed, then, may be considered to be responding to a total of approximately 350 USP units of activity as a minimum estimation. This represents a recovery of 70% of added parathyroid hormone activity. The reasons for inability to obtain complete recovery of added activity in work of this type have been discussed previously in connection with the addition of parathyroid extract to rat plasma.

It is interesting to note at this time, that Fraction II-III of human plasma is the fraction with which plasma ACTH activity (Bethune, 1957), plasma gonadotrophic activity (McArthur et. al., 1956) (Antoniades, 1957), plasma insulin activity (Beigelman, 1956 a) and plasma thyrotrophic hormone activity (Queredo and

Lameyer, 1956) have been found associated.

Cold Ethanol Fractionation Of Normal Human Plasma. Concentrates of unfractionated normal human plasma have been shown to lack sufficient parathyroid hormone potency to be detected by our analytical system. In view of this, and considering the results obtained in the previous section, it was now decided to apply cold ethanol fractionation techniques to normal plasma and assay the resulting concentrated plasma protein fractions for parathyroid hormone activity. In this way relatively large volumes of normal plasma could be utilized for plasma parathyroid hormone analysis.

Experiment 1. 560 mls of normal human plasma were obtained from two normal male donors (Caucasian, ages 23 and 42) at the Cook County Blood Bank of the Cook County Hospital,Chicago. The plasma was then subjected to cold ethanol fractionation in the usual manner. The resulting fractions were lyophilized and stored in the freezer until used for assay, at which time they were warmed to room temperature in a warm water bath and reconstituted tosufficient volume so that each animal of the assay group received no more than 5 ml. aliquots of the reconstituted solution. This was possible in all cases except that of Fraction V (albumin). Enough of this fraction was utilized for analysis so that each animal of a nine animal assay group received the maximum permissible dosage of protein, 1.5 gram/150 gram animal. The

protein analysis of the various fractions are listed in Table 20, the biological assay data are shown in Table 21 A.

The results indicate detection of endogenous plasma parathyroid hormone activity in concentrates of protein Fraction II-III of normal human plasma. No activity was detected in any of the other fractions assayed. This agrees with the results obtained in the previous section where exogenous parathyroid activity was also primarily associated with Fraction II-III.

Experiment 2. In order to confirm the previous results, the following experiment was performed.

550 ml. of normal human plasma were obtained from two normal male, Caucasian donors (ages 22 and 34) at the Cook County Blood Bank, Cook County Hospital, Chicago. This plasma was subjected to cold ethanol fractionation in the usual manner, with the resulting plasma protein fractions being lyophilized and placed in the freezer until utilized for assay. In this experiment, Fraction II-III was assayed, and Fraction I, IV-I, IV-4 and V were combined and then assayed as a combined fraction.

The experimental results are outlined in Table 21 B.

TABLE 20

PROTEIN ANALYSIS OF PLASMA FRACTIONS OBTAINED FROM NORMAL HUMAN PLASMA BY THE COLD ETHANOL FRACTIONATION METHOD OF COHN ET. AL.

Fraction	gm.% Protein	% Total Protein	gm./560 ml.	Animals per Assay Group	Dose/Animal mg. Protein	Plasma cc. Equivalent/Animal
I	0.30	5.4	1.70	5	330	112
II-III	1.51	27.2	8.46	8	1,060	70
IV-I	0.35	6.3	1.97	5	394	112
IV-4	0.38	6.8	2.14	5	429	112
V	3.03	54.5	17.10	9	1,500	38
Total	5.57					
Whole Plasma	5.71					
% Recovery	98					

TABLE 21 A

BIOLOGICAL ASSAY OF PROTEIN FRACTIONS FROM NORMAL HUMAN PLASMA

Fraction I

Dose: 112 plasma cc. equivalents of Fraction I protein/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.4	7.4	0.0
2	7.0	7.1	0.1
3	6.7	6.6	-0.1
4	7.3	7.5	0.2
5	7.8	8.0	0.2
		Mean and Standard Error	0.1 ± 0.1

Fraction II-III

Dose: 70 plasma cc. equivalents of Fraction II-III protein/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.4	8.4	1.0
2	7.9	9.2	1.3
3	7.1	7.9	0.8
4	6.6	7.6	1.0
5	7.3	8.1	0.8
6	6.8	8.3	1.5
7	6.8	7.3	0.5
8	7.6	8.7	1.1
		Mean and Standard Error	1.0 ± 0.2

TABLE 21 A (Cont'd.)

Fraction IV-1

Dose: 112 plasma cc. equivalents of Fraction IV-1 protein/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	6.2	6.4	0.2
2	6.7	6.6	-0.1
3	7.2	7.2	0.0
4	6.1	6.2	0.1
5	7.7	7.9	0.2
		Mean and Standard Error	0.1±0.1

Fraction IV-4

Dose: 112 plasma cc. equivalents of Fraction IV-4 protein/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.0	7.0	0.0
2	6.3	6.7	0.4
3	6.8	7.1	0.3
4	7.3	7.4	0.1
5	7.0	7.2	0.2
		Mean and Standard Error	0.2±0.1

TABLE 21 A (Cont'd.)

Fraction V

Dose: 38 plasma cc. equivalents of Fraction V protein/animal

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	8.2	8.3	0.1
2	7.9	8.2	0.3
3	8.3	8.3	0.0
4	7.6	8.0	0.4
5	7.0	7.4	0.4
6	8.6	8.7	0.1
7	7.5	8.0	0.5
8	7.1	7.3	0.2
9	7.6	7.6	0.0
		Mean and Standard Error	0.2 ± 0.1

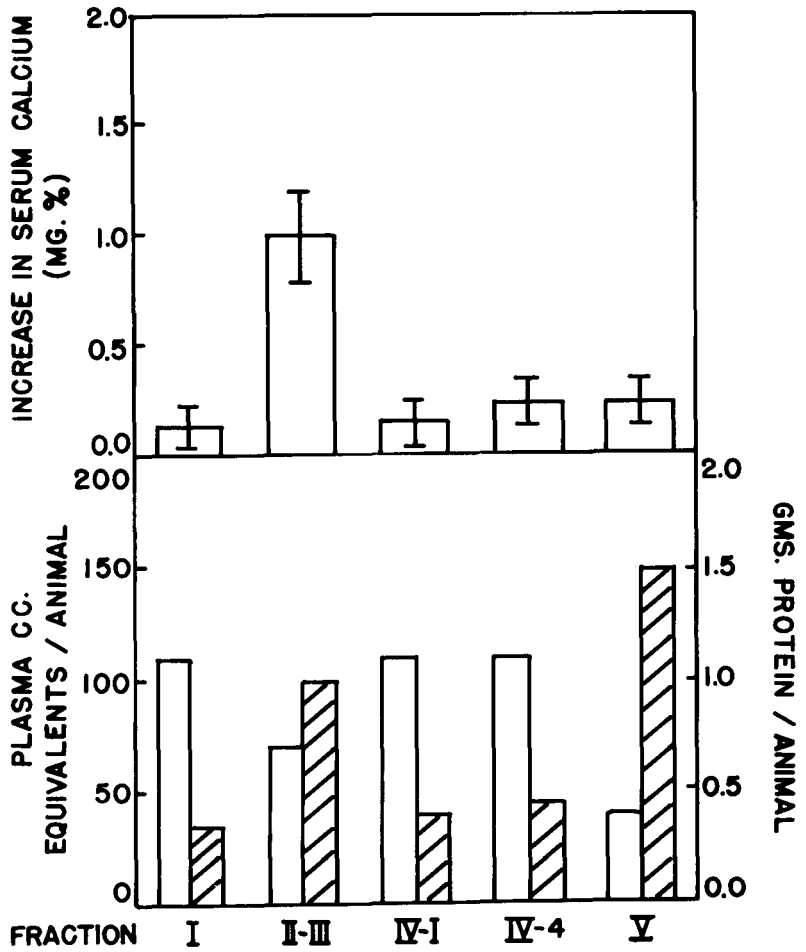

FIGURE 11

DISTRIBUTION OF PARATHYROID HORMONE ACTIVITY IN PLASMA PROTEIN FRACTIONS OBTAINED FROM NORMAL HUMAN PLASMA

TABLE 21 B

BIOLOGICAL ASSAY OF PROTEIN FRACTIONS FROM NORMAL HUMAN PLASMA

Experiment 2

Protein Analysis

Fraction	gm.%	gm/550 ml. Plasma	Animals in Assay Group	Dose/Animal gms. Protein	Plasma cc. equiv./animal
II-III	1.5	8.3	8	1.03	69
Combined Fractions I, IV-I, IV-4, V	4.0	23.6	12	1.5	37
Total	5.5				
Whole Plasma	5.9				
% Recovery	93				

Biological Analysis

Fraction II-III

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.0	8.5	1.5
2	8.3	9.4	1.1
3	6.2	8.1	1.9
4	5.9	5.9	0.0
5	7.8	7.8	0.0
6	8.5	9.9	1.4
7	9.0	10.5	1.5
8	6.8	7.7	0.9

Mean and Standard Error 1.0 ± 0.3

TABLE 21 B (Cont'd.)

Assay of Combined Fractions I, IV-1, IV-4, V

Animal Number	Initial Serum Calcium, mg.%	Final Serum Calcium, mg.%	Change In Serum Calcium, mg.%
1	7.7	7.8	0.1
2	6.4	6.9	0.5
3	6.9	6.8	-0.1
4	8.4	8.7	0.3
5	11.6	11.8	0.2
6	7.8	7.9	0.1
7	6.9	6.9	0.0
8	6.7	6.6	-0.1
9	5.9	6.4	0.5
10	8.3	8.7	0.4
11	6.9	7.1	0.2
12	7.6	7.6	0.0
		Mean and Standard Error	0.2±0.1

The results observed in experiment 2 corroborate those obtained in experiment 1, that is, endogenous plasma parathyroid activity has been detected in protein Fraction II-III of normal human plasma. It will be noted that the magnitude of the mean response of the assay group of both experiments are strikingly similar, and are of the order which one would expect from an assay group responding to doses of 25 USP units of parathyroid activity per animal. We have, then, the basis for an initial semi-quantitative extimation of circulatory levels of endogenous plasma parathyroid hormone activity in humans. This will be considered in detail in the following section.

CHAPTER VII

DISCUSSION AND CONCLUSIONS

For purposes of discussion, this dissertation may be considered to consist of two main sections. The first of these concerns the description and experimental evaluation of the biological assay system to be used, and the second deals with the application of this analytical system to the study of plasma parathyroid hormone activity. The former aspect has been considered in detail in Chapter IV and Chapter V. This discussion, then, will concern itself primarily with the data recorded in those experiments designed to investigate the properties of the parathyroid hormone as it exists in the plasma.

Since it has been shown in preliminary work that normal rat plasma lacked sufficient parathyroid hormone potency to be detected by the biological assay system, it was necessary, in order to study plasma parathyroid hormone activity, to develop a technique for the preparation of rat plasma with sufficient hormone potency for detection by the analytical system in use. This was accomplished by the administration of large doses of parathyroid extract to rats intraperitoneally, followed by exanguination after one hour. The efficacy and reproducibility of this method have been repeatedly demonstrated throughout this dissertation. The intraperitoneal route of administration was chosen because evidence is available to indicate that intravenously

administered protein hormones are rapidly inactivated by the host animal. (Greenspan et.al. 1950) (Pincus et.al. 1952) (Sydnor et.al. 1953) (Wolstenholme and Millar, 1957) The mechanism of this in vivo inactivation of protein hormones by the body has, in itself, been the subject of much investigation. A possible explanation of this phenomenon has been suggested in a report by Mirsky et.al. (1959). These workers confirmed the findings of White and Grass (1957) that the plasma proteolytic enzyme, plasmin, was capable of rapidly hydrolyzing ACTH and further observed the enzyme to similarly effect glucagon and somatotrophin. Interestingly, Insulin was shown to be resistant to such inactivation, a fact which tends to confirm current thought that Insulin is inactiva primarily in the liver and not in the circulation. (Mirsky et.al. 1949) (Mirsky et.al. 1953) (Mirsky et.al. 1955)

In addition to the removal of excess hormonal activity from the plasma by a destruction of the active molecule as suggested above, there are other, more subtle concepts, which could be visualized. The organism could, for example, render excess hormone physiologically inocuous by some reversible oxidation-reduction phenomena, or, perhaps, by a binding of the active hormone to plasma proteins or other tissue in such a manner as to prevent it from exerting its biological potency. Under such conditions the body would have excess hormone available in the blood stream at all times, ready for instant conversion to a physiologically active form

in response to any emergency. Ingle (1959), for example, has pointed out that the rapid disappearance of endogenous or exogenous ACTH from the blood need no longer be attributed directly to a destruction of the ACTH molecule in the circulation, since ACTH has been shown capable of binding to tissues, espicially the kidney (Sydnor and Sayers, 1953) and of being released from these sites by other peptides which it is thought, either displaces it by competing for possible binding sites, or forces it out in some unknown manner.

In view of the oft proposed association of parathyroid hormone function with that of the kidney (Albright, 1948) (Greep and Kenny, 1955) (Goldman et.al. 1958) (Buchanan et.al. 1959) (Talpers and Stein, 1959), a parathyroid hormone-kidney binding phenomenon similar to that observed with ACTH appears an intriguing possibility.

At the present time there is no direct evidence available concerning the mechanism or rate of inactivation of parathyroid hormone in the circulation. There is, however, some data which suggests that the plasma parathyroid hormone is not rapidly inactivated or destroyed as are some of the other plasma hormones. Davies and Fraser (1956) and Davies (1958) have demonstrated parathyroid hormone activity to be present in urine from normal and hyperparathyroid individuals. The possibility exists, therefore, that excess or unutilized plasma parathyroid hormone activity is ultimately excreted in the urine.

It has been demonstrated that the technique of massive intraperitoneal administration of parathyroid extract to rats results in a plasma containing detectable levels of parathyroid hormone activity up to three hours subsequent to its intraperitoneal injection. Apparently, then, excess parathyroid hormone is not rendered physiologically inactive as rapidly as are excess amounts of other protein hormones.

It is commonly accepted, and indeed seems axiomatic, that any hormone present in excess quantities in the circulation must be rapidly destroyed or rendered inactive if the body is to maintain homeostasis. The exogenous parathyroid hormone which enters the circulation subsequent to intraperitoneal injection, then, must either be not present in levels capable of upsetting homeostasis, or if present in such levels, must be prevented from exerting its physiological effects by some as yet unknown mechanism. If this is so, the next logical assumption would appear to be that the sustained high levels of parathyroid activity noted in rat plasma one and three hours after injection of the parathyroid extract, reflect a slow, steady diffusion of hormonal activity into the rat circulatory system, in levels which permit detection by the biological assay system, but which are not sufficiently high at any one time to upset homeostasis or trigger a parathyroid hormone inactivating mechanism. Such a slow passage of exogenous hormone into the circulatory system would seem more

compatable with the objectives of this investigation then, for example, the massive flooding of the blood with large quantities of exogenous hormone by such methods as intravenous injections. In the latter case there is reason to believe that the excess hormone would bind to plasma protein fractions with which it was not normally associated (Roberts, 1957) (Robbins and Rall, 1957). In the former situation, that of intraperitoneal injection of exogenous hormone, it was felt that a flooding of binding sites on the plasma protein normally associated with endogenous hormone would not occur, or would be much less likely to occur.

The technique developed for preparation of plasma containing detectable levels of parathyroid hormone activity (hormonally active plasma) then, would seem to afford the best available method for introduction of excess hormone into the circulation in such a manner as to assure its association with the same plasma protein as the endogenous secretions of the parathyroid glands.

The question now arises as to whether the hormonally active molecular species present in the parathyroid extract utilized in this investigation is identical with the endogenous secretions of the parathyroid glands?

It is, of course, extremely difficult and dangerous to make categorical statements in such matters. One can,however, make reasonable assumptions based upon documented experimental observati In general, we can state that it has been conclusively demonstrated

that administration of parathyroid extract reverses the biochemical changes of hypoparathyroidism. Also, administration of excess parathyroid extract to animal and man produce a chemical syndrome identical to that observed in hyperparathyroidism (Howard, 1956). Observations such as these have historically been considered ample to indicate presence of a true hormone in a test preparation and, indeed, are the basis of the science of endocrinology. It seems logical to assume, therefore, that the parathyroid extract employed in this work contains to at least some degree, the active secretion of the parathyroid gland.

Although hormonally active rat plasma preparations were, therefore, available for chemical fractionation studies, it was necessary to determine whether or not partition of the plasma parathyroid activity among plasma protein fractions was a feasible undertaking. The classical ammonium sulfate fractionation procedure was applied in order to ascertain if a crude partition of hormonal activity could be attained.

Inherent in any ammonium sulfate fractionation work is the problem of subsequent removal of the salt by dialysis. It became necessary, then, to observe the effect of dialysis upon the activity of our parathyroid preparations. The results which we recorded show quite definitely that under the conditions of our experiments, dialysis of parathyroid extract results in a dramatic reduction of biological potency, but that the identical dialysis procedure does

not alter the biological potency of parathyroid extract when in the presence of rat plasma, nor does dialysis affect the potency of hormonally active rat plasma.

The practical effects of these observations were primarily that we could now proceed with the initial crude ammonium sulfate fractionation of hormonally active rat plasma. The theoretical implications of these observations, however, merit further consideration. Two main questions come to mind in this regard, namely, a) what was the nature of the inactivation of the parathyroid extract by dialysis and b) how does one explain the "protective effect" of the plasma proteins upon this phenomenon. Loss of activity of the parathyroid extract upon dialysis was, in itself, a rather surprising observation. The parathyroid hormone has historically been considered to be protein in nature and, indeed, dialysis has been utilized as a preparative and purification technique, although under conditions different from those employed here (Melius, 1956).

There appear to be three main theories which could be forwarded to explain the loss of activity of the parathyroid extract upon dialysis. 1) The dialysis procedure could have inactivated the active principle of the extract due to changes in the physical environment within the dialysis bag, such as changes in pH or loss of dialyzable cofactors such a metal ions. The change in pH subsequent to dialysis of the extract, however, was slight and the

final pH after dialysis was not one known to result in a loss of biological activity by the parathyroid extract. Rasmussen (1959) has recently suggested the possibility that the parathyroid hormone may require a metal ion as a cofactor, but this possibility has, as yet, received no experimental verification. 2) The second possible explanation for the inactivation phenomena observed subsequent to dialysis is that the active principle of the extract became immeshed in the fibers of the dialyzing membrane. There have been a number of reports, however, dealing with dialysis and ultra-filtration of parathyroid preparation with no mention of such a situation occurring. 3) Another possibility is that the active principle passed through the dialyzing membrane employed. Davies and Gordon (1953) (1954) reported that they had observed the hormonal potency of their parathyroid preparation to pass through cellophane membranes upon ultrafiltration. Rasmussen and Westfall (1954) reported that up to 95% of the calcium mobilizing activity of their hydrochloric acid extract had passed through a cellophane membrane upon ultrafiltration. Kenny et. al. (1956) reported both the calcium-mobilizing and the phosphaturic activities of his parathyroid extract preparations to be dialyzable to "a considerable extent." Further, the active principle obtained from the parathyroid glands by acid extractions has recently been assigned a molecular weight of about 10,000 (Rasmussen, 1957). Craig et. al., (1958) in a detailed review of the technique of

dialysis, has reported that under various conditions they have observed such molecular species as Trypsin (MW 20,000), Pituitary Lactogenic Hormone (MW 26,000) and Ovalbumin (MW 50,000) to be capable of passing through visking dialyzing membranes to various degrees. The evidence presented here, therefore, would seem to strongly suggest that what has been observed in this work was a passage of the active principle of the extract through the dialyzing membrane.

If this is so, the next logical inference to be drawn from the data is that plasma proteins in some way prevent this dialysis from occurring. Roberts (1957) recovered corticosteroid releasing activity in dialyzed, lyophilized rat plasma proteins and considered this as indicating that the hormonal moeity was either protein or protein-bound. Daughaday (1956) found that the rate of dialysis of cortisone was slowed down markedly by the presence of plasma proteins and concluded that the difference in rates of dialysis was due to a binding of the hormone to plasma proteins. Heller and Lederis (1957) on the basis of similar data, suggested a binding of the pressor and oxytocic principles of the anterior pituitary to plasma proteins. Pitt-Rivers (1957) was unable to separate thyroxin from certain plasma proteins by dialysis and reported this to be an indication of binding of the hormone by a plasma protein. These reports seem to demonstrate that the binding of a hormone to plasma proteins is capable of decreasing

its rate of dialysis and conversely, a decreased rate of dialysis of a hormone when in the presence of plasma proteins is usually indicative of a binding of the hormone to the plasma protein. It is felt, therefore, that the strong presumptive evidence exists to suggest that what has been observed in this investigation was a binding of the parathyroid principle to a plasma protein, with a resultant inability of the bound hormonal moeity to pass through the dialyzing membrane.

The information obtained from the dialysis experiments confirmed the feasability of applying salt fractionation to the study of plasma parathyroid hormone activity. Ammonium sulfate fractionation of hormonally active rat plasma resulted in a definite partition of hormonal activity in the "globulin" precipitate. There was no activity observed in the supernate or albumin fraction.

Since some success has been realized through use of this classical but relatively crude ammonium sulfate fractionation, it was next decided to utilize the more refined cold ethanol fractionation techniques in attempts to localize parathyroid activity in rat plasma protein fractions.

Hormonally active rat plasma was subjected to cold ethanol fractionation and parathyroid activity was found localized in Fraction IV, which consists primarily of alpha-globulins. No activity was detected in Fraction I (fibrinogens), Fraction II

(beta and gamma globulins), Fraction SCA (beta-globulins) or Fraction V (albumins). It will be recalled that ammonium sulfate fractionation of hormonally active plasma had indicated an association of parathyroid activity in the plasma "globulin" precipitate. The results observed upon cold ethanol fractionation of hormonally active plasma, therefore, were not surprising and appeared a confirmation of the validity of the experimental approach.

Cold ethanol fractionation of rat plasma to which had been added parathyroid extract, in vitro, however, resulted in an association of hormonal activity not only with the alpha-globulin fraction as in the previous instances, but also with the plasma albumin fraction.

What is the explanation and significance of this variation in the distribution pattern of parathyroid activity among plasma protein fractions?

Roberts (1957 C) has reported that exogenous thyrotrophic activity appeared primarily in the albumin fraction of rat plasma, but also "spilled over" into the other protein fractions. Robbins and Rall (1955) studied the binding of thyroxin by thyroxin binding protein (TBP) in human plasma. These workers added thyroxin to human plasma, in vitro, and concluded that the thyroxin binds preferentially with TBP until all of the binding sites are saturated, after which it binds to other proteins.

Roberts (1957 A) utilizing cold ethanol fractionation techniques, added ACTH to rat plasma in vitro, and compared the distribution of hormonal activity in the various plasma protein fractions with that observed in the protein fractions of plasma collected from rats which had been infused with ACTH intravenously prior to exanguination and fractionation. It was found that hormonal activity in the latter case was associated primarily with the albumin fraction, but that in the former case, where the ACTH had been added to the plasma in vitro, hormonal activity was associated not only with the albumin fraction, but with the other protein fractions as well.

On the basis of these observations, Roberts (1957 A) concludes that "endogenous circulating ACTH in the rat is transported mainly or exclusively in the albumin fraction of the plasma proteins. The exogenous hormone also binds to the albumin fraction, but when present in large quantities, may bind to certain of the other plasma proteins."

It would appear, therefore, that the phenomenon observed in this investigation is similar to those reported by Robbins and Rall (1955) and by Roberts (1957 A) (1957 C). It would seem reasonable to consider the distribution of parathyroid activity among the protein fractions of hormonally active plasma as approaching that of the endogenous hormone more closely than does the distribution pattern observed in the case of the in vitro

parathyroid extract-plasma preparation. Cold ethanol fractionation of hormonally active plasma, however, resulted in a detection of parathyroid hormone activity exclusively in Fraction IV, which consists of alpha-globulins, while similar fractionation of rat plasma to which had been added parathyroid extract in vitro, resulted in detection of activity in Fraction IV, but also in Fraction V, which consists of the plasma albumins. We may assume in this latter case that there was present in the parathyroid extract-rat plasma mixture large excesses of parathyroid hormone molecules. Since no mechanism was available under such in vitro conditions for removal of this excess activity, it can be suggested that the hormonal molecules present first occupied all the available binding sites on the alpha-globulin proteins, and then spilled over and became associated in the albumin fraction in an unphysiological manner. A similar situation could not occur in the case of hormonally active rat plasma, since its preparation involved a slow steady diffusion of exogenous hormone into the rat circulatory system under conditions which permitted inactivation or removal of excess hormonal activity by enzymatic hydrolysis, excretion in the urine or in some other unknown manner. Upon exanguination, then, such plasma would contain detectable levels of exogenous hormone bound in a physiological manner to only one plasma protein fraction, Fraction IV.

The above theory is based upon the assumption that the

extract employed contains only one hormonally active molecular species. This preparation is, however, an acid extract of bovine glands and as such, it would not be surprising if it contained at least two molecularly different but hormonally potent chemical species. Such a situation has been suggested by the work of Handler, Cohn and Dratz (1954) who subjected parathyroid extract to column chromatography on IRC-50 resin and recovered 5 fractions, each containing similar biological potencies.

The distribution pattern obtained from rat plasma to which had been added parathyroid extract, in vitro, then, could represent the affinity of the various hormonally potent chemical species for specific plasma protein fractions. When the extract is administered intraperitoneally, however, the more unphysiological molecules, presumably the species that binds to the albumin fraction, is inactivated. destroyed or excreted. The hormonal moieties remaining in the circulation could then bind to the fraction normally associated with the endogenous secretions of the parathyroid glands, Fraction IV, to give the activity patterns actually observed.

Another possibility may be considered here. Let us assume that the parathyroid extract contains a number of physiologically active, but structurally distinct chemical species, one of which is strongly similar to, or identical with the endogenous plasma parathyroid hormone molecule. The in vitro distribution pattern

would, again, reflect the affinity of the respective moieties to specific protein fractions. When the extract is administered intraperitoneally and diffuses slowly into the circulation, however, it is conceivable that the unphysiological molecules, which presumably possess an affinity for the albumin fraction when added to plasma in vitro, were chemically altered in the circulation so as to approach more closely the structural configuration of the true endogenous hormone.

It should be pointed out at this time, however, that the possibilities just discussed were presented as explanation for the differences noticed in the parathyroid hormone distribution patterns of rat plasma protein fractions obtained after cold ethanol fractionation of hormonally active rat plasma. The fact should not be overlooked that in the case of the hormonally active plasma, which represents the situation most closely reflecting the physiological environment of the endogenous hormone, all the activity recovered was localized in a single plasma protein fraction.

In situations containing the inherent complex paremeters such as those with which we are faced here, it is always difficult to draw categorical conclusions from the experimental data. If the distribution of parathyroid hormone activity among plasma protein fractions obtained from biologically active plasma had been found in more than one plasma protein fraction, the

danger of associating endogenous plasma parathyroid hormone activity to any particular fraction would be greatly compounded. In this work, however, ammonium sulfate fractionation of biologically active rat plasma has indicated an association of plasma hormone activity with the plasma globulin fraction, and this has been confirmed by the cold ethanol fractionation of hormonally active rat plasma, in which case all recovered plasma parathyroid hormone activity was found associated with the Fractio IV, or alpha-globulin fraction.

It would appear, then, that on the basis of the observations reported here, we can assume with reasonable certainty, that rat plasma parathyroid hormone activity is normally associated with the plasma alpha-globulin fraction, but when present in large excesses, may bind to certain other plasma proteins.

The problem of associating human plasma parathyroid hormone activity with specific plasma protein fractions was approached in a slightly different manner due to the availability of relatively large amounts of normal human plasma, a situation which we did not obtain with rat plasma. As has been pointed out, the limiting factor in the detection of hormonal activity in normal human plasma was the amount of plasma protein which could be administered to each assay animal without production of protein toxicity. A series of studies were conducted to define the limits of plasma protein which could be administered to the test animal, and this was found

to be 0.01 grams of plasma protein per gram of body weight or about 1.5 grams of protein per 150 grams assay animal.

Sufficient normal human plasma was lyophilized to provide the maximum permissible amount of plasma protein for administration to each member of an eight animal test group. This amounted to injecting 25 plasma cc. equivalents of normal human plasma protein to each assay animal. The analytical data recorded demonstrated clearly that no plasma parathyroid hormone activity could be detected in these test doses.

It has been demonstrated that our analytical system is sensitive to at least 25 USP units of parathyroid hormone activity. In this work 25 plasma cc. equivalents of total plasma protein failed to elicit a response in the rat preparations utilized for biological assay, and, therefore, presumably contained less than 25 USP units of plasma hormone activity. If this is so, then 100 ml. of normal plasma contains less than 100 USP units of parathyroid activity. Estimations of this type based upon similar analytical data have been reported in the case of a number of protein hormones (Wolstenholme and Millar, 1958).

Next, it was decided to study the distribution pattern of exogenous parathyroid hormone activity among protein fractions of normal human plasma obtained through use of the cold ethanol fractionation techniques of Cohn et. al. (1946). This approach had been employed with some success by Gemzell et. al. (1955) for

growth hormone and by Bethune et. al. (1955) in studies on plasma ACTH. The latter report was of particular significance to this investigation, since it was found that the distribution pattern of ACTH activity among protein fractions of human plasma to which had been added ACTH in vitro, was identical to the distribution pattern of endogenous ACTH activity.

Gemzell et. al. (1955) found exogenous Growth Hormone activity to be recovered in Cohn Fractions IV-I and IV-4, of normal human plasma and also observed the endogenous growth hormone activity of young pigs and a calf to be in the same fraction.

In the work reported here, parathyroid extract was added to normal human plasma which was then subjected to cold ethanol fractionation. The distribution pattern observed indicated that all the recovered activity was located in Fraction II-III, which consists mostly of beta and gamma globulins (Cohn et. al., 1946). No significant amounts of activity were detectable in any of the other fractions obtained.

Localization of parathyroid activity in this fraction was an extremely interesting situation, since Fraction II-III is the same fraction with which plasma ACTH activity (Bethune et. al., 1957), pituitary gonadotrophic activity (McArthur et. al., 1957), plasma thyrotrophic activity (Querido and Lameyer, 1956) and plasma insulin activity (Beigelman et. al., 1956 B), were found

to be associated following cold ethanol fractionation studies.

Since plasma hormone activity could not be detected in 25 plasma cc. equivelants of total plasma protein, it was now decided to employ cold ethanol fractionation as a method of concentrating specific plasma protein fractions and presumably such endogenous parathyroid hormone activity as may be present in these fractions. 560 ml. of normal human plasma was fractionated in this manner.

In the previous work it had been possible to administer the maximum dose of plasma protein to each animal of a test group. In the situation encountered here, however, this was impossible . The maximum permissible protein dosage per animal was, as indicated previously, 1.5 grams of protein per 150 gram animal. In addition, it was necessary to have at least 5 animals in each assay group. In order to prepare sufficient amounts of plasma Fraction I, IV-I and IV-4 protein for use at the maximum protein dose level, it would be necessary to fractionate over 10 liters of normal human plasma, and such large quantities werenot available to us. Further, if we were to assay all the available Fraction V protein (albumins) at the 1.5 gram/150 gram animal ratio, it would involve use of many more experimental animals than required for significant evaluation, due, of course, to the large % of albumin in normal human plasma. In order to solve this problem it was decided to administer each fraction at the

maximum protein level which would permit use of at least 5 animals in the assay group. In this way it was possible to assay 112 plasma cc. equivalents of Fraction I (fibrinogen) on each of five animals, 112 plasma cc. equivalents of Fraction IV-I (alpha globulins) on each of five animals, 112 plasma cc. equivalents of Fraction IV-4 (alpha and beta globulins) on each of five animals, 70 plasma cc. equivalents of Fraction II-III (gamma and beta globulins) on each of eight animals, and 38 plasma cc. equivalents of Fraction V protein on each of nine animals. In view of the limitations imposed upon our experimental procedures by the assay system employed, this represented the maximum amounts of each plasma protein fraction possible.

The results obtained indicated the presence of endogenous plasma parathyroid hormone activity in Fraction II-III, the same fraction with which the exogenous activity of the previous experiment had been associated. Bolinger (1959) has stated that the localization of exogenous hormone in the same fraction which contains endogenous hormone-like activity may be interpreted as evidence in favor of the assumption that the activity noted is actually due to the endogenous hormone and not to an artifact. Since no constituent of plasma is known to ape the effect of parathyroid hormone, and since the negative results obtained with Fraction V at a higher protein test dose level rule out a non-specific response due to the presence of plasma proteins, it would seem reasonable to conclude that the effect elicited in

the assay system by Fraction II-III protein was due to the presence of endogenous plasma parathyroid hormone.

The mean response of the test group employed in the analysis of the potency of Fraction II-III is of the order which one would expect from about 25 USP units of parathyroid activity. Since this response was obtained from 70 plasma cc. equivalents of Fraction II-III protein, it may be estimated that normal human plasma contains in the order of 40 USP units of detectable endogenous parathyroid hormone activity per 100 ml. of plasma.

In view of the fact that all the evidence recorded in the experiments discussed had indicated localisation of parathyroid hormone activity in Fraction II-III, this fraction was again separated from 560 ml. of normal human plasma, the other fractions being also isolated but combined prior to analysis. In confirmation of the previous work, activity was detectable in 69 plasma cc. equivelants of Fraction II-III protein, while no activity could be detected in 37 plasma cc. equivelants of combined Fractions I, IV-I, IV-4 and V. The estimated level of endogenous activity in this experiments is strikingly similar to that observed in the previous work, about 40 USP units of activity per 100 ml. of normal plasma.

This is, to the authors' knowledge, the initial successful demonstration of endogenous parathyroid hormone activity in normal human plasma, and the initial association of such activity with

a specific plasma protein fraction. Further, there is, as far as can be determined, no information available concerning the level of plasma parathyroid hormone activity other than that presented here.

Since endogenous plasma parathyroid hormone seems to be primarily associated with Cohn Fraction II-III, let us look at the exact chemical composition of this fraction in more detail. The Fraction II-III precipitate contains essentially all the gamma-globulins (antibodies), most of the beta-globulins, including the beta-lipoprotein, as well as small amounts of albumin, alpha-globulin and fibrinogen. In addition, prothrombin, isoagglutinins and plasminogen are also present in this fraction. (Cohn, 1946)

In previous discussions frequent reference has been made to the "association" of hormonal activity with particular protein fractions. Just what is actually implied by this term?

We have, in this dissertation, presented evidence based on dialysis studies which strongly suggests a binding of plasma parathyroid hormone activity to plasma proteins. In addition, a co-precipitation of plasma hormone activity has been associated with certain specific plasma protein fractions in the case of in vitro addition of parathyroid extract to rat and human plasma, and with a single plasma protein fraction in the case of hormonally active rat plasma and normal human plasma.

Pitt-Rivers (1957) concluded that hormonal iodine was bound to plasma proteins because it could not be separated by dialysis and was co-percipitated by protein precipitants. Heller (1957) suggested that co-precipitation of ACTH and thyrotrophic hormone with specific plasma fractions is indicative of binding. Bethune et.al. (1957) suggested that the occurence of ACTH activity in Cohn plasma protein Fraction II-III, indicated ACTH existed in human plasma as a protein-polypeptide complex. Roberts (1957A) felt that co-precipitation of rat plasma ACTH activity justified assumption of a binding type of phenomenon of this activity with specific plasma protein fractions for transportation purposes. These are only a few of many reports which imply that co-precipitation can be regarded as evidence in favor of binding of the hormone with the co-precipitated proteins. Since the data reported here is of a similar nature, it is felt we have justification in proposing a binding of plasma parathyroid hormone to theprotein in fractions with which it has been co-precipitated.

Assuming a binding of parathyroid hormone to plasma protein does occur, what is its raison de etre ? Although it may be philosophically dangerous to assume that everything that occurs normally in the body is for a beneficial purpose, it would seem that some reason should be forwarded to explain why a hormone-protein binding would be desired by the organism. A possible clue may be found in the work of Brown et.al. (1955) who studied the

interaction of corticotrophin with Bovine Albumin. This worker found that corticotrophin bound to the albumin and further, that this binding increased the potency of his corticotrophin preparations.

A binding of hormone to plasma protein to form a complex, cound be visualized as helping to prevent the inactivation of the hormone by the organism. This could be accomplished, for example, by denying the active site of the hormone contact with inactivating enzymes, such as plasmin. In addition to preventing "active" hormonal molecules from being too rapidly inactivated in vivo, and, thereby rendering them biologically more effective, protein binding could also conceivably render "active" hormone molecules inactive without actually destroying them. In this way the body could preserve homeostasis and at the same time have on hand some "reserve" hormone for emergencies. There are many interesting and useful possibilities which could be considered a consequence of a hormone-protein binding phenomena.

In conclusion, it is felt that we have definitely demonstrated an association of plasma parathyroid hormone activity with specific rat and human plasma protein fractions. Strong circumstantial evidence has been presented which suggests that the association of hormonal activity and plasma protein fraction is in the nature of a hormone-protein binding phenomenon, and initial estimations have been made of the circulating level of

endogenous plasma parathyroid hormone activity.

CHAPTER VIII

SUMMARY

1. A biological assay system has been described suitable for use in the evaluation of parathyroid hormone activity in plasma and in plasma protein fractions.
2. Freshly collected rat plasma has been shown to lack a parathyroid hormone inactivating system, in vitro.
3. Nembutal has been shown capable of markedly decreasing the sensitivity of the biological assay system to parathyroid extract.
4. A technique has been described for the preparation of rat plasma with sufficiently high levels of parathyroid hormone activity to be detected by the biological assay system. Plasma prepared in this manner has been designated as "hormonally active plasma."
5. It has been demonstrated that dialysis of commercial parathyroid extract results in a virtual elimination of hormonal activity. This loss of activity has been found not to occur when the extract is incubated with plasma prior to dialysis. Evidence has been presented to suggest that the latter phenomenon can be explained on the basis of a binding of the hormone to plasma proteins.

6. Commercial parathyroid extract is inactivated by oxidation with hydrogen peroxide, but this inactivation is not observed to occur if the extract is incubated with plasma prior to treatment.

7. The biological potency of hormonally active rat plasma is not affected by either dialysis or treatment with hydrogen peroxide. The latter observation would seem to preclude oxidative inactivation of plasma parathyroid hormone activity from being of significance, in vivo.

8. Ammonium sulfate fractionation of hormonally active rat plasma resulted in detection of parathyroid activity in the globulin precipitate.

9. Cold ethanol fractionation of freshly collected rat plasma to which had been added parathyroid extract, in vitro, resulted in a localization of hormonal activity in Fraction IV (alpha-globulin) and in Fraction V (albumin).

10. Cold ethanol fractionation of hormonally active rat plasma resulted in a localization of parathyroid activity in Fraction IV but <u>not</u> in Fraction V.

11. Evidence is presented to suggest that Fraction IV is the fraction with which endogenous rat plasma protein activity is normally associated.

12. Lyophilized protein from 25 cc. of normal human plasma was shown not to contain detectable levels of endogenous plasma parathyroid hormone activity.

13. Cold ethanol fractionation of normal human plasma to which had been added parathyroid extract, in vitro, resulted in an association of activity with protein Fraction II-III, which contains primarily beta and gamma globulins.

14. Cold ethanol fractionation of large volumes of normal human plasma resulted in a detection of endogenous plasma parathyroid hormone activity in Fraction II-III, the same fraction in which exogenous hormonal activity had been detected. Fraction II-III is the fraction with which the plasma activity of Insulin, ACTH, Thyrotrophic Hormone and Pituitary Gonadotrophins have been shown to be associated.

15. On the basis of the data presented, the level of circulating endogenous human plasma parathyroid hormone activity has been estimated to be in the order of 40 USP units of activity per 100 ml. of plasma.

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APPENDIX

TECHNIQUE OF PARATHYROIDECTOMY BY CAUTERIZATION

The following technique was routinely employed to parathyroidectomize female albino rats of the Sprague-Dawley strain weighing 120-130 grams.

Ether was used as the anesthetic during the operation, which was carried out in the hood to facilitate removal of ether vapors. The animal was anesthetized in an ether jar and removed when, after rolling over on its back, the animal ceased struggling to right itself. After removal from the jar the animal was placed on its back on a wooden board, approximately five inches by ten inches, and secured by placing wide adhesive tape over each limb. A rubber band was placed about the incisor tooth in such a manner as to make the neck of the animal slightly taut, the incisor becoming the apex of a triangle formed by it and two nails imbedded in the board about two inches above the head of the animal. A small piece of cotton was made damp to the touch with ether and placed close to, but not in contact with, the nose of the animal. Ether was kept on hand and periodically during the operation was added to the cotton, not more than five drops at a time. If during the course of the operation the animal developed pronounced ataxic breathing, the cotton plug was removed from the nose area until breathing returned to normal. If, however, respiratory difficulties, characterized by a wheezing or gasping sound developed and did not cease after a minute or two, the cotton plug was replaced

and the operation completed as rapidly as possible. After the animal had been secured on the operating board, an incision was made along the midline of the neck from the cephalic boarder of the sternum to the mandible. Similar incisions are made through the various subcutaneous layers of connective tissue, and hemostats were used as needed to hold the cut tissue to one side. Care was taken to avoid damage to the submaxillary salivary glands situated bilaterally to this midline incision. Any damage to these glands resulted in profuse bleeding. It was found convenient to feel for the trachea with a finger before making the incision, and then cutting along what was felt to be the midline of the trachea. In this way the initial incision sometimes separated the glands present right away. Once the muscle layer covering the glands and trachea had been exposed, it was gently separated at the midline through use of a sharp probe or scalpel. The muscle flaps were retained by hemostats, and the exposed trachea cautiously freed of fascia. A muscle was noted lying adjacent to each lobe of the thyroparathyroid apparatus. This was separated by blunt dissection. Once this muscle had been retracted, it was usually possible to observe the parathyroid gland as a noticeable lighter-colored,bulbular projection located at the upper lateral edge of the thyroid. In all cases identification was greatly facilitated through use of a 2.75X binocular loupe (Magni-Focuser). The parathyroid was

then cauterized with the eye tip of a cautery pencil. Caution must be exerted here to eliminate danger of inflammation of the ether saturated cotton plugs. To insure complete removal, it was found best to cauterize the general area where the parathyroids were usually located. Ifthe parathyroid glands glands could not be located, cauterization of the general area with which the glands were normally found associated was shown to be generally effective in producing a parathroidectomy. Care must be taken not to use too hot an eye tip, since this would burn into the trachea or destroy some other vital structure. Insertion of a blunt probe uder the trachea, which was used to gently lift the thyroparathyroid apparatus, proved helpful in allowing the operator to better distinguish and cauterize the parathyroid gland mass. Immediately after cauterization, all limb restraints were removed, and thewound was closed with from two to four wound clips. The animal revived in a minute or two following a return to its cage. If the animal developed a pronounced gasping or wheezing which did not subside within 15 minutes, it was destroyed.

CARDIAC PUNCTURE TECHNIQUE

The following method was routinely employed in obtaining rat plasma for serum calcium analysis.

The animal is anesthetized with ether and placed on the operating board in the same manner as described in the previous section. The cardiac puncture is made utilizing a one cc. Luer type syringe, with a 25 or 26 guage needle. A larger needle is not advisable as itmay seriously damage the heart. The needle is inserted at a point slightly lateral to the point of maximum palpitation as determined by touch. If the animal is prepared in the same manner for each cardiac puncture attempt, it is often possible to "hit" the heart even though some difficulty is encountered in feeling for the point of maximum palpitation. If the heart is missed in the initial puncture attempt, one should not probe around with the needle, but apply gentle lateral pressure to each side to see if the impulse of the heart can be felt on the needle. This will aid in judging the site of the next attempt. It is felt that for a beginner it would be of great benefit to insert the needle into the animal, then destroy it with ether and observe the in situ location of the heart in relation to the position of the inserted needle. This serves as an invaluable orientation. A number of rats withstood up to six cardiac punctures with no noticeable ill effects, and after some practice relatively few fatalaties are incurred.

CALCIUM ANALYSIS OF SERUM. METHOD OF NATELSON AND PENNIALL.

The reagents and materials used were prepared exactly as described by Natelson and Penniall (1955). The following is a detailed account of the technique shown to give reproducible results when employed for serum calcium analysis.

One ml. of water is placed in a ground glass stoppered 12 ml. capacity centrifuge cone. To this is added 50 lambda of serum, usinga 50 lambda micropipette. The micropipetting seemed to be the source of most of the initial difficulties encountered in obtaining reproducible results. The following procedure was found to be acceptable. The micropipette is attached to a tuberculin syringe by means of some rigid attachment, such as a plastic nozzle or a thick walled pressure tubing, about 3/4 inch in length. It should be possible to control the pipette using the syringe and only one hand. The micropipette is inserted into the sample, and the sample is drawn up to the point a bit above the 50 lambda mark. This sample is then discarded. The size of the plasma sample will almost always be large enough to permit this procedure with a 50 lambda or smaller pipette. The pipette is reinserted into the sample and again the sample is drawn up to a point slightly above the mark. The outside of the micropipette is wiped off with Kleenex, and in this way the sample is usually drawn down to the mark by capillary action. The sample is then ejected into the water in the centrifuge cone. The mixture in the

centrifuge cone is used to rinse the micropipette twice. The micropipette is finally rinsed twice with distilled water, the rinse beingejected into the centrifuge cone. This procedure assures removal of all the serum from the walls of the micropipette. Pipetting of a second serum sample is accomplished in the same manner as above. After use, the micropipette is cleaned with detergent, distilled water, concentrated nitric acid and again with distilled water. It is not necessary to dry the pipette if the above procedure is used. To the cones containing the serum and water, 2 ml. of Triethanolamine and then 6 ml. of the alizarin-octanol reagent are added. It was found convenient to add these reagents by burette for speed and accuracy. The tightly stoppered centrifuge cones are placed on the shaking machine and shaken for 20 minutes. Following this,the cones are centrifuged for five minutes at 2000 rpm in the clinical centrifuge If cloudly supernates are obtained, the cones are re-centrifuged for another five minutes. It is important, however, that all the cones in a run are centrifuged for the same length of time. The supernate is drawn off using a 5 ml. graduated pipette controlled by mouth suction. About 6 ml. can usually be drawn off, but a minimum of 4 ml. are required for the colorimetric measurement in standard size colorimeter tubes. The color developed in the supernate is measured at the Klett-Summerson colorimeter, using a number 56 filter (560 mu.). This method involves a modification

of the original Matelson and Penniall technique in that 50 lambda of sample are utilized instead of 20 as suggested by these workers, and 6 ml. of the alizarin-octanol reagent are used instead of the original 3 ml. After use, the cones are cleaned with detergent distilled water, acetone, dilute nitric acid and distilled water. The colorimeter tubes are cleaned with detergent, distilled water, dilute nitric acid and again with distilled water.

APPROVAL SHEET

The dissertation submitted by Leo Edmund Reichert, Jr., has been read and approved by five members of the faculty of Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

December 3 1959
Date

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