INVESTIGATION OF A METHOD OF FRACTIONATION OF ANTERIOR PITUITARY EXTRACT

by

MARY N. COURTRIGHT

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THESIS

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TABLE OF CONTENTS

Page No.

HISTORICAL INTRODUCTION 1

THE NUMBER OF PITUITARY HORMONES 9

The Metabolic Factors 11

Carbohydrate Metabolism 12
Protein Metabolism 13
Fat Metabolism 14

THE GROWTH HORMONE

Existence of a Growth Hormone 16
Early Studies on Pituitary-Growth Relationship 17
Chemical Properties of the Growth Hormone 20
Biological Properties of the Growth Hormone 20
Assay Methods for the Growth Hormone 22
Preparation of the Growth Promoting Substance 24

THE THYROTROPHIC HORMONE

Early History 28
Chemical Properties of the Thyrotrophic Hormone 30
Biological Properties of the Thyrotrophic Hormone 31
Assay Methods for the Thyrotrophic Hormone 32
Preparation of the Thyrotrophic Hormone 33

PROLACTIN OR THE LACTOGENIC HORMONE

Early History 37
Assay Methods for Prolactin 40
Chemical Properties of Prolactin 42
Biological Properties of Prolactin 44
Purification of Prolactin 45

THE CORTICOTROPHIC PRINCIPLE

Early History 48
Biological Properties of the Corticotrophic Principle 50
Chemical Properties of the Corticotrophic Principle 51
Assay of the Corticotrophic Principle 52
Preparation of the Corticotrophic Principle 56

Page No.

THE GONADOTROPHIC PRINCIPLES OF THE ANTERIOR PITUITARY

Early History 59
The Actual Number of Pituitary Gonadotrophins 61
Biological Properties of Gonadotrophic Factors 63

Properties of FSH 64
Properties of ICSH 65
Combination of FSH and ICSH 66

Assay Methods for ICSH 67
Assay Methods for FSH 69
Assay of Pituitary Gonadotrophins in Unfractionated Extracts 70
Chemical Properties of ICSH 70
Chemical Properties of FSH 72
Preparation of Gonadotrophic Principles of the Anterior Pituitary 73

EXPERIMENTAL

INTRODUCTION 77

PURPOSE OF THE INVESTIGATION 77

Figure I - Cohn Method
Figure II - Application of Cohn Method to Anterior Pituitary Extract

PREPARATION OF MATERIAL

Dissection and Extraction 79
Precautions Observed 79
Fractionation 80
Preparation of Extracts 81
Yield of Lyophilized Material 82

Preparation of Reference Extracts 82

Use of Standardized Preparations 84

Page No.

STUDY OF THE VARIOUS FRACTIONS

Nitrogen Content 85
Table I
Contamination with Posterior Pituitary 86
Table II
Sensitization of Guinea Pigs 88
Table III
Activity of Lyophilized Crude Anterior Pituitary Extract 90
Table IV

ASSAY OF GROWTH HORMONE 92

Reference Material 94
Table V
Table VI
Lyophilized Fractions 96
Graph I
Discussion 98

ASSAY OF THYROTROPHIC PRINCIPLE 100

Procedure 101
Table VII
Reference Dose 102
Table VIII
Graph II
Yield of Activity 105
Discussion 107

ASSAY OF PROLACTIN 109

Table IX

Reference Material 110
Lyophilized Material 111
Discussion 111

ASSAY OF THE CORTICOTROPHIC PRINCIPLE 113

Maintenance Method 114
Table X
Table XI
Reference Material 115
Lyophilized Fractions 117
Graph III
Yield of Activity 119
Discussion 121

Page No.

GONADOTROPHIC ASSAY

Method 123
Reference Dose 124
Table XII
Table XIII
Lyophilized Fractions 126
Graph IV
Yield of Activity 127
Assay for ICSH 129
Method 129
Table XIV
Yield of Activity 130
Discussion 131

SUMMARY AND CONCLUSIONS 134

Table XV 143

BIBLIOGRAPHY

HISTORICAL INTRODUCTION

Our knowledge of the hypophysis cerebri or pituitary gland dates back to the early anatomists. Galen, who considered that the gland acted as a filter for the mucous fluid of the brain which was secreted in some way into the naso-pharynx, adopted the name glandula pituitaria. It is interesting to note that the name has persisted to the present although the early theory of its function has been discarded. These ideas of the specific function of the pituitary were based on theoretical speculation and observation rather than experimental experience and consequently were not easily corrected. Other theories of its function were upheld from time to time, but they did not threaten the theory of Galen and his followers very seriously. With the advent of the microscope systematic investigation of the gland was undertaken by Rathke (1838) and for the next fifty years anatomists studied its embryological development, and described its structure.

In the early eighties of the last century when the whole science of endocrinology was just beginning to take form the clinical observations of Pierre Marie (118) in cases of acromegaly and their association with pituitary tumors established a relationship between the

pituitary and body growth. The work of Rogowitsch (144) on thyroidectomy also led to the establishment of a relationship of the pituitary with the thyroid. However, the experimental phase of pituitary investigation which ushered in the modern era, albeit slowly, started with the successful removal of the pituitary from two dogs by Horsley in 1886. (86)

From the time of Horsley's removal of the gland until Smith described its successful removal in rats forty years later the specific symptoms resulting from hypophysectomy were never adequately or fully described. Workers too numerous to mention hypophysectomized cats and dogs and reached no unanimous opinion of the symptoms definitely associated with removal of the gland and their compatability with life. All the investigation on experimental hypophysectomy prior to 1910 formed the basis of a review by Cushing and co-workers (39). The operative techniques employed in removing the gland from various species and the resulting symptoms as well as the conclusions reached by the numerous investigators on the physiological essentiality of the pituitary are contained in the review. Cushing on the basis of his experimental work expressed the opinion that the anterior lobe was responsible for the preservation of physiological equilibrium. The difficulty of the operative technique resulted

in injury to the brain tissue in the region of the hypophysis and this gave rise to symptoms which we now recognize as not being due to loss of the pituitary. Similarly the idea that pituitary removal was incompatible with the survival of the animal resulted from the death of the animals through inanition. The muscles employed in mastication were destroyed by the operative approach to the pituitary. Aschner who employed the buccal route of hypophysectomy succeeded in keeping his animals alive and has given an excellent review of the knowledge of pituitary function as it existed in 1912 (9).

While clinical evidence and evidence of the reciprocal action of other endocrines on the pituitary, as well as experimental knowledge of pituitary function were gradually accumulating, definite proof of the role played by the pituitary, especially with regard to the trophic influence it exerts on other endocrine glands, was lacking. Smith's work in 1926 on the results of hypophysectomy upon growth, the thyroid, adrenals and gonads was the long sought answer of anterior pituitary research (171). The early historical evidence for the pituitary's relationship with other endocrines before Smith's famous publication, and a complete survey of the established anterior pituitary principles are treated

under separate headings.

A few years prior to Smith's investigation of pituitary function evidence from another source was important in its contribution to pituitary research. After many failures to produce results by oral administration of the pituitary to mammals Evans and Long (45) were successful in preparing an extract of anterior pituitary which on injection stimulated growth in normal rats. They subsequently obtained evidence that this extract influenced other endocrines apart from its growth effects. Other workers were also engaged in the preparation of pituitary extracts and the study of their effects.

By 1930 the stage was set as a result of Smith's work and the preparation of extracts by others for the investigation of pituitary function by replacement therapy. The gradually accumulated evidence, both clinical and experimental, then formed the basis for the successful solution of pituitary problems. In the relatively short space of time which has elapsed since then the greatest strides have been made in pituitary investigation. The ability of pituitary extracts to prevent the changes characteristic of hypophysectomy and to restore those structures where regression had occurred led workers to investigate the principles present in the extracts responsible

for these effects. Although the existence of factors in the extracts distinct from those which influenced growth and the gonads was speculated upon, their existence still remained to be proved. The efforts of investigators in this field were not only rewarded by the establishment of the existence of these factors, but others whose existence was not even suspected were shown to be definite entities.

The protein or protein-like nature of thevarious principles and the difficulty in separating specific factors uncontaminated with other active principles greatly handicapped pituitary investigation. Despite all efforts the active principles could not be accepted as definite substances until they could be prepared in a chemically pure form. In the investigation of other hormones of the endocrine system the work had been particularly successful. Not only were the substances prepared in the pure state, but they were successfuly synthesized. Pituitary research was veritably at a standstill. Various workers who appreciated the protein nature of the anterior pituitary principles set themselves the task of purifying the active principles and studying their chemical properties. As a result of their efforts they have succeeded in preparing the active principles in either a chemically pure form or with only the slightest

contamination. The physical and chemical characteristics have been studied and as a result of purification our knowledge of the biological properties has greatly improved. The appreciation of the protein-like nature of these principles and the application of methods employed by physical chemists in the study of structure has been of untold advantage in the final elucidation of the problem.

The problem of isolating and studying the principles in the pure state was undertaken in two ways. Workers either set about preparing a single factor or else they attempted the fractionation of anterior pituitary extracts to isolate as many as possible of the active principles. The majority of workers have employed the former method. The extraction procedures employed were such that they ruled out contamination with other factors as much as possible. In a word, they were selective, and the other principles were discarded. After suitable extraction and isolation they then proceeded to purify the active substance and to obtain it in as active a form as possible. The means taken to effect this were the use of isoelectric precipitation or salting-out procedures.

More recently the tendency of workers has been to isolate and prepare as many as possible of the active principles from an anterior pituitary extract. Such a method is not new. It was successfully applied by Collip ten years ago (34). However those now using this method of preparation and investigation have the advantage of knowing the physico-chemical properties of the active substances.

The main procedures used in fractionation are isoelectric precipitation and salting-out the protein by use of $(NH_4)_2 SO_4$. While isoelectric precipitation was formerly most popular, the salting-out procedure appears to be the method most generally used in the fractionation of extracts and the preparation of purified principles.

The protein nature of the active principles of the anterior pituitary necessitates care in handling the material prior to extraction. Although acetone desiccated or frozen glands can be used as a source material it is advisable to use fresh anterior pituitary tissue. The species of the gland employed in investigation is also important. Beef glands are recognized as a poor source

of gonadotrophic principles and workers interested in the preparation of these factors use either sheep or swine pituitaries. Recent work has shown that the gonadotrophic principles extracted from different species while possessing similar biological properties are slightly different chemically.

The chemical procedures used in the preparation of hormones are as numerous as the workers in this field. The main procedures in the preparation of any hormone or hormones are the extraction, isolation and purification. The main methods employed for each hormone will be found under the separate headings.

THE NUMBER OF ANTERIOR PITUITARY HORMONES

It is impossible to state the number of hormones actually secreted by the anterior lobe of the pituitary gland. The various physiological and pharmacological effects produced by injection of the cruder extracts of this tissue have led to the postulation of a great number of active principles. These principles are said to exert a control over the various endocrine glands as well as a direct or indirect control over general body metabolism. It is inconceivable that one tissue could actually be the source of so many separate and distinct factors, and there is a great deal of speculation on the possibility. However, the complete answer to the problem will be attained only when investigators have succeeded in isolating the various factors of the anterior hypophysis in pure form. Within recent years a number of fractions have been obtained which are almost completely free of contamination with other principles. The hormones of the anterior hypophysis have been the subject of so much investigation and study, yet, they, of all the hormones of the endocrine system, are most resistant to isolation.

All investigation both physical and chemical points to the protein or protein-like nature of these substances. It

has been suggested by Collip that the various active fractions must of necessity be derived from or be altered products of large protein molecules produced by the cells of the living gland. He believes that all the physiological activities of the anterior lobe are represented by different groupings of two or three protein substances secreted by the gland. "It would be possible to visualize the act of secretion of the normal gland in situ in one or two ways. Either the individual physiologically active groups may be liberated by some hydrolytic process, probably enzymatic, from the native protein carrier, or else the secretion consists of native proteins each carrying many active groups. For example, in the former case relatively small molecules could be visualized, representing the corticotrophic substance being set free in the circulation and being picked up by and acting upon the cells of the adrenal cortex; in the latter case, it would be inferred that different peripheral structures are acted upon by different groups in the hormone molecule, a specific group always having a specific physiological effect" (35).

Investigation to date allows for the conclusion that there are six separate physiologically active principles secreted by the anterior hypophysis. These are the growth, thyrotrophic, corticotrophic, lactogenic and gonadotrophic

fractions. The last fraction is divided into a follicle stimulating factor and a luteinizing or interstitial cell stimulating factor. The history of these factors will be treated in detail under separate headings. In the present investigation these are the only principles whose separation was sought by the fractionation procedure employed and upon which assays were subsequently conducted.

THE METABOLIC FACTORS

In addition to these six effects, anterior pituitary extracts have been credited with other activities of a metabolic nature, and the principles supposedly producing them are generally grouped under the heading metabolic hormones. Since Houssay first noticed the anti-insulin effect of anterior pituitary extracts many investigators have turned to a study of the control over carbohydrate, fat and protein metabolism exerted by the anterior hypophysis. It should be mentioned that the existence of some of these factors is questioned, and in the majority of cases the principles in anterior pituitary extracts responsible for them have not been clearly identified. Some of the effects ascribed to separate metabolic principles may be either a function of one or more of the already accepted factors, or else result from breakdown products of pituitary proteins which arise during preparation.

In this investigation the various metabolic factors now mentioned have not been studied. They are reviewed here rather briefly to complete the anterior pituitary picture. The role in metabolism ascribed to the anterior hypophysis is an important one, and as reports on this particular topic continue to be published one is amazed at the variety of functions governed by this relatively small structure.

CARBOHYDRATE METABOLISM

Prior to Houssay's report in 1929 the posterior lobe of the pituitary was considered the important factor in the regulation of carbohydrate metabolism. At this time Houssay and Potick (87) demonstrated the great sensitivity of hypophysectomized animals to insulin. The following year Houssay announced the ameliorating effects of hypophysectomy upon pancreatic diabetes. This phenomenon is now generally recognized by the name of its discoverer (88). In 1932 Evans et al (50) reported the blood sugar raising effect of anterior pituitary extracts. A few years later Young (192) (135) reported that certain anterior pituitary extracts were capable of producing a permanent diabetes in dogs. The mechanism by which such effects are produced by the extracts has been studied by Campbell and Best (26). These authors find regressive changes in the islet cells of the

pancreas accompanied by a fall in their insulin content. The diabetogenic and anti-insulin effects of anterior pituitary extracts are but a few of the many ascribed to this gland which control one phase or another of carbohydrate metabolism. Russell (147) described a factor responsible for the retention of carbohydrate stores in muscle and Young has described a similar one for liver (193).

The role played by the adrenal cortex in carbohydrate metabolism, and the regulation of its function by the anterior pituitary was reported by Lukens and Dohan (198). The intimate relation of carbohydrate and protein metabolism has been extensively studied since that time and interest has shifted from the pituitary to the adrenal cortex. The net result of these studies is the postulation of a direct and indirect role of the pituitary in carbohydrate metabolism.

PROTEIN METABOLISM

The effects of anterior pituitary extracts upon protein metabolism, particularly those rich in growth activity were studied by Teel and Watkins (181). Gaebler and Galbraith and others (182, 75). The nitrogen retaining effect is demonstrated by a fall in the non protein nitrogen of the blood and a decrease in urinary excretion.

Although most workers consider this an effect of the growth principle there are a few workers who think a specific principle is responsible for the effects on nitrogen metabolism.

A recent report of Young et al (41) would seem to indicate that they consider some factor in pituitary extracts distinct from the growth principle responsible for nitrogen retaining effects. Speculation is still rife as to the method in which the pituitary controls protein metabolism (131). Evans et al ascribe control of some phases of nitrogen metabolism to their purified growth preparation (71). The pituitary also exerts an indirect effect upon protein metabolism through the adrenal cortex (109).

FAT METABOLISM

Carbohydrate, fat and protein metabolism are so closely interrelated within the organism that it is inconceivable that an alteration in one component would not affect the others. A separate factor of the anterior hypophysis which influences fat metabolism has been designated as the ketogenic factor. Burn and Ling (199) first described a property of anterior pituitary extracts which caused increased ketosis in animals on a high fat diet. This factor has been extensively studied by Neufeld and Collip, (128) (129) Shipley (160) and others. Various workers report that

their more purified growth preparations possess ketogenic activity (20) (190). Shipley distinguishes the two activities by differences in stability to heat.

As workers continue to prepare more purified fractions of the various principles it is hoped that the identity of the metabolic hormones will be established satisfactorily and that separate principles will be postulated or they will be associated with the already existing ones.

THE GROWTH HORMONE

EXISTENCE OF A GROWTH HORMONE

Of the six separate physiologically active principles of the anterior hypophysis which have been postulated the growth hormone is the one whose existence is questioned. Among the foremost workers who deny that the pituitary secretes a specific growth-promoting substance one can mention Riddle (12) and Turner (16). It seems paradoxical that the function first associated with the pituitary should have been one of growth. The observations of Marie (118) in which acromegaly was associated with pituitary tumors drew attention to the role of the pituitary in the regulation of body growth.

The different criteria used by various investigators in judging growth is responsible for the confusion attached to this term. A mere increase in body weight is considered by some a true indication of growth, while others insist that an increase in skeleton size is a necessary criterion. In the strict sense other factors such as nutrition and heredity, not to mention the influence of other endocrine glands such as the thyroid, exert a control over growth. It is the belief of some that "the hormone stimulus of growth is not vested in a single pituitary factor, but rather results from a synergistic effect of several pituitary factors which

influence directly and indirectly general metabolism and the metabolism of fat, carbohydrate and protein" (16). Such objections were understandable in the early days of pituitary investigation when adequate test animals were not available and the growth-promoting extracts were contaminated with other pituitary principles. However, with the advent of newer physico-chemical methods of extraction and the preparation of a growth principle contaminated to only the slightest extent with other anterior pituitary factors (69) the denial of a growth hormone is absurd. The pituitary is the most important and essential regulator of growth.

EARLY STUDIES ON PITUITARY-GROWTH RELATIONSHIP

Although clinical evidence first supplied an indication of the probable role of the pituitary in growth regulation the experimental stage lagged far behind because of the difficulty of the operative technique in hypophysectomy and the failure of animals to withstand the operation. The experimental investigation of growth regulation may be said to have been inaugurated by the successful removal of the pituitary in puppies by Aschner in 1912 (9). The excellent review by Cushing (39) on the earlier attempts at hypophysectomy has been mentioned previously. The studies of Cushing (40) and Smith (171) firmly established that the removal of the pituitary results in an abrupt

cessation of growth.

In the years intervening between Aschner and Smith investigators turned their attention to a study of growth regulation in flies, worms, fowl, amphibia and mammals. The relative ease of ablating the hypophysis in tadpoles suggested their value in the study of growth. Investigators seeking the substance in pituitary tissue responsible for growth administered either the whole gland or the anterior lobe to normal animals and hypophysectomized amphibia. Feeding rather than the parenteral mode of administration was used. The results of these investigations were rather confusing, but the main conclusion seemed to be that feeding pituitary was ineffective in accelerating growth except in the case of amphibia.

Aldrich (4) in 1912 reported that growth acceleration was not produced in mammals by feeding pituitary. His work was amply confirmed by other investigators such as Evans and Long (46) and most conclusively by Smith (173) in 1927. A retardation rather than an acceleration of growth was the result of feeding the anterior pituitary to fowls. This was reported by Wulzen (191), Pearl (132) and Maxwell (123). The results of feeding in amphibia differed from those in the other species. Smith (167) fed fresh anterior lobes to normal and hypophysectomized tadpoles and

produced increased growth. Uhlenhuth (184) later confirmed these results in salamanders.

Evans and Long (45) first demonstrated that the growth rate of normal mammals could be increased by the injection of an alkaline extract of the anterior pituitary gland (1921). Their extract was the first growth-promoting extract although it was contaminated with a variety of pituitary principles. Since that time the efforts of this group have been devoted to the systematic investigation of methods of extraction and purification of the specific principle. It was not until Smith was successful in demonstrating growth stasis in hypophysectomized rats and the restorative effects of anterior pituitary implants (171) and Pituitary extracts (175) that final evidence for the pituitary's effect on growth was presented.

While the growth produced by pituitary extracts was not denied, nevertheless, the crude extracts employed did not rule out the possibility that the effects were not due to a specific growth-promoting substance. Even the purest extracts available tended to stimulate other endocrines when administered in high dosage (35). Recent progress in the purification of this principle and the specific results produced by these products definitely proves the existence of a specific growth promoting substance (69, 61, 20).

CHEMICAL PROPERTIES OF THE GROWTH HORMONE

Growth promoting extracts may be extremely labile under certain conditions. Heating an extract in alkaline medium at 80 deg. C. for 15 minutes destroys growth activity. The growth principle does not dialyze through collodion and other membranes. It is euglobulin in nature and can be precipitated from 0.25 M $(NH_4)_2 SO_4$ solution at pH 7 by dialysis or increased concentration of $(NH_4)_2 SO_4$. Growth hormone is largely precipitated by 1.6 M $(NH_4)_2 SO_4$ and completely by 1.8 M (61).

Treatment with cysteine allows the growth hormone to be separated from other active pituitary principles. The isoelectric point of cysteine treated growth hormone lies between pH 6.2 to 7.1. Untreated fractions precipitate over a wider range, pH 4.6 to 9. The presence of S-S bonds is indicated by loss of activity when treated with urea and cysteine (69).

BIOLOGICAL PROPERTIES OF THE GROWTH HORMONE

As in the case of other pituitary principles more effort has been devoted to a study of the biological properties of growth hormone than to attempts at isolation and purification. An active growth preparation when administered to an immature hypophysectomized animal enables it to grow at a normal rate. Growth in this instance being judged by

both the increase in body weight and skeleton size.

The increase of body weight produced by growth extracts is indicative of metabolic changes within the animal. The studies of Teel and Watkins (181) Teel and Cushing (182) and Gaebler (75) show changes in the nitrogen-containing constituents of the blood represented by changes in urinary nitrogen excretion. The fall in the blood non-protein nitrogen is largely due to a reduction in the amount of urea and amino acids. The reduction in the excretion of urine nitrogen shifts the nitrogen balance in a positive direction. Recent publications from Evans' laboratory (71, 121) shows that nitrogen retention is produced by the injection of a purified growth hormone preparation. Young (41) seems to be of the opinion that growth and nitrogen-retaining properties are represented by separate anterior pituitary principles. His argument rests on the destruction of nitrogen-retaining activity after treatment with 85% methanol. Paschkis (131) also suggests the existence of two factors but on different evidence. However, the bulk of evidence still points to the association of growth and nitrogen-retaining properties.

The most potent as well as the least contaminated extracts recently prepared all report an association of growth activity with diabetogenic as well as ketogenic activity, the

latter particularly (69) (20). However, the recent report by Shipley strongly suggests the separate identity of the growth and ketogenic factors (160).

Assay of Growth Hormone

Growth hormone can be assayed by two main methods both of which depend upon a weight increase response. Normal female rats which have reached a period of growth stasis or hypophysectomized rats are used in assaying this principle.

USE OF PLATEAUED RATS

When female rats reach an age of five or six months they cease to grow and their body weight becomes stationary. Such animals are known as "plateaued" rats. Injection of a growth stimulating extract into plateaued rats causes a resumption of growth and the activity of the preparation is judged by the weight increase. In distinction the male rat does not attain a plateau, but continues growth at a slow rate. It is therefore an unsatisfactory test object in judging growth response.

Evans et al (47) introduced the use of plateaued rats for a rapid and satisfactory method of assay. Workers interested in the investigation of the growth principle continue to use normal animals despite the introduction of hypophysectomized rats for assay purposes. Evans and co-workers

(53) conduct their assay over a period of twenty days; the total number of injections during this time being seventeen. More recently they have employed a fifteen day period of assay which proves to be equally satisfactory (120). Other workers have introduced their own modifications into the assay procedure. Van Dyke et al (27) recommend a ten day assay period while Light, de Beer and Cook find a five day period satisfactory (103). The longer assay period is probably more satisfactory since daily weight variations in the animals do not assume as much prominence.

USE OF HYPOPHYSECTOMIZED RATS

Hypophysectomized rats, about ten days postoperative, are really the more satisfactory animals for assay of the growth principle. The use of such animals, although more difficult to procure, has many advantages over that of normal animals. Animals of either sex can be used since the same response is given by both. Hypophysectomized rats are ten to twenty times more sensitive than normal rats to growth extract and their use rules out the possibility of an augmenting action of the animals' own pituitary. An interesting correlation and comparative study of the two assay methods has been made by Evans (120).

Van Dyke and Wallen Lawrence (187) introduced the

hypophysectomized rat in the assay of the growth principle. Their period of assay was only three days. Collip et al (31) employed hypophysectomized (100 gms.) rats ten days post operative and recommended a fifteen day assay period. Evans et al (120) use immature hypophysectomized rats ten to twelve days after operation. They claim that at an age of 26 to 28 days growth continues for only a few days after hypophysectomy. These workers formerly recommended a ten day assay period, but it is interesting to note that a fifteen day period as suggested by Collip is now found more satisfactory. Bulbring also uses hypophysectomized animals but her assay period is only five days (24).

Freud (200) has introduced another criterion of growth apart from body weight response. He uses the increase of tail length in the assay of growth hormone. Griffiths and Young (81) have found this a satisfactory method since tail growth in the rat is proportional to the increase in body weight. It seems unlikely that this method will replace the weight response methods.

PREPARATION OF GROWTH PROMOTING SUBSTANCE

Until 1933 attempts at purification of a potent growth promoting extract were confined to a few workers. Investigation of this problem was carried on in Evans' laboratory from 1921, and by 1929 he and co-workers (48)

had prepared a potent alkaline extract from which the bulk of inactive protein had been removed. The main problem which then confronted them was the necessity of removing all specific contaminants as well as increasing the growth stimulating potency of extracts. The alkaline extract was the starting point for further purification both by use of precipitation methods and organic solvents (49). Teel (180) who had originally worked with this group introduced ammonium sulphate in the preparation of the growth stimulating substance from anterior pituitary tissue. Several other workers immediately introduced modifications of this technique which is still the most widely used method in the fractionation of anterior pituitary hormones (187; 23). About the same time Janssen described the preparation of a potent acetone powder (92). Even in the early days of hormone purification there was difficulty in evaluating the potency of preparations due to the introduction of modifications in assay procedure.

The ability of $Ca_3(PO_4)_2$ to adsorb the growth hormone from a pituitary extract was utilized by Collip (33) in the preparation of a growth extract contaminated to only the slightest extent with other principles. The poor yield of active substance, in some cases as low as 5 per cent, showed the impracticability of such a method of preparation. Within the last few years rapid strides have been made in the

preparation of growth promoting substance contaminated to only the slightest degree with one or two other principles. As knowledge of the chemical nature of the growth hormone is gradually acquired by investigators it is immediately applied to advantage in future work. Fevold et al (61) recognizing the euglobulin nature of this factor applied the principles of protein chemistry to its fractionation. They separated the growth factor by careful control of the ammonium sulphate concentration and the pH of the solution from the other principles which were present. Their final product represented the total activity of the original tissue and a hundred and sixty-fold increase in potency. The material appears to be free of contaminants and assays 115 units per mg. of dried powder (Lee's method) (61).

The cysteine treated growth preparation of Evans (68) is essentially free from the thyrotrotrophic, lactogenic and corticotrophic and follicle stimulating hormones. It is contaminated with the 1 C S H principle to the extent of ten per cent. The cysteine treated preparation assays 100 hypophysectomized rat units per mg. (Evan's method) (120).

These two preparations represent the most potent preparations to date. It is interesting that Fevold uses sheep glands while Evans uses the customary beef glands.

The main principles in the separation of this factor are the alkaline extraction of either fresh or acetone desiccated glands, the former being preferable and the isolation of this principle from the extract by suitable regulation of ammonium sulphate concentration and pH. Evans recommends high alkalinity pH 11.5 to 11.7, in a calcium oxide solution. He claims it has a denaturing effect on inactive protein so that less appears in the neutralized extract. Fevold uses a $(NH_4)_2OH$ solution at pH8 for extraction. The success of the work of investigators in this field appears to be due to modifications in the general technique which permit maximum extraction coupled with a great increase in the growth activity of the final product, and freedom from other pituitary principles.

THE THYROTROPHIC HORMONE

The existence of the thyrotrophic hormone as a separate physiologically active principle of the anterior hypophysis is generally accepted. In 1889 Rogow itch (144) noted cell changes and enlargement of the anterior pituitary of thyroidectomized dogs and rabbits and wrongly attributed these changes to the vicarious action of the hypophysis. The clinical and experimental findings of other investigators have confirmed his observations of pituitary changes after thyroidectomy (104) (22). As a result of Adler's (3) successful ablation of the pituitary in amphibia (1914) Allen and Smith studied the results of hypophysectomy in larval amphibia (5; 166) (166). They observed atrophic changes in the thyroid gland and associated them with the failure to metamorphose. In 1912 Gudernatsch (83) had produced precocious metamorphosis in tadpoles by feeding thyroid. The theory of a pituitary-thyroid relationship in amphibia was strengthened by these observations and further confirmed by the thyroid repair Smith (169) produced by the implantation or injection of anterior pituitary extracts in hypophysectomized tadpoles. The injection of anterior hypophyseal extracts into amphibia by Spaul (176) in 1924 produced acceleration

of metamorphosis associated with hypertrophy of the thyroid. Uhlenhuth and Schwartzbach (185) confirmed his findings a few years later.

The investigation of the pituitary-thyroid relationship was soon extended to mammals. Aron (196) and Loeb (105) induced hyperplasia of the guinea pig thyroid by injection of anterior pituitary extracts (1929). Subsequent to their reports numerous investigators observed hyperplasia of the thyroid in a variety of laboratory animals: Janssen and Loeser (93) and Junkmann and Schoeller (96) in the guinea pig, Houssay et al (89) and Putnam, Benedict and Teel (14) in the dog, and Riddle and Polhemus (137) in the pigeon; Anderson and Collip (6) in the rat and Schockaert (152) in the duck. The result of this work was a stimulation to investigate further the principle present in anterior pituitary extracts responsible for the thyroid effects. The existence of a specific principle was confirmed by its separation from other pituitary factors and subsequent investigation has resulted in the preparation of potent fractions almost completely free of contamination.

THE CHEMICAL PROPERTIES OF THE THYROTROPHIC SUBSTANCE

The thyrotrophic hormone is soluble in water and dilute solutions of acid and alkali. It is soluble in aqueous solutions of ethyl alcohol, acetone and pyridine, but is insoluble in concentrated solutions of organic solvents, a property that has been used to advantage in purification of the active principle. Cohn et al (61) have shown that the hormone appears to be completely precipitated when the concentration of ammonium sulphate reaches 2.4M at pH 7. When an extract is heated in neutral solution to 100 deg. C its activity is destroyed. Chemical analyses of an almost pure fraction show that the protein contains 13% nitrogen; 3.5% carbohydrate and 2.5% glucosamine. Cysteine treatment, under conditions favorable for the reduction of S-S bonds was found to inactivate the hormone (72).

THE BIOLOGICAL PROPERTIES OF THE THYROTROPHIC HORMONE

The biological properties of the thyrotrophic hormone have been amply studied both in normal and hypophysectomized animals. The administration of a suitable amount of this principle to a normal animal produces hypertrophy of the gland associated with histological changes indicative of hyperactivity (196) (105). This results in an increase in the metabolic rate (161) and increase in the heart rate (151) reduction in the iodine content of the gland (152) and exophthalmus (107). This principle repairs the effects of hypophysectomy on the thyroid gland.

A great species variation exists in the response to thyrotrophic hormone. The guinea pig is the most sensitive mammal while the normal rat is almost insensitive to injected extract. It is interesting to note that a study of the thyrotrophic content of these two species shows that the more sensitive animal contains the least hormone (34).

The specific effects produced by the injection of the thyrotrophic hormone are credited as an activity of the interstitial cell-stimulating or luteinizing hormone (94;95). The results obtained by Fraenkel-Conrat (72) with an

almost purified preparation of thyrotrophic hormone as well as those of Cohn et al (61) do not support the contention that these two hormones are identical. Similarly the studies of Van Dyke on selective enzymatic inactivation shows their separate identity (28). Marx, Evans and Simpson (119) found that the purified thyrotrophic hormone enhanced the growth effect of a purified growth hormone.

METHODS OF ASSAY OF THE THYROTROPHIC HORMONE

The various biological effects of this principle are the basis for the numerous methods used in the assay of the thyrotrophic substance.

The most popular method of assay is based on the weight increase of the thyroid gland produced by the thyrotrophic principle. The use of guinea pigs for this test was introduced by Rowlands and Parkes (145). More recently the thyroid weight increase of newly hatched chicks has been introduced by Smelser (164; 165). The chick is more sensitive to the action of the hormone and the method is gaining widespread use in the assay of thyrotrophic activity. Smelser's work has been confirmed by Turner and others (16; 37).

Since the histological changes wrought in the thyroid gland by the pituitary principle were among the first effects noted by the early investigators it was only natural that they should serve as a criterion of response. Among those using such methods in the estimation of activity are Loeb and Bassett (106), Aron (197), Junkmann and Schoeller (96) and Heyl and Laqueur (85).

Another biological effect of the active principle is the decrease in the iodine content of the gland. Stimmel (177) makes use of this effect as an index of activity. The increase in metabolic rate produced by the thyrotrophic hormone has been used by Anderson and Collip in assaying the hormone (7).

PREPARATION OF THE THYROTROPHIC HORMONE

Despite all the investigation devoted to a study of the thyrotrophic substance the literature on the preparation of this principle is in a confusing state. Very few investigators have attempted to prepare the principle in a pure form uncontaminated by other factors, and the majority of those who report active preparations neglect to give all the details of procedure. The variety of assay methods

available and the failure to correlate newer methods with the already accepted ones serves to complicate the picture further.

Smith (175), Schockaert (153) and Foster, Gutman and Gutman (66) made use of fairly crude extracts in their investigation of the thyroid-pituitary relationship. A saline extract of beef anterior pituitary, contaminated by a variety of pituitary principles, was used by these workers. Putnam, Benedict and Teel (14) as well as Houssay and co-workers (89) used modifications of the alkaline extract originally described by Evans. Loeb (105) showed that the same effect was produced in the thyroid by use of either acid or alkaline extracts.

The neutralization of either acid or alkaline extracts caused the removal of inactive protein material from the solution. Attempts were also made to free thyrotrophic material from other active contaminants. Uhlenhuth (186) brought his acid Ringer extract to the boil, filtered and injected the extract immediately. The heating process was effective in removing other pituitary principles, he claimed. The use of iron hydroxide as an adsorbent resulted in a highly purified preparation. Junkmann and Schoeller, are, (96) however, vague concerning the details of procedure. Anderson and Collip (7) prepared a potent extract, free from

growth, by repeated saturation with ammonium sulphate and subsequent washing of the precipitate and precipitation of the active principle by alcohol. Differences in solubility in higher concentrations of alcohol served to separate the thyrotrophic from the adrenotrophic principle with which it was associated. Rowlands and Parkes (145) by means of pyridine extraction removed thyrotrophic and gonadotrophic principles from growth and prolactin. Greep(77) succeeded in separating the thyrotrophic from the gonadotrophic principles of the anterior pituitary, particularly the follicle stimulating hormone with which it was closely associated as a result of the particular extraction procedure. Lambie and Trikojus (97) describe a potent preparation by precipitation with tungstic acid and subsequent removal of the active principle by alcoholic benzoic acid. Fraenkel-Conrat (72) has recently reported the preparation of the thyrotrophic hormone in a practically pure state. The final product represents a hundredfold purification with recovery of about one-third the total thyrotrophic activity of the starting material. It was found to contain about 10 per cent of the interstitial cell-stimulating hormone, but only small amounts of growth, prolactin, adrenocorticotrophic and follicle stimulating hormones. The gain in weight of the chick thyroid has been used in assaying the potency of the

preparation. An excellent correlation of this method with five other well-known methods of assay is given by these authors.

Beef glands because of their high thyrotrophic content are used in the preparation of the thyrotrophic principle. Rowlands (145) has shown their superiority over sheep glands.

Prolactin or The Lactogenic Hormone

The study of prolactin or the lactogenic hormone, has proved the most successful in the whole field of pituitary investigation. Prolactin differs from the other pituitary factors which exert a trophic influence on their specific end-organs. It is merely a secretagogue causing initiation of lactation in suitably prepared mammary tissue. This active principle has been prepared as a crystalline substance as well as an amorphous powder, and investigation by electrophoretic and other methods has shown them to be homogeneous substances. The physico-chemical as well as the biological properties will be treated separately in greater detail.

Before the report of Stricker and Grueter in 1928 (178) a relationship of the pituitary with milk secretion had been hinted at. Ott and Scott in 1910 observed increased lactation in a goat after intravenous injection of an extract of the posterior lobe (130). Stricker and Grueter, however, showed a relationship between the anterior pituitary and lactation when they produced milk secretion in a pseudo-pregnant rabbit by injections of an anterior lobe extract. Within the next year they extended their observations to dogs and swine (179;82). Other workers such as Turner and Gardner (183), Nelson and Pfiffner

(126) and Corner (38) were quick to confirm their results in different species such as the rat, guinea pig and goat.

The first studies on lactation were intimately tied up with studies of the gonadal pituitary relationship. Following high doses of oestrus-producing substances lactation was noted in guinea pigs by Laqueur and co-workers (98). The necessity of the presence of the gonads in mammals so that their secretions could produce the proper conditions in mammary tissue for prolactin to be effective was soon recognized. Generalizations along this line were impossible since a species differentiation was soon recognized. Some species merely required the action of oestrin on mammary tissue (73) while others required progesterone (21) in addition.

Riddle (136) in 1931 while investigating the effects of an alkaline pituitary extract on pigeons noted stimulation of the pigeon crop gland. The only recognized pituitary hormones at that time were the growth and gonadotrophic factors, and Riddle thought the latter was probably responsible for the effects produced. Subsequent study by Riddle, Bates and Dykshorn (139) showed that a separate pituitary factor, which they called prolactin, was responsible for the initiation of milk secretion in mammals, and pigeon crop gland stimulation. Lyons and

Catchpole (110) confirmed the existence of a separate pituitary factor responsible for lactogenic function and named the principle mammotrophin.

Gardner and Turner (76) described the changes in mammary tissue of the rabbit during lactation and ascribed the name galactin to the effective agent. The identity of these three substances is now definite.

Apart from the work of various investigators in the preparation of the active principle and suitable assay methods others turned their attention to a study of the mechanism and control of milk secretion. The role of the pituitary was investigated by Collip and co-workers (33) who showed the necessity of this structure for continued lactation. The effects of ovariectomy and Caesarian section late in pregnancy, as well as the part played by suckling, was studied by these investigators and greatly contributed to our knowledge of the interrelationship of pituitary and mammary tissue in lactation (156;157).

Within recent years the efforts of investigators in this field have been directed to the preparation of the active principle in a pure form, uncontaminated by other factors, and possessing a high degree of potency. In this they have been highly successful as will be shown.

Assay Methods for Prolactin

Since the establishment that the principle responsible for crop gland stimulation in the dove or pigeon is the same as that causing milk secretion in the prepared mammary tissue of mammals, two general methods of assay may be used. One is based upon crop gland response in pigeons and the other on the production of lactation in mammals. Various modifications of the two methods have been introduced.

The more suitable method of assay would appear to be that based on crop gland stimulation since pigeons of either sex can be used whereas the female mammal must be employed. The mammary tissue of the mammal must be suitably prepared before lactation will result, but stimulation of the pigeon crop gland requires the observance of only a few simple precautions. The response in pigeons can be strictly quantitative especially if judged by weight increase while it is rather difficult to judge the amount of lactation, particularly in a small animal.

Riddle, Bates and Dykshorn (139) introduced a crop response method based upon the weight increase of the crop gland of doves and pigeons after a uniform period of injection with an anterior pituitary extract. These authors excise the stimulated portion of the crop wall,

the crop gland, and compare its weight with normal controls. Selected birds 2 to 3 months old are injected intramuscularly once daily for four days with autopsy about 96 hours after the first injection. They found that a linear relation exists between the crop weight and the logarithm of the dosage, and their unit is the extrapolated value for the threshold dose in an average sample of their doves of many races.

McShan and Turner (115) propose as their pigeon unit "the total amount of hormone injected during a period of four days which will cause a minimum, but definite proliferation of the crop glands of 50 ± 11 per cent of 20 common pigeons weighing 300 ± 40 gms." Lyons (113) uses a similar minimal stimulation unit. Positive stimulation is indicated by the presence of typical parallel strands of thickened mucosa. This method of assay is subjective whereas that of Riddle and Bates is objective. Lyons and Page (111) introduced a more sensitive assay method by modification of the route of injection. The method is essentially a minimum stimulation method, but by injection of the extract intracutaneously over the crop sac a response can be evoked with 1/1000 of the systemic dose. Others using local stimulation methods are Burrows and Byerly (25) and Reece and Turner (134).

As far as lactation in mammals is concerned Gardner

and Turner (76) proposed the use of the pseudo-pregnant rabbit in assaying the active principle. Nelson (127) uses the hysterectomized pregnant guinea pig and finds that it only takes 1/5 of the dose required to produce a similar response in the pseudo-pregnant rabbit.

At the Third International Conference on the Standardization of Hormones it was agreed to set up an international standard preparation (36). Lyons (114) reports that his purified prolactin preparation is twice as active in guinea pigs and three times as active in pigeons as the standard preparation.

CHEMICAL PROPERTIES OF PROLACTIN

Prolactin was the first pituitary hormone that investigators have succeeded in preparing in a pure state. The solubility of prolactin as well as its loss of potency when treated with pepsin and trypsin (116) and its thermal instability at various hydrogen ion concentrations is an indication of its protein nature (190). Electrophoretic studies by Li, Lyons and Evans (101) on a product which they consider pure shows it to be homogeneous and possessing a molecular weight of approximately 25,000. Analysis of their product shows that it contains 2.5% tryptophane, 8.31% arginine, 3.0% cystine, 5.7% tyrosine and 1.79% sulphur. These figures agree quite well with the

results of White, Bonsnes and Long (190) whose crystalline preparation contains 5.5% tyrosine, 1.3% tryptophane and 3.3% cystine. They report the molecular weight of their preparation as 32,000.

Prolactin was formerly reported insoluble in all fat solvents, however, the recent report of Fleischer (64) indicates its solubility in 99.8% methanol and 95% ethanol at a pH below its isoelectric point. Prolactin is soluble in H_2O except at its isoelectric region pH5-6. The isoelectric point values of pH 5.6 (101), 5.7 (100), 5.73 (159) have been reported for both amorphous and crystalline prolactin fractions. White et al (190) report the isoelectric point to be between pH 5.65 and 5.70.

The data collected on both the amorphous and crystalline fractions indicate the identity of these two products on the results of bioassay, solubility, sedimentation and electrophoretic studies (190). Riddle and Bates (143) have shown that a prolactin solution may be heated at pH 8 to 100 deg. C for one hour or at 60 deg. C for five hours. White et al (190) using their highly purified prolactin report it to be stable to heat in 0.04% solutions of pH 1 to 9 for 15 minutes in a boiling water bath. With a 30 minute heating period there is a definite destruction of prolactin activity at all pH.

BIOLOGICAL PROPERTIES OF PROLACTIN

Certain well-known effects are definitely associated with prolactin. Most notable among these are the effects upon lactation, crop-sac, gonads and certain organs as well as metabolic effects.

The crop sac of pigeons is markedly stimulated during the last week of brooding and for three weeks after hatching. This results in the formation of crop milk which is regurgitated by both parents to feed their offspring. Injection of prolactin into pigeons can bring about all these effects and its action would appear to be a direct one on the crop gland (139). Similarly in mammals prolactin exerts a direct effect upon suitably prepared mammary tissue so that milk secretion results (76).

Riddle et al (12) have observed a splanchnomegaly in pigeons treated with prolactin. There is a definite overgrowth of liver, and increase in length of the intestinal tract. These authors have also been successful in inducing broodiness in fowl of suitable genetic constitution. The testes of adult pigeons are reduced in weight by injection of prolactin (140) and the ovaries stop production of yellow ova, and those already formed are resorbed (11). Riddle, Smith, Bates, Moran and

Lahr (141) noticed a marked calorigenic action in pigeons. Prolactin exerts a definite effect on carbohydrate metabolism. It has been found to increase the blood sugar in doves, pigeons and rabbits (142), but Young (194) has shown it is not the factor responsible for pituitary-induced diabetes of normal dogs and rabbits.

PURIFICATION OF PROLACTIN

Prolactin was the first pituitary hormone to be prepared in the pure state. Riddle (138) and Turner (76) showed that the active principle could be extracted from fresh or acetone-desiccated glands by means of an aqueous acid or alkaline solution. The use of non-aqueous media for extraction was soon found to result in clearer extracts. Lyons and Catchpole (110) introduced the use of 66% acetone in acid medium pH 2, and Bates and Riddle (10) used 60 to 70% ethanol in an alkaline medium pH 9-10. The latter procedure was reported (15) to give the best extraction of the active principle both from the point of view of yield and unitage per mg of extracted solids. The acid acetone procedure described by Lyons in 1937 (112) has subsequently proved the most efficient method of extraction. In the hands of many investigators (190; 101; 159) it has been the starting

point for the crystallization and preparation of highly purified fractions. A recent publication by Fleischer (64) reports the preparation of a highly purified fraction by rather an unusual procedure. The active principle was removed from pituitary tissue and separated from the majority of other principles by acidic chloroform. Extraction of prolactin from the chloroform gel by acid methanol (99.8%) and subsequent purification by salt fractionation results in recovery of thirty five per cent of the original activity and a thirty fold increase in the activity of the final product.

Subsequent to any extraction procedure it is necessary to isolate the prolactin from other pituitary factors, particularly the corticotrophic factor with which it is closely associated. The extraction procedures generally employed result in the presence of a minimum number of active principles in the extractive. The separation of prolactin and the corticotrophic principle is generally accomplished by isoelectric precipitation. The isoelectric point of the corticotrophic factor was formerly thought to be pH 6.5, while that of prolactin, as previously mentioned, is pH 5.65. It is interesting to note the points of minimum solubility between acid and alkaline extracts. The isoelectric point of acid extracts is around pH 6-7

(110;138) while in alkaline extracts it is between pH 5-5.6 (139;76).

After isolation from contaminating principles the prolactin is ready for further purification. This is usually accomplished by reprecipitation from salt solution or treatment with a mixture of pyridine and acetic acid or precipitation with acetone in an acid medium. The product that results from these procedures is either an amorphous preparation or a crystalline one. White et al (189) first reported the preparation of crystalline prolactin which they considered highly active. Riddle and Bates later found that their amorphous powder contained 10 Riddle-Bates units per mg. as compared with 6.5 similar units in White's crystalline preparation. The purification of prolactin has advanced rapidly within the past few years. The recent crystalline and amorphous preparations have been found to possess the same activity. A comparative study of the various purified prolactin preparations showed their identity and similar activity (190). The majority of preparations possessed 30-31 international units per mg. (36)

THE CORTICOTROPHIC PRINCIPLE

The corticotrophic or adrenotrophic principle refers to that factor in anterior pituitary tissue which has as its special function the maintenance of the adrenal cortex. This factor was the last of the six accepted pituitary factors to be identified and established as a specific entity. Its close association with both the thyrotrophic and prolactin fractions of anterior pituitary extracts was responsible for the delay in its recognition.

The stimulating action of the pituitary on cortical tissue was first reported by Smith. Hypophysectomy was observed to cause atrophy of the cortical, but not the medullary tissue of tadpoles (168). Smith in his renowned study of the effects of hypophysectomy in the rat reported regression of the cortex (171). In a more complete report a few years later he showed that implants of anterior pituitary were effective in restoring the atrophied cortex to normal while saline extracts of bovine anterior pituitary were without effect (175). Evans and associates reported that their growth extracts were capable of restoring the atrophied cortex of hypophysectomized rats, but gonad stimulating preparations from pituitary origin were ineffective (51).

The number of non specific agents capable of causing adrenal cortical hypertrophy was borne in mind and while

most investigators thought that a specific principle could exist they lacked final proof in the matter. The production of cortical adenoma in experimental acromegaly produced in dogs by pituitary extracts strengthened the argument as did the reports of adrenal hypertrophy in other animals. The work of Houssay (90), Anselmino, Herold and Hoffman (8) and Emery (44) reported stimulation in normal animals. Clinical evidence of a pituitary-adrenal relationship also existed (148).

From the observations of those interested in the study of the thyrotrophic hormone the adrenal stimulation was considered a secondary effect of this principle. Loeser (108) claimed that his thyrotrophic extract induced adrenal hypertrophy in all his treated guinea pigs and quoted this as evidence that this principle was the causative agent. He then retracted his statement. Friedgood (74) observed that the hyperthyroidism of guinea pigs produced by partially purified alkaline extracts was accompanied by adrenal hypertrophy.

Collip et al (32) while working on a thyrotrophic preparation succeeded in separating from it a factor responsible for cortical stimulation and repair. They presented evidence for the existence and separate identity of this specific principle. Riddle and associates (143) in their studies of prolactin observed adrenal stimulation, but con-

sidered it one of the numerous effects of prolactin. They discredited the evidence of Collip for the existence of a specific principle until Lyons (112) succeeded in separating an adrenotrophic fraction from prolactin. Moon (124) described the preparation of this principle and its biological effects.

BIOLOGICAL PROPERTIES OF THE CORTICOTROPHIC PRINCIPLE

It is generally accepted that the only function of this principle is the maintenance of the adrenal cortex. The regressive changes initiated by hypophysectomy can be restored to normal by the injection of the active principle (32). There is a restoration of the cytoplasm of the cortical cells, an increase in the fat content and the disappearance of pycnotic nuclei. The injection of an active preparation into normal immature rats produces an increased fat content throughout the whole cortex, but particularly, in the normally fat-free zone between the glomerulosa and fasiculata. There is hypertrophy and hyperplasia of the cortical cells particularly in the zona glomerulosa and outer zona fasiculata (124).

The adrenotrophic principle has been reported to partly restore the work capacity which is subnormal in hypophysectomized rats (91). Jensen and Grattan (95)

showed that the adrenotrophic fraction of the anterior lobe has an anti-insulin action and increases liver glycogen. Extracts from the adrenal cortex and crystalline corticosterone acetate produce definite glycotrophic effects so one can conclude that these actions of the corticotrophic principle are mediated through the adrenal cortex.

CHEMICAL PROPERTIES OF THE CORTICOTROPHIC PRINCIPLE

The corticotrophic principle is the last of the six accepted pituitary factors whose separate identity has been established. It, like the other hormones, is protein or protein-like in nature. The study of the chemical properties of this hormone has been retarded by the relatively small yields of purified material which have been obtained. Recent reports indicate that two laboratories have succeeded in preparing pure corticotrophic substance and the results of chemical study will be published shortly (102; 150).

The chemical properties of this principle resemble very closely those of prolactin with which it is usually associated. Like prolactin the corticotrophic principle is soluble in 60-70 per cent ethanol even in its isoelectric region (32). Its high solubility at pH 3.5 in the presence of H_2SO_4 differentiates it from prolactin. Lyons reported that the isoelectric point of the corticotrophic principle is pH 6.5 (112). Riddle et al criticize Lyons description of this protein as a typical aqueous isoelectric-

ally insoluble one (13). They found that at the reported isoelectric point some active material remained in the supernatant. Solubility of this principle at pH 6.5 has been intimated by two investigators (133; 95). In view of this the recent reports of Evans et al (102) and Sayers, White and Long (150) are of interest. Both groups have obtained the principle in a pure state as indicated by electrophoretic studies and solubility tests and they report the isoelectric point at p H 4.7.

The tryptophane content is ten times less than that of prolactin which is 2.5 per cent. The hormone was reported by Collip to be very stable on heating in aqueous solution over a wide p H range (35). Even in the purified state the heat stability of this principle is exceptional. Its biological activity is not diminished when a 1 per cent solution in p H 7.5 phosphate buffer is heated on a water bath at 100 deg. C for 2 hours.

ASSAY OF THE CORTICOTROPHIC PRINCIPLE

Several methods of assay may be used for the corticotrophic principle. They are based upon the hypertrophy of the adrenal cortex in normal animals treated with the specific principle and on the maintenance or restoration towards normal of the atrophic cortex after hypophysectomy.

In the opinion of most investigators the use of the hypophysectomized rat is the most satisfactory method for

the assay of this principle. A period of two weeks after hypophysectomy is sufficient for regressive changes to take place in the cortical tissue. This layer becomes thinner, the cells become disorganized and there is a loss of their fat contents. The injection of the active principle brings about a restoration so that the cortex assumes its normal appearance. Collip (32) introduced the use of hypophysectomized rats for assay purpose when he reported the existence of a specific pituitary factor exercising a trophic effect on the adrenal cortex. The assay consists in the removal of the left adrenal from rats ten days to two weeks after operation. Injections of the active principle is carried on for six days when the right adrenal is sectioned and weighed. The difference in weight between the right and left adrenals, together with the histological picture is indicative of the degree of stimulation. The use of a large number of hypophysectomized animals in the assay procedure eliminates the removal of one gland prior to treatment since the degree of regression in animals of the same sex and age is fairly constant. Evans (102) uses immature rats fourteen days post operative and after four days of injection kills his animals on the fifth. The degree of activity is judged by the histological picture of repair. White et al (150) employ mature rats as suggested by Collip. The activity is calculated on the minimum dose

effecting cortical repair by both Evans and White.

Modifications in the weight of the animals at hypophysectomy have been introduced in the maintenance method of assaying the corticotrophic principle. The animals are treated with the active substance immediately after hypophysectomy for a period of two weeks. The minimum dose which is effective in maintaining the adrenal weight of these animals at the level of normal controls of the same age is determined. The histological picture is also an important criterion (102; 150).

The variety of agents which lead to a stimulation of the adrenal cortex in a normal animal does not allow one to consider hypertrophy of the gland under the influence of an extract as a specific effect. Moon (124) introduced the use of twenty-one day old normal rats for assay of the corticotrophic principle. Injections are made intraperitonally for three days and the production of a 100 per cent increase in adrenal weight above that of untreated animals is considered a unit response. More recently he has introduced the use of four day old rats (125). Other workers have introduced modifications particularly with reference to the number of injections per day (20).

Bates, Riddle and Miller (13) make use of white Leghorn cockerels in their assay of the active principle. The chicks are two days old at the start of injections which

PREPARATION OF THE CORTICOTROPHIC PRINCIPLE

Since the announcement by Collip in 1933 of the existence of a corticotrophic principle of anterior pituitary which has as its specific function the maintenance of the adrenal cortex, this hormone has been almost ignored. It has not received the amount of concentrated investigation directed toward the other pituitary principles, and very few investigators have turned their attention to its preparation and study. The dearth of interest might have been due to lack of foresight of the possibility of clinical application, but the stress recently placed upon the importance of the adrenals seems to have overcome the lethargy in its investigation.

As with the other hormone preparations the method employed in extraction determines the procedure to be used in its isolation and purification. As a result of the extraction procedures employed by the investigators of the thyrotrophic principle the biological actions of the corticotrophic principle were wrongly associated with it before Collip (32) succeeded in establishing the separate identity of the two factors. The corticotrophic principle was separated from the thyrotrophic factor by virtue of its solubility in 70 per cent ethanol and 50 per cent

continue for five days. Their unit is the total amount causing a 25 per cent increase in weight when injections are made three times daily. Riddle et al claim that non-specific agents are incapable of causing a response in baby cockerels under similar conditions. It must be admitted that normal animals whether rats or cockerels are more easily available for routine assays than hypophysectomized animals two weeks postoperative.

acetone. After removal of the thyrotrophic precipitate Collip worked up the alcohol and acetone filtrates. After adjusting the pH of an aqueous alkaline extract to 6 and allowing it to stand at 0 deg. C isoelectric precipitation of the active principle took place. It is interesting to note that Collip described its solubility at pH 3, a property recently used to advantage by Riddle et al in its purification (13).

In the extraction procedure employed by Lyons (112) a more selective extraction of the active principles of the anterior pituitary took place. He used a high concentration of acid-acetone which resulted in the extraction of both the corticotrophic and prolactin fractions. By adjustment of an alkaline aqueous extract to pH 6.5 he succeeded in separating the corticotrophic factor as an isoelectric precipitate. It was only with its successful separation from prolactin that Riddle et al accepted the existence of a corticotrophic factor distinct from prolactin.

The extraction procedure introduced by Lyons has been successfully employed just as described above or slightly modified by those who have succeeded in preparing the corticotrophic principle in the pure state. Sayers, White and Long (150) used Lyons original method of extraction in the preparation of their highly purified fraction.

Evans and associates use acidified 80 per cent acetone as the extraction medium. Isolation of the cortico-trophic principle from its chief contaminant, prolactin, is accomplished by precipitation with saturated ammonium sulphate. Frequent reprecipitation of the principle results in a highly active preparation. Evans uses sheep glands as a source material while Sayers et al use hog glands. The preliminary studies indicate that the active protein of the two species has the same isoelectric point. Biological tests also show their similarity of activity.

Riddle, Bates et al have worked on the prep-aration of highly purified extracts for some time. They use alkaline alcohol as an extraction medium. Beef anterior lobes are extracted with 65 per cent ethanol at pH 9.5. This was their extraction procedure in the preparation of prolactin. Their extracts have not the same degree of purity possessed by the more recent preparations described above.

THE GONADOTROPHIC PRINCIPLES OF THE ANTERIOR PITUITARY

The history and study of the gonadotrophic principles of the anterior pituitary constitute one of the most interesting chapters in the whole of endocrine investigation. The proof of the existence of two factors, the so-called follicle stimulating (FSH) and the interstitial cell stimulating (ICSH) or luteinizing hormone (LH), their relationship with the gonadotrophins extracted from the blood and urine of normal men and women, and their similarity with gonadotrophic substances from other sources are some of the main features of investigation in this field. Interesting as they are, it is impossible to deal with the complete study of gonadotrophic substances. Only those factors obtained directly from anterior pituitary tissue have been treated here.

That a pituitary-gonadal relationship of some sort existed was shown by the clinical evidence of Cushing (40) and the observations of Addison (2) and others. They described changes in the number of basophil cells of the anterior lobe after castration, as well as typical changes in their appearance, giving rise to what is known as castration cells. Evans and co-workers (45) in their early investigation of growth-stimulating extracts observed that continued injection of these products into rats caused extensive

luteinization of their ovaries and disturbances in their oestrus cycles. As early as 1923 they reported separation of an ovary stimulating principle from the growth factor in anterior pituitary extracts (65).

The real proof that the pituitary exerted a stimulating effect upon the gonads was that introduced by Smith in 1926 (170) and by the European workers Zondek and Ascheim (195). They succeeded in producing precocious sexual maturity in immature rats and mice by implantation of pituitary tissue. Smith (172) then showed that the atrophic genitals of hypophysectomized rats could be restored by pituitary implants. His report of negative results on injection of bovine alkaline pituitary extracts in similarly operated animals was rather disconcerting, particularly when it had proved effective in tadpoles (175). American and European investigators continued their study of the gonad-stimulating substance of the pituitary, and as the conflicting reports gradually accumulated it became evident that two effects were being produced in the gonads. The great variation in species response, as well as the different results produced by modifying the route of injection and the number of injections, to say nothing of the source of the pituitary tissue employed, produced a great deal of confusion. The existence of two pituitary factors was

postulated by many, but denied by others. In 1931 Fevold and Hisaw offered evidence for their existence and succeeded in separating the F S H and L H (57). This was the first great step in the solution of the problem that had tantalized investigators for a decade. Efforts to effect the complete separation of the two pituitary principles and to purify F S H and I C S H or L H, and to obtain a clear concept of their specific function have occupied investigators to the present day.

THE ACTUAL NUMBER OF PITUITARY GONADOTROPHINS

The number of postulated pituitary gonadotrophins has been greater than two. Evans et al (52) formerly believed their I C S H to be a separate factor. Recent investigation has proved the identity of Evans' I C S H and Fevold's L H (99; 158; 63). Despite the actual preparation of two separate gonadotrophic principles from anterior pituitary and the strong evidence of their specific biological properties and separate chemical identity there are some who refuse to accept the existence of the F S H and I C S H. These workers believe that all the biological effects attributable to two factors can be produced by a single factor, and they deny that I C S H possesses any specific function. The majority of their arguments rest upon the regulation and rate

of dosage of unfractionated pituitary extracts or purified F S H alone.

In 1934 Maxwell (122) showed that by manipulating the rate at which the unfractionated pituitary extract was allowed to reach the ovary, lutein changes could be abolished or made made to appear. The action of salts such as zinc and copper sulphate, and other non-specific substances in augmenting the effect of injected F S H is generally accepted by the proponents of the single and dual theory. (201; 54; 18; 19) However, there are some who claim that the augmenting effects of non-specific substances when administered with F S H produce greater augmentation than when I C S H is administered in combination with it (149). The F S H used by these workers has since been shown to have been contaminated with I C S H, and that further augmentation with this substance was impossible. Bischoff (19) is one of the foremost proponents that F S H is responsible for all the multiple hormone effects.

It is interesting to note that all the arguments on the augmenting effects of non-specific substances are based upon evidence in female animals. Non-specific substances have not been shown capable of simulating the effects of I C S H on the interstitial cells of the testes, and the accessory sex glands of the male rat. The experiments of

Rowlands (146) and the evidence of Du Shane, Levine and Pfeiffer (43) give further support to the dual theory. The purification of two separate principles by numerous workers (158;122;67;68) seemsto give definite proof of the existence of two distinct pituitary gonadotrophins.

BIOLOGICAL PROPERTIES OF GONADOTROPHIC FACTORS

The description of the biological properties of the two separate gonadotrophic principles must take into account the synergistic action of these two hormones in mammalian physiology. Each hormone plays a particular role in the maintenance of the genital system whose normal function is dependent upon the proper balance of the two gonadotrophic principles. It was not possible to obtain a clear picture of the specific biological properties of each hormone while they were contaminated with one another. Effects formerly attributed to the action of one factor are now known to result from a combination of both factors. The advent of highly purified preparations of both principles has allowed a detailed and more clearly defined study of the biological properties of both the F S H and I C S H.

In considering the biological effects of injection one must bear in mind the sex of the animal, the length of

treatment, the site of injection, the rate of adsorption, the dosage, and above all, the purity of the injected material. The age of the animal, whether immature or adult, and its condition, whether normal or hypophysectomized are also important sonsiderations.

The properties of each factor will be considered separately and then their combined effects.

PROPERTIES OF F S H

When administered to an hypophysectomized female rat a pure preparation of F S H causes growth and development of numerous follicles associated with an increase in ovarian weight (67). The complete absence of any accompanying lutein changes is dependent upon its freedom of contamination with I C S H. Pure F S H does not initiate any oestrogenic effects on the accessory sex organs (158;80). Fevold et al (58) intimated that the development of the accessory sex organs formerly believed to result from an increased oestrogen secretion of the ovary was due to contamination of the injected F S H with ICSH.

The results of injection of F S H into the hypophysectomized male are analagous to those in the female. The epithelium of the seminiferous tubules is maintained or repaired and sperm formation is at least carried to the

stage of secondary spermatocytes. (67;68). The accessory sex glands as well as the interstitial tissue of the testes are not influenced by F S H administration.

It would seem that follicular development within the ovary of the normal female and maintenance of the seminiferous epithelium in the normal male are the specific functions of F S H.

PROPERTIES OF ICSH

When I C S H is administered to the hypophysectomized male rat it effects repair of testicular interstitial tissue, but tubular stimulation does not result. Some accessory development occurs as a result of secretion of testosterone (32;68;162). The administration of I C S H immediately after hypophysectomy in the male rat maintains the interstitial tissue of the testes and the accessory sex organs. Sperm formation in the adult rat is maintained under these circumstances (162).

In the hypophysectomized female rat I C S H can maintain ovarian interstitial tissue or restore it when atrophy occurs as a result of the operation. (54; 67) It does not affect follicular development. In the normal adult rat I C S H increases the amount of lutein tissue, prolongs the dioestrus interval and delays parturition in

pregnant animals.

COMBINATION OF FSH AND ICSH

The synergistic action of F S H and I C S H was recognized by Fevold and Hisaw (57). The augmenting action of I C S H when combined with F S H is well known and has been used as a basis of assay (58; 163)

When I C S H is combined with F S H and administered subcutaneously to hypophysectomized rats synergism is observed. The ovarian hypertrophy and follicular development is increased above that resulting from the administration of F S H alone, and the interstitial tissue is repaired. The amount of I C S H required is small and luteinizing effects are not produced by this amount. At higher levels of I C S H administration luteinization results (54). Similar effects are observed in normal immature rats (68). Pronounced uterine development is observed when a combination of F S H and I C S H is administered to both types of animals (80).

The antagonistic action of I C S H and F S H when administered intraperitoneally to hypophysectomized rats is rather generally accepted. Rather than augmenting the action of F S H follicular development is inhibited (62) Evans et al (162) claim that the antagonism of purified

I C S H against gonadotrophins from other sources cannot be demonstrated against pituitary F S H.

ASSAY METHODS FOR I C S H

The fact that two pituitary gonadotrophins act upon the gonads to produce physiologically related effects makes it absolutely necessary to use as a criterion of activity some response which is absolutely specific for each factor. With the advent of purified F S H and I C S H it was soon realized that effects formerly attributed to the action of a single factor were in reality due to the combination of gonadotrophic factors.

There are various methods available for assaying ICSH. Some utilize normal rats while others use hypophysectomized rats. Assays are not limited to rats, chickens and pigeons may also be used. The sensitivity of the test differs in the various methods employed. Evans et al have successfully prepared I C S H in a purified form and assayed it on hypophysectomized immature female rats (163). The criterion of activity is the minimal dose that initiates repair in the ovarian interstitial tissue which has atrophied as a result of hypophysectomy. Fevold (58) suggested that the augmentation of F S H by I C S H be used as a method of assay. Evans et al use the augmentation effect as an assay procedure on immature female rats hypophysectomized 6-8 days previously. They find that I C S H activity can be recognized with 1/5 the amount necessary to produce interstitial repair (163). For accurate assay results an uncontaminated preparation of F S H is demanded.

The other assay procedures make use of males rather than females. Fevold (60) uses normal immature male rats for I C S H assay. The response is measured by the increase in the weight of the seminal vesicles. It should be mentioned that all strains of rats are not suitable for this assay (68). Greep et al (79) also use the response of the secondary sex organs of the male for assay of I C S H. These workers use immature male rats two days after hypophysectomy and measure I C S H activity by the increased weight of the ventral lobe of the prostate. Intraperitoneal injections are much more sensitive than subcutaneous injections in this assay.

Contrary to the effects of I C S H in the hypophysectomized female this factor is only partially effective in restoring the interstitial tissue of the testes after degenerative changes have taken place (162). However, it can maintain the size and function of the testes if injected immediately after hypophysectomy. Simpson, Li and Evans plan to standardize the results in hypophysectomized mature rats and use them as a basis of assay.

Riddle first reported the great sensitivity of the pigeon testis to pituitary gonadotrophins (136). Quantitative data are however lacking, although weight response occurs on injection with I C S H (163). Evans and

associates suggest this as a method of assay and present evidence of its sensitivity which is superior to chick methods. Riddle (154) doubts the ability of pure I C S H to stimulate testis weight in normal and hypophysectomized pigeons, and Greep (80) also offers evidence to the contrary. White leghorn chicks can also be used for I C S H assay. The criterion of response is the increment of testis weight (17). Methods of assay for I C S H employing hypophysectomized females are more sensitive than methods employing male rats.

ASSAY METHODS FOR F S H

Fevold introduced an assay method for F S H based upon the ovarian weight increase in immature female rats treated with this fraction (59). When one realizes the augmenting action of I C S H in such a procedure the necessity of a pure F S H preparation is obvious. Evans recommends the use of hypophysectomized animals for assay of pituitary gonadotrophins since the possibility of interference by the animals own pituitary is obviated. For assay of F S H Evans et al (68) use the hypophysectomized immature female rat. The minimal dose which causes resumption of ovarian follicular development in such an animal is the standard response.

ASSAY OF PITUITARY GONADOTROPHINS IN UNFRACTIONATED EXTRACTS

To test the gonadotrophic activity of unfractionated pituitary extracts the older and less specific methods of assay can be used. The use of the immature female rat according to the method of Van Dyke and Wallen Lawrence (188) is probably most general. The increase in the ovarian and uterine weight is the index of gonadotrophic activity. All investigators have employed this test and introduced their own modifications. The mouse assay is also widely used (84). The criterion of activity is the increase in the uterine weight of mice. One day old cockerels are also used for assay of unfractionated extracts. J.S. Evans et al (56) have compared the sensitivity of these three methods of assay of unfractionated pituitary extracts. They find the mouse uterus about sixty-six times as sensitive as the rat ovary and ten times as sensitive as chick testes.

CHEMICAL PROPERTIES OF I C S H

Within recent years investigators have succeeded in completely separating the I C S H from F S H. The resulting preparations appear to be homogeneous on the basis of electrophoretic, solubility and ultracentrifugation tests, and evidence for the identity of the hormone and protein is presented (158;29;99). Sheep or swine pituitaries are

used as the source material. The isoelectric point of I C S H prepared from sheep glands is reported by Evans et al to be pH 4.6 to 4.8, and Chow and associates estimate it to be about 4.8 to 5.0. The latter authors use swine pituitaries as their source material and report the isoelectric point to be pH 7.45. They suggest that I C S H from sheep and swine glands are different proteins. Their biological activity appears to be similar. Chow estimates the molecular weight of his protein to be about 100,000.

I C S H gives positive biuret, xanthoproteic; Millon and ninhydrin reactions. The carbohydrate content of I C S H is less than that of F S H, a property that may contribute to it being less soluble. Chemical analyses show that the active protein contains 4.45 per cent hexose, and this appears to be mannose. Fevold et al (61) show that the globulin can be precipitated from solution by 1.6 to 2.0 M $(NH_4)_2SO_4$ at pH 7. The active protein appears to contain free amino groups since inactivation occurs on treatment with ketene. The action of proteolytic enzymes on I C S H has also been studied. Crystalline trypsin destroys the activity of this protein and ptyalin is without effect (1; 117).

CHEMICAL PROPERTIES OF FSH

The chemical nature of F S H has not been studied to the same extent as I C S H. It has been prepared in fairly pure form by a number of investigators (78; 54; 70) who give evidence of its protein nature.

The most striking characteristic of F S H is its high solubility. It is the most soluble of all the active fractions of the pituitary. It is soluble in water and in the absence of electrolytes in 50 per cent acetone and 70 per cent alcohol. Fevold et al report its globulin nature and precipitate it from 2.4 to 2.8 M $(NH_4)_2SO_4$ solution at pH 7 (61).

Evans et al (55) have studied its carbohydrate content and find that their preparation contains 13 per cent and 8% glucosamine. Cysteine which attacks S-S bonds inactivates F S H. Crystalline trypsin destroys the activity of this protein and so does ptyalin (1).

PREPARATION OF GONADOTROPHIC PRINCIPLES OF ANTERIOR PITUITARY

In the twenty years since Evans and Long noted the gonadal effect of their crude pituitary extracts, and more particularly since the establishment of a pituitary-gonadal relationship by Smith and Engle and others, the literature that has accumulated on the preparation of gonadotrophic extracts from pituitary tissue and isolation of the active principles is voluminous. It is impossible to do more than generalize on the principal procedures.

The effective, although crude, separation of two pituitary gonadotrophins by Fevold and Hisaw gave the impetus to further investigation. The majority of workers have concentrated their efforts since that time on the separation and purification of the active principles. The contributions of some workers have been spasmodic and not particularly valuable, however, the continued investigations of the principal workers have been attended with success. Those who have contributed most to the particular problem are Fevold and associates, Chow and Van Dyke, and Evans and co-workers.

It was soon realized that the main difficulty in separating the two factors was their great solubility. The two proteins are chemically similar and their solubilities

in any medium tend to overlap so that a clear-cut separation cannot be effected with a simple precipitation procedure. Numerous reprecipitations of either factor are necessary to free it of its contaminant.

The deficiencies of bovine pituitary in the gonadotrophic factors has led to the use of sheep and hog pituitaries as starting material. Either acetone desiccated or fresh glands may be used. Numerous extraction procedures have been employed but they have gradually narrowed down to the use of aqueous alkali, or 2 per cent NaCl or 40 per cent alcohol.

All the principal workers have used aqueous alkali as a method of extraction at one time or another. Fevold continues to use aqueous pyridine, but he now uses 2 per cent rather than 50 per cent. It has the advantage of serving as a preservative. Any pH above 9 tends to destroy activity so dilute aqueous solutions are employed. Van Dyke et al formerly used $(NH_4)OH$ in dilute solution while Evans and his group made use of $Ba(OH)_2$. The tendency in recently published procedures seems to be the use of 2 per cent saline solution. This has been employed with success by Van Dyke et al. Extraction at pH 6 has been reported by Fevold to lead to no loss of activity. The employment of dilute alkaline solutions

and neutral saline has the tendency to bring down a great deal of protein material which makes isolation and purification of the active principles more difficult. To avoid this Evans uses 40 per cent alcohol as an extraction medium and lessens the content of other protein material.

With whatever method of extraction used the bulk of inactive protein material is removed subsequent to extraction. This allows the active fractions to be isolated more easily. Evans et al then remove the active fractions from the 40 per cent alcohol powder by extraction with acetic acid pH 4. The separation of F S H and I C S H is then accomplished by regulation of the degree of saturation of the solution with ammonium sulphate. The precipitates obtained at the appropriate saturation point are then redissolved and reprecipitated several times to yield purified preparations. The yield of activity does not represent much more than 10 per cent of the original activity, but the potency of the preparations is increased.

While the initial extraction procedure of Van Dyke and his associates is different they also isolate the active factors by control of ammonium sulphate concentration. Separation is effected by precipitating I C S H with 1 M acetate buffer pH 4.41 and 2 volumes of 41 per cent Na_2SO_4. The F S H which remains in solution is then

precipitated with $(NH_4)2SO_4$.

Fevold et al use benzoic acid to separate the active principles and then precipitation with ammonium sulphate. The protein fractions obtained by the principal workers have been studied by electrophoretic, solubility and ultracentrifugation tests, and the purity of the I C S H fractions in particular is established. The minimum effective dose with hypophysectomized rats as test objects is a fraction of a milligram and the activities of the preparations are about equal.

E X P E R I M E N T A L

INTRODUCTION

The protein or protein-like nature of the various principles of the anterior hypophysis is borne in mind when fractionation methods are applied to extracts of this tissue. The use of dilute aqueous alkali, among other media, is satisfactory for complete extraction of all the active principles of the anterior pituitary. Methods of separating the principles present in the extraction medium are usually based upon precipitation in various concentrations of inorganic salts, or precipitation by alterations in hydrogen ion concentration or by the use of organic solvents. The method used in the present investigation is essentially a combination of the last two. It is based upon a method for the preparation of normal human serum albumin by E.J. Cohn. The various protein components of blood plasma as well as certain pigments are separated in various stages by virtue of differences of solúbility in increasing concentrations of alcohol and hydrogen ions.

PURPOSE OF THE INVESTIGATION

It was suggested that Cohn's method for blood proteins might be successfully applied to the separation of the

protein, or protein-like principles in an extract of anterior lobe tissue. This study was undertaken primarily to ascertain the value of the Cohn procedure in preparing fractions of the anterior pituitary. The present work includes assays on the fractions obtained by the application of Cohn's method in an attempt to ascertain :

1. What activity is associated with each fraction.
2. The percentage recovery of such activity as compared with the original tissue which was used throughout as reference material.

and finally

3. The effectiveness of separation of specific hormonal activities.

Dr. Cohn kindly granted permission to Dr. Collip to apply the blood plasma method to fractionation of an extract obtained from anterior pituitary tissue.

Fig. 1 shows a draft of the Cohn method for the preparation of normal human serum albumin and the products resulting at each stage of the precipitation procedure.

Fig. 2 shows the application of the Cohn method to an anterior pituitary extract and the fractions obtained at each step in the procedure.

FIGURE I

COHN METHOD FOR PREPARATION OF NORMAL HUMAN SERUM ALBUMIN

Material:- Blood Plasma

Add 0.231 l. of 53.3% ethanol or 129.6 cc. of 95% ethanol at -5 degree C. per l. of plasma at 0 degree C. slowly and through a capillary. The temperature retained at -2 to -3 degree C. The ethanol concentration of the solution is 10% and the pH,7. The solution is allowed to stand and is then centrifuged.

PPT I largely fibrinogen
covered by 10% ethanol
containing 0.5% Na.Cit.
stored at -5 deg.C.

SUPERNATANT

Add 0.530 l. of 53.3% ethanol or 297.5 cc. of 95% ethanol at -5 deg.C. per l. of supernatant at -1 deg.C. through a capillary. The ethanol concentration of the solution is 25% and the temperature must not rise above -1 deg. C. The solution is allowed to stand for a period before being centrifuged.

PPT II-III mostly γ globulins
most of the prothrombin
and certain β globulins
stored at -5 deg.C.

SUPERNATANT

Add 1.13 l. of 53.3% ethanol or 634.5 cc. 95% ethanol at -5 deg.C. and 0.076 l. of acetate buffer I at -5 deg.C. per liter of supernatant through a capillary at about 100 ccs. per minute. The ethanol concentration of the solution is 40% and the temperature must not rise above -5 deg.C.

The pH should be 5.75 on five-fold dilution with H_2O. If pH reaches 5.75 before all the solution is added the addition of ethanol only is continued. The solution is allowed to settle for 24 to 36 hours.

PPT IV α & β globulins
also cholesterol and carotene

SUPERNATANT
(0.8% albumin solution)

Buffer II is added through capillary until the pH is 4.7 after five-fold dilution with H_2O

PPT V albumin

SUPERNATANT

Precipitate V should be 97% albumin. If in the presence of 0.06 N sodium acetate buffer the salt content is too high then it is dissolved at 0 deg.C. in aqueous 0.02 N sodium acetate buffer (II) at pH 4.7 and brought to 15% ethanol, using sufficient solvent to give a protein concentration of 3%. The solution is agitated gently for several hours at 0 deg.C. and the precipitate centrifuged at ϕ deg.C.

One may also use vacuum distillation at low temperature after precipitate IV has been removed with a pH of 5.5 - 5.9. Proteins are in solution and the pH and high salt content render this an excellent starting point for the crystallization of albumin.

The mother liquor from precipitate V may also be reduced to 1/50 the volume at 17 deg.C. It is then dialysed and the alcohol concentration of 15 to 40% gives fraction VI which consists mostly of albumin, some α globulins and follicle stimulating hormone.

BUFFER I

250 ccs. 4 M. sodium acetate (136.07 gms)
40 ccs.10 M. acetic acid (23.04 cc.)
420 ccs.95 % ethanol diluted to 1 l. with H_2O
On twenty-fold dilution with H_2O pH - 5.50 - .02 @ 25 deg.C.

BUFFER II

50 ccs. 4 M. sodium acetate (27.214 gms.)
100 ccs.10 M. acetic acid (57.6 ccs.)
420 ccs.95 % ethanol diluted to 1 l. with H_2O
On twenty-fold dilution with H_2O pH - 4.00 - 0.02 @ 25 deg.C.

FIGURE II

METHOD OF PREPARATION OF FRACTIONS

1235 g. A.P. calf

48 hrs. | .5 l. 0.9% NaCl
pH 8.7 @ 0 deg.C
vol. 1 in 5

RESIDUE

SUPERNATANT

48hrs | To 5 l. added
650c.c. 95% EtOH
pH 7.0 @ 0 deg.C
= 10% alcohol

PRECIPITATE I

R_1 = 13 gms.
Lyophilized 48 hrs.

SUPERNATANT

48hrs | To 5.5 l. added
1632 cc. 95% EtOH
pH 7.0 @ -1 deg.C
= 25% alcohol

PRECIPITATE II

R_2 = 11.5 gms.
Lyophilized 48 hrs.

SUPERNATANT

48hrs | To 6.7 l. added
4440 cc. 95% EtOH
and 55 ccs. BUFFER I
pH 5.75 on 5-fold dil'n
with H_2O @ -1 deg. C
= 40% alcohol

PRECIPITATE III

R_3 = 13 gms.
Lyophilized 48 hrs.

SUPERNATANT

48hrs | To 10 l. added 200 cc.
BUFFER II until
pH 4.70 on 5-fold dil'n
with H_2O
= abt. 41% alcohol

PRECIPITATE IV

R_4 = 1 gm.
Lyophilized 48 hrs.

SUPERNATANT

96hrs | volume reduced to
300 ccs. on high
vac. pump
DIALYSED for 48 hrs
LYOPHILIZED for 48 hrs

PRECIPITATE V

R_5 = 3.6 gms.
Lyophilized 48 hrs.

PREPARATION OF MATERIAL

DISSECTION AND EXTRACTION

Calf pituitary glands which had been stored in a frozen state in an atmosphere of hydrogen were carefully dissected when frozen and the anterior lobes processed as indicated in Fig. 2.

The dissected anterior lobes were thoroughly ground in a chilled mincer with the addition of a small amount of dry ice. They were then transferred to a chilled container and alkaline physiological saline at pH 9 was added. The pH of the mixture was then adjusted to pH 8.7 by the addition of I N NaOH and allowed to stand in an automatically controlled refrigerator at -3 deg. C for 48 hours. The solution was stirred frequently and the pH adjusted during this period. All pH determinations were made with a Beckmann glass electrode apparatus. The final pH of the solution was 7.0 and the supernatant was removed from the residue by centrifugation.

PRECAUTIONS OBSERVED

Since temperature and hydrogen ion regulation are important in the preparation of protein fractions, all precautions were observed to maintain the conditions stipulated in Cohn's procedure. Prior to centrifuging the metal cups and

glass bottles were chilled thoroughly and chopped dry ice was placed in the centrifuge during the fifteen-minute rotation at high speed. These precautions prevented the temperature from rising more than a few degrees. The same procedure was followed in all subsequent centrifugation.

FRACTIONATION

The successful removal of the active principles of the anterior pituitary when a slightly alkaline extraction medium was used has been reported (61). It was, therefore, assumed that under the conditions employed in the present procedure the supernatant after extraction at pH8.7 would contain all active principles of the anterior pituitary. The addition of alcohol to the supernatant obtained by centrifugation and adjustment of the pH caused the precipitation of a protein fraction (R_1). After its removal various fractions were obtained by higher concentrations of alcohol and increase in acidity.

The resulting precipitates were suspended in a small amount of distilled H_2O and equal volumes of the suspension were added to small Erlenmeyer flasks. These containers were frozen rapidly in an ether-CO_2 mixture and placed in a lyophilizing unit. A forty-eight hour period in this apparatus insured complete drying. CO_2 was then introduced and the flasks stoppered in this inert atmosphere. They were sealed with collodion immediately and stored in the refrigerator

at a temperature from 3 - 10 degrees below zero Centrigrade.

While all precautions have been taken to observe the conditions set forth by Cohn, it is realized that our laboratory facilities compare poorly with his. The advantages of a cold room and automatically cooled centrifuge are obvious when one considers the importance of preserving a low temperature in the protein solutions.

PREPARATION OF EXTRACTS

The dry powders obtained by lyophilizing the fractions of anterior pituitary extracts have been labelled R_1; R_2; R_3; R_4; R_5. It should be noted that there is a slight change from the corresponding Cohn fractions. Fraction R_2 corresponds with Cohn's precipitates II and III and the subsequent numbers are changed accordingly.

For assay purposes the various fractions were weighed on an analytical balance and then dissolved in physiological saline at pH 8.5. It was difficult to obtain complete solution of these dried powders, particularly when high dosage was employed. Since the majority of injections were subcutaneous, a fine suspension of the material to be tested was used for injection. When not in use these solutions were stored in the refrigerator, and since most assays were conducted over a lengthy period, it was decided to make up fresh

preparations every three days.

YIELD OF LYOPHILIZED MATERIAL

A record was kept of the amount of powdered material removed from the various flasks. Since the same volume of material had been added to each flask before drying, it was possible to calculate the total yield of each powdered fraction, and to compare this with the original fresh material.

The total yield of dried material was roughly 45 gms. (Fig. 2); this allows for loss of precipitate clinging to the sides of flasks and centrifuge bottles. The weight of starting material was 1235 gms. of calf anterior lobes. The yield of dry material, 45 gms. therefore represents 3.64 per cent of the original material, or each gram of dried material is equivalent to 27.4 gms. fresh anterior pituitary tissue.

PREPARATION OF REFERENCE EXTRACTS

500 gms. of the original batch of calf pituitary glands were preserved in the frozen state at -10 deg. C. An extract was prepared as needed by carefully dissecting the anterior lobes and grinding with sand in a mortar until the tissue was completely macerated. Alkaline saline was then added to the mixture, pH 8.7, and the extraction allowed to proceed overnight in the ice-box at 4 deg. C. The final concentration of the extract was 250 mgs. of fresh anterior pituitary tissue per cc. of solution. The sand and glandular

residue were removed by centrifugation and solutions of suitable concentration were stored in the ice-box. As in the case of lyophilized material it was customary to prepare fresh extracts every three days.

It is an accepted fact that there is a variation of the potency of the active principles from one batch of glands to the other, and for this reason, fresh tissue extracts were prepared from the original shipment of calf anterior pituitary glands. The use of such material allowed one to compute the yield of activity in the lyophilized fractions in terms of the original tissue.

The use of the original pituitary tissue over a period of several months, despite its preservation in the frozen state, is open to criticism. The lyophilized material was less apt to deteriorate since the protein was free from moisture, and an atmosphere of CO_2 was used for storage. The container in which the calf pituitaries were stored was opened frequently, and the variations of the temperature of the refrigerator over a period of several months may have permitted slight thawing. Dry ice, when available, was added to the container, but an atmosphere of CO_2 was not maintained throughout the storage period. The use of acetone desiccated glands would perhaps have been more advisable under the circumstances. It is generally accepted that glands treated in this way suffer

no great loss of potency in any of the active principles.

Other alternatives would have been the use of an acetone powder from an alkaline saline extract of the gland similar to the one fractionated by the Cohn method. And finally, an alkaline saline extract, after dialysis, could have been lyophilized in the same manner as the fractions obtained by Cohn's procedure. This last alternative was followed and the results will be given subsequently.

USE OF STANDARDIZED PREPARATIONS

The use of standardized preparations of the various principles of anterior pituitary in conducting the assays would have allowed the expression of results either in terms of international units or as standardized units set up by numerous investigators. The value of the Cohn procedure in fractionation would then have been evaluated in comparison with other methods. The preliminary assay tests showed that the dose of any fraction necessary to produce changes indicative of activity was so much larger than the dose of standardized preparations necessary to produce comparable results that future assays were not based on any standardized preparations. The assays were conducted along standard lines, but the results were not expressed even as arbitrary units. The yield of activity in terms of original tissue was calculated by a comparison of these assay results with those produced by the use of a crude saline extract of original tissue.

STUDY OF THE VARIOUS FRACTIONS

NITROGEN CONTENT

The micro-Kjeldahl method was used to determine the nitrogen content of the various fractions.(Table I) Unfortunately the total yield of R_4 was so small that material could not be spared for this determination.

20 mgs. of each fraction were dissolved in 100ccs. of distilled H_2O with the addition of a drop of alkali to facilitate solution. To 2 ccs. of each solution 2 ccs. of digestion mixture were added and the usual procedure followed. All determinations were made in duplicate.

Determinations of the ash content were made by combustion of the dry powder in a muffle furnace. It was possible to determine the true nitrogen value of the dried fractions from the ash content.

TABLE I

NITROGEN CONTENT OF LYOPHILIZED PITUITARY FRACTIONS

Expressed as Mgs per cent N per 100 Mgs Dried Powder

	NITROGEN	% ASH	TRUE NITROGEN
R_1	12.10 12.81 av. 12.45	8.35 8.77 av. 8.56	13.63
R_2	14.20 14.40 av. 14.30	4.74 5.66 av. 5.15	15.10
R_3	14.10 13.90 av. 14.00	3.20 2.68 av. 2.95	14.42
R_5	9.30 9.40 av. 9.35	10.00 13.10 av. 11.55	10.58

CONTAMINATION WITH POSTERIOR PITUITARY

It has been the experience of all investigators that no matter how carefully the anterior lobes are separated from the posterior lobes their preparations are contaminated to the slightest extent with posterior pituitary principles. All precautions were observed to keep the anterior lobes free from posterior tissue during dissection but it was thought advisable to investigate if the dried fractions were contaminated to any extent with posterior pituitary principles, and if so, what fractions were affected.

The pharmacological effects of posterior pituitary principles are quite striking. The most notable effects are the contraction of smooth muscle, particularly that of the uterus, under the influence of the oxytocic principle, and the diminution of urine excretion after treatment with the anti-diuretic principle.

The oxytocic effects of the various lyophilized fractions were assayed on the virgin guinea pig uterus according to the method of Dale and Laidlaw [42]. A standard oxytocic preparation was used as a reference dose. Table II contains the results of these assays. Because of the small yield of fraction R_4 it was not investigated. Fractions R_1 R_2 and R_3 in the dosage employed failed to produce any contraction of the uterine muscle. Apart from the possibility of contamination of R_4 the oxytocic principle was confined

TABLE II

OXYTOCIC ASSAYS ON LYOPHILIZED ANTERIOR PITUITARY FRACTIONS

Standard Oxytocic Preparation Used

Preparation	Dose	Response	Remarks
R_1	0.1; 0.2; 0.4 mgs.	None	
R_2	0.2 mgs	None	
R_3	0.2 mgs	None	
R_5	0.1 mg	Corresponded to .01 u of standard	Fraction R_5 possesses oxytocic activity equivalent to .1 u per mg. dry powder

to fraction R_5 which represented the bulk of material remaining in solution after treatment with 41 per cent ethanol at pH 4.7. Assays were not conducted for the pressor and antidiuretic content of the various fractions.

The degree of contamination with the posterior lobe oxytocic principle is rather small. Fraction R_5 possesses oxytocic activity equivalent to 0.1 unit per mg. of dry powder.

SENSITIZATION OF GUINEA PIGS

The usefulness of any protein preparation is limited if it is capable of sensitizing an animal. The guinea pig is generally used for sensitization tests of this sort. The methods and modifications of methods that have been introduced by various investigators are too numerous to mention; however they are all more or less similar. The majority of methods do not allow for study of a faint reaction should this be necessary. The injection of the extract to which the animal has been sensitized results in a spectacular seizure which culminates in immediate death. In the present investigation a method was employed which allowed for the study of slight reactions by fall in rectal temperature if it were necessary.

METHOD

Fairly mature female guinea pigs were injected intraperitoneally with 5 mgs. of the various lyophilized fractions three times at three day intervals. Four weeks later 250 mgs. of the same material was injected intravenously. The results of these sensitization tests are contained in Table III . It was not necessary to study the reaction by rectal temperature. In all cases the animals died during the intravenous injection. Fraction R_4 was not tested due to the scarcity of material. It should be

TABLE III

SENSITIZATION OF GUINEA PIGS

GUINEA PIG	FRACTION	DOSE	AFTER 4 WEEKS	RESULTS
# 10	R_1	5 mgs 3 times - 3 day intervals	250 mgs	Died during injection
# 14	R_2	5 mgs 3 times - 3 day intervals	250 mgs	Died during injection
# 18	R_3	5 mgs 3 times - 3 day intervals	250 mgs	Died during injection
# 30	R_5	5 mgs 3 times - 3 day intervals	250 mgs	Died during injection

mentioned that in preparation of the 250 mgs. dose for intravenous injection the customary suspension was not used. The powder was dissolved in saline and allowed to extract overnight. It was centrifuged and filtered before use to prevent any occlusion of blood vessels with suspended particles.

It is realized that 5 or 6 guinea pigs should have been employed in testing each fraction; however, these few results indicate the presence of some sensitizing substance in the fractions separated from an anterior lobe extract by the Cohn procedure. It is generally accepted that the crude saline extract which was used as the standard reference material is incapable of sensitizing guinea pigs. It is unfortunate that such material was not investigated in this case. However, granting that the general assumption is correct, the question then arises concerning the agents in these fractions responsible for the effects produced. Denaturation of the protein may have resulted in the many steps of the Cohn procedure or in the drying process. However, the various activities possessed by these fractions would not seem to indicate denaturation of the active material to any great extent, and particularly so when one considers the great lability of some of the anterior lobe principles. Sensitization of these animals may have resulted from the presence of serum proteins in the lyophilized fractions. Since the method of fractionation was an adaptation of a specific method to precipitate the proteins of blood plasma, there is a possibility that the dried fractions were contaminated in this way.

LYOPHILIZED CRUDE ALKALINE ANTERIOR PITUITARY EXTRACT

Since all the lyophilized fractions obtained by the application of Cohn's procedure to an anterior pituitary extract were to be tested against a similar extract of original tissue, it was decided to attempt the lyophilizing of such an extract and to determine if it could serve as satisfactory reference material.

PROCEDURE

150 gms. of calf anterior pituitary were ground with sand in a large mortar while still in the frozen condition and extracted with alkaline saline for 24 hrs. At various intervals during the extraction period the solution was stirred and the pH adjusted to 8.7. The final concentration of the solution was 250 gms. of anterior pituitary per cc. The residue was removed by centrifugation and all precautions were taken to avoid any great rise in temperature. The supernatant was placed in a collodion membrane and dialysed against running tap-water (3-4°C) for 24 hrs. The dialysed solution was equally distributed into 125 cc. Erlenmeyer flasks and lyophilized. Each mg. of the resulting powder was equivalent to 10 mgs. of fresh tissue.

RESULTS

The effects produced by injection of this lyophilized crude extract were compared in a few assays with the effects produced by a fresh extract of the same tissue. The activity

TABLE IV

LYOPHILIZED CRUDE ANTERIOR PITUITARY EXTRACT

ASSAY	Equivalent of Dose in Mgs. Fresh Tissue	Effect of Fresh Tissue Extract	Effect of Lyophilized Tissue Extract
		Expressed as Mgs. Crop Sac/100 Gms. Body Wt.	
Prolactin	100	120 80 Av. 100	63 59 Av. 61
		Expressed as Percentage Increase of Thyroid Wt.	
Thyrotrophic	60	62% 50% 89% 70% Av. 68.0%	15% 24% 9% Av. 16%
		Wt. Ovaries in Mgs./100 Gms. Body Wt.	
Gonadotrophic	100	63.3 (4)*	40; 32.4 38.7; 30.9 Av. 35.5

* Number of Animals.

of this material was found to be less than that possessed by the fresh tissue extract. Since the yield of activity of the lyophilized fractions obtained by the Cohn method was to be compared with that possessed by the original tissue it would have been unsuitable to substitute the lyophilized crude alkaline extract. Unfortunately the small number of animals available for assay purposes did not permit an extensive comparison of the dried extract. A few of the results obtained are given in Table IV.

The same extraction procedure was used in the crude saline extract which has been used throughout as the reference material and the crude saline extract which was subsequently lyophilized. Loss of half the activity in the lyophilized extract can hardly be attributed to incomplete extraction under these circumstances. The loss of activity can only be explained as having taken place during the drying process. Precautions similar to those employed in drying the fractions were observed when drying the material. The dry powder was not exposed to the atmosphere, and it was used in some assays after a few days' storage.

ASSAY OF GROWTH ACTIVITY

It was decided to use the more sensitive test animal in assaying the growth activity of the various fractions obtained by the application of Cohn's method to an anterior pituitary extract. Normal rats require much larger doses of extract and were not available in sufficiently large numbers within suitable age limits.

METHOD

Male or female hooded rats weighing about 130 gms. were hypophysectomized and used for assay ten days later. In addition to the standard laboratory diet of purina they were allowed a supplement of rolled oats. During the post-operative period the weight of the animals was checked every three days and any rats that showed an increase were discarded. At the end of the post-operative period the majority of animals showed an average loss of 25 or 30 gms. body weight. Only those animals that appeared in good condition were employed for assay. Because of the fine suspension rather than solution that resulted when physiological saline was added to the powdered fractions the injections were administered subcutaneously. The growth response has been found the same after subcutaneous or intraperitoneal routes of injection (120). A total of seventeen

injections was made in the period of twenty days; a longer period than is usually employed for hypophysectomized rats, but it was thought advisable to allow for optimum response should adsorption of the powder have been slow. During the assay it was customary to weigh the animals at the same time every three days.

At the end of the assay period the rats were autopsied and the sella checked under a binocular dissection eye-piece for pituitary remnants. If any were found the results were discarded from the assay. Due to the presence of a certain amount of corticotrophic activity the adrenal weights could not be used as an indication of total hypophysectomy. The weights of the adrenals and gonads were recorded in some cases to indicate which fractions possessed these specific activities.

The five dried powders were injected in rather high dosage to determine whether growth stimulating activity was confined to a single fraction or spread over a few. When the results of the assays indicated which fractions possessed growth activity, assays were conducted for more definite information. It should be mentioned that the high mortality of hypophysectomized animals in the interval before injection cut down the number of available animals to a considerable extent. All doses of a given fraction could not therefore be

assayed at the same time.

REFERENCE MATERIAL

A crude alkaline saline extract (pH8.7) of original tissue was used as the standard reference dose. The use of a daily dose of this extract equivalent to 2.5 mgs. of fresh tissue failed to produce any weight increase. The animals receiving such a dose either died or their weight remained stationary. A dose equivalent to 5.0 mgs. produced a weight response, but higher doses (10 and 15 mgs.) caused the death of the animals. The normal animal is much more resistant to any toxic substance than the hypophysectomized animal. Death on high dosage may have resulted from toxic effects of the extract in operated animals.

The failure of the 2.5 mg. dose to produce a weight increase was surprising when one considers the reported sensitivity of hypophysectomized animals to the growth factor. Collip (35) reported that a crude extract containing the equivalent of 0.5 mgs. of tissue may produce a positive growth response in the hypophysectomized rat.

To explain the unexpected results with the 2.5 mg. dose three reasons presented themselves:-

1. The dose was so large that any weight increment was neutralized by the toxic effects of overdosage. However, the production of weight gain by a 5.0 mg. dose overruled this argument, and particularly so when larger doses caused

TABLE V

GROWTH ASSAY - CONDUCTED ON HYPOPHYSECTOMIZED RATS
TEN DAY POST-OPERATIVE - CRUDE SALINE A.P. EXTRACT

Daily Dose Equiv. Orig. Tissue Mgs.	Total Increase in Body Weight During Assay(1) Increase in Grams per Day(2)	Average
2.5★	19; 15; 15; 14; 13 (1) .95;.75;.75;.70;.60 (2)	= 0.76 Gms./Day Increase
5.0	24; 30; 27; 32; 32; 18;21; 44;20;26.7;26.7; 28;18; 28; 28; 34 1.2;1.5;1.35;1.6;1.6;0.9; 1;2.2; 1;1.3; 1.3;1.4;.9;1.4;1.4;1.7;	= 1.36 Gms./Day Increase.
5.0★	40; 41; 39; 36 2;2.25;1.95;1.8	= 2 Gms./Day Increase

★ FRESH SHIPMENT OF CALF ANTERIOR PITUITARY.

the death of the animals during the injection period.

2. Complete extraction of the growth material may not have been effected under the conditions employed. It would have been interesting to determine whether a more strongly alkaline medium would have yielded a more potent extract. Since the conditions of extraction were to resembleas closely as possible the extraction procedurè used in the preparation of the lyophilized material the use of a medium at p.H 10 or 11 was not feasible. Some investigators have employed highly alkaline media in the preparation òf the growth principle. However, recent reports ($\frac{61}{190}$) show successful extraction at pH 7 and 8, and it was therefore assumed that the extraction procedure in the present investigation was capable of removing the growth principle.

3. The only remaining explanation that can be offered for the necessity of such high dosage was the deterioration of growth activity in the original glands. It should be remembered that the glands were stored in the frozen state for a length of time, and were subject to changes in temperature despite all precautions. To test the possibility of deterioration a fresh shipment of glands was obtained from the same source and the response to their injection wascompared with the original glands.

From Table V it will be seen that the 2.5 mg. dose of the fresher tissue did not result in the death of the animals; however, the response did not equal a growth increase of 1 gm. per day. The higher dose of 5.0 mgs. did not produce a response

TABLE VI

GROWTH ASSAY - LYOPHILIZED FRACTIONS OF ANTERIOR PITUITARY (COHN METHOD)

Conducted on Hypophysectomized Rats - 10-Day Post-operative - Daily Subcutaneous Injections
Total 17 Injections in 20 Days

Daily Dose Mgs. Fraction	Total Gain in Body Weight - Gms. Gain Gms./Day = Average ⁂No. of Animals	Adrenal Weight ⁂⁂ Adrenal Weight Expressed as Mgs./100 Gms. Body Weight = Average	Weight Gonads ⁂⁂ Gonad Weight Expressed as Mgs./100 Gms. Body Weight Ovaries	Uterus	Testes
R_1 .3	26.0; 1.3;1.2;1.2 = 1.2 (3)⁂				
R_1 .5	48.5; 25;36.5;39 2.42;1.25;1.82;1.95 = 1.86 (4)	16 ;14 10.3;14.4 = 12.3 (2)⁂	22.5;9.5 21.2;9.8	70;59 60.6;60.9	
R_1 1.0	43.5; 52;35 2.17;2.6;1.75 = 2.17 (3)	12; 11;12 12.2;10.6;12.2 = 11.9 (3)	18.5;18 18.5;17.3	58;65 58;62.5	475 475
R_1 2.5	66; 45;52.5;39.5;48.5; 3.0;2.2;2.6;1.98;2.42; = 2.44 (5)	18.5; 16; 20;17 17.4;13.2;17.4;15.6 = 15.9(4)			
R_1 10.0	25;42 1.25;2.12 = 1.68 (2)				
R_1 20.	Evidently toxic; 4 animals died in 3 days.				
R_1 50.	Evidently toxic; 3 animals died in 3 days.				
R_2 1.	11;14;12.5 .5;.7;.63 = .61 (3)	15.5;13.5;13.5 14.9;12.8;11.5 = 13.0 (3)	12.5;19;15.5 12.0;17.8;13	65;56;53.5 62.5;53;45	
R_2 2.5	22.5; 21; 28;24.5;37.5;14 1.12;1.05;1.4;1.2;1.9;.7=1.23(6)	13; 12; 11;12; 10;11 11.6;11.2;10.9;12;10.4;10.2 = 11.0 (6)			850;600
R_2 10.0	34;41.5;36 2;2.42;2.1 = 2.17 (3)				
R_2 20.	48;26 2.4;1.3 = 1.8 (2)				
R_3 2.5	Animals failed to gain weight - 4 dead.				
R_3 5.0	11.5;15.5;15. .57;.775;.75 = .63 (3)	13; 14;9.0 12.4;13;8.6 = 11.3 (3)			1.2;1;1.4 Gms./100 Gms.Body Wt.
R_3 10.0	13. .65	17. 16.2			
R_3 20.0	8;26 .4;1.3 = .8 (2)				
R_3 50.0	26;13 1.3;.6 = .95 (2)				
R_4 5.	No increase in 5 animals.				
R_5 2.5	No increase in 4 animals.	No autopsy.			
R_5 10.0	No increase in 4 animals.	No autopsy.			

⁂⁂ Normal Adrenal Wt. = 18-20 Mgs./100 Gms. Body Wt.
Normal Ovarian Wt. = 27-30 Mgs./100 Gms. Body Wt.
Normal Uterine Wt. = 300 Mgs./100 Gms. Body Wt.
Normal Testis Wt. = 2500 Mgs./100 Gms. Body Wt.

which exceeded a 2 gm. per day increase, and such an effect had been produced with the older glands in some instances. The fresher glands had been stored in the frozen state in an atmosphere of hydrogen before delivery to the laboratory.

One can only conclude that the growth activity extracted from both gland shipments was not as great as expected, and that some deterioration of growth activity had taken place in the original glands during storage at the laboratory.

LYOPHILIZED FRACTIONS

The growth response obtained with various doses of the lyophilized powders is contained in Table VI. The results produced by high doses of the various fractions indicated that growth activity was not limited to a single fraction.

Fractions R_4 and R_5 failed to produce any growth response with the dosage employed, and the animals succumbed during the assay period. The extremely high doses of fraction R_3 (50 mgs.) necessary to produce a slight response indicated that the amount of growth activity present was negligible. Fractions R_1 and R_2 obviously possessed the significant growth activity and the greater portion was in fraction R_1.

From Table VI it can be seen that R_1 in daily doses of 10 mgs. and over was evidently toxic and such results were not considered. Similarly the results with 20 mgs. of R_2 were discarded.

YIELD OF ACTIVITY

The discrepant values in the growth activity extracted from the old and new shipments of beef anterior pituitaries led

to confusion in the choice of a reference value. Coupled with this difficulty was the paucity of data on various doses of the crude extract. In an attempt to overcome the latter difficulty and yet employ all data to the best advantage, it was decided to plot the growth in grams per day resulting from treatment with R_1 and R_2 against the logarithm of the dose of these fractions in milligrams.

Graph I shows the regression lines for fractions R_1 and R_2. It will be noted that fraction R_2 gives a straight line while R_1 gives a slightly curved line.

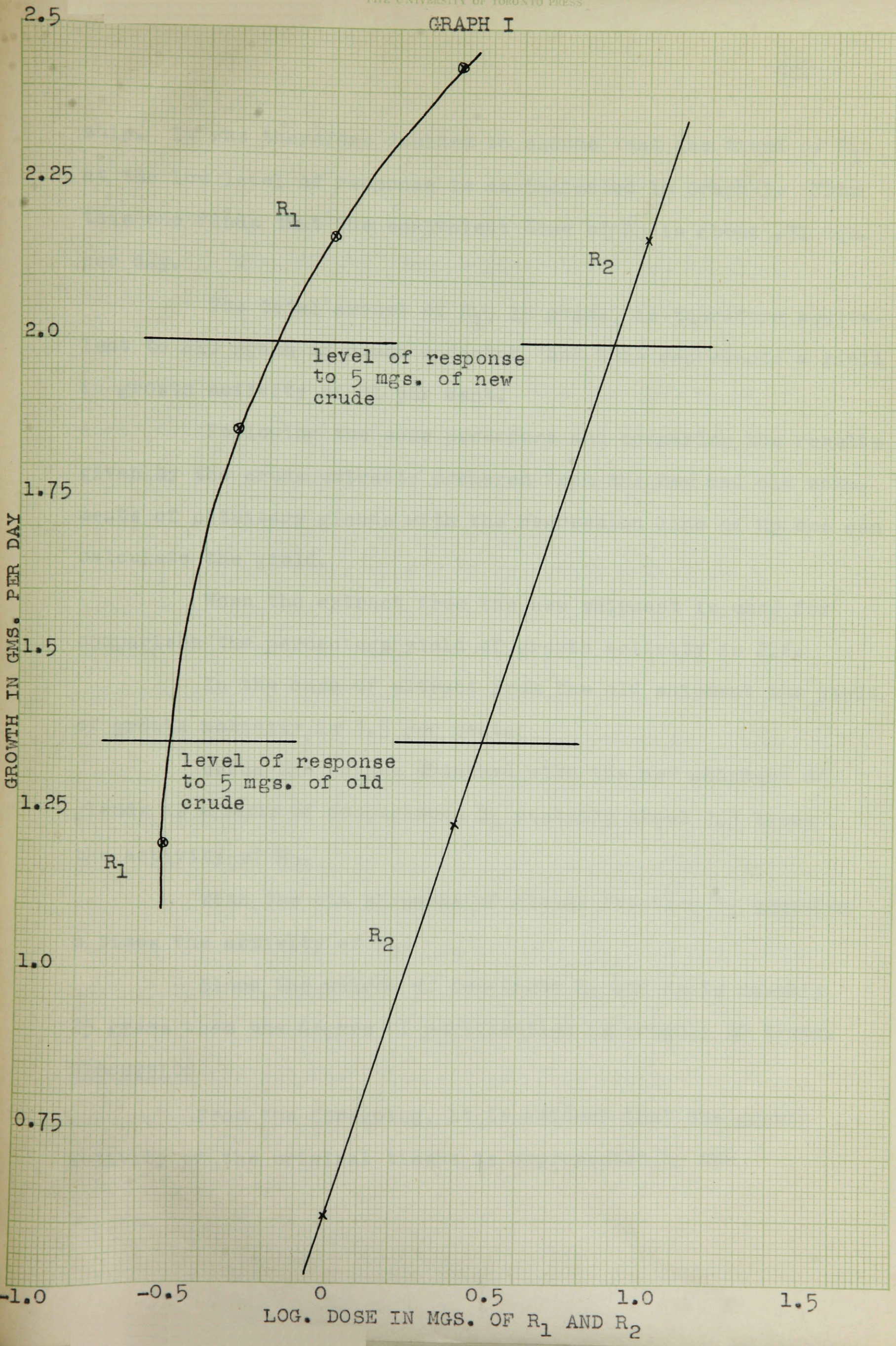
If we take the results given in Table V for the 5 mg. daily dose of extract prepared from the fresh shipment of glands and intersect the regression line of R_1 at the 2 grams per day level, we find that the equivalent dose of R_1 is 0.66 mgs.

The total weight of R_1 was 13 grams and this is equivalent to $\frac{13 \times 5}{0.66}$ or 98.5 grams of crude material.

And the yield of growth activity is therefore $\frac{98.5}{1235} \times 100$ or roughly 8 per cent.

If on the other hand we use the values resulting from dosage of the crude extract prepared from the old shipment of glands, we find that the regression line is intersected at a point where its trend is rather uncertain. One wonders if perhaps line R_1 should not be more parallel to R_2 at this point. When the grams increase per day with fractions R_1 and R_2 were plotted against the log. log. dose of these fractions in milligrams there did not appear to be any striking improvement in their relation-

GRAPH I



ship. It was therefore decided to assume that the trend of R_1 at the low level of response is as indicated in Graph I. From this one finds that the equivalent dose of R_1 is about .31 mgs. per day.

The total amount of R_1, 13 grams, is therefore equivalent to 210 grams of crude material, and this represents a yield in growth activity of 21 per cent.

Following the same procedure and comparing the results given by the crude extracts prepared from the new and old shipments of pituitary glands with the regression line of R_2 one can calculate the yield.

When the extract from the new shipment is used for comparison the percentage yield of growth activity is 2.8.

In the case of extract from the old shipment the yield of growth activity is 3.5 per cent.

Using the values obtained with the new shipment of glands as a basis of comparison, R_1 contains about 2.5 times the activity of R_2.

With the old shipment of glands fraction R_1 contains 6 times the activity of R_2.

Since the weight of fractions R_1 and R_2 is roughly 25 grams then the degree of purification is roughly 50 times.

DISCUSSION

From the foregoing, it is evident that the growth activity of the original tissue is represented by the

lyophilized fractions R_1 and R_2 which resulted from the application of the Cohn fractionation procedure to an extract of this tissue. This answers the first question of association of activity with a particular fraction. The second question of yield of activity in terms of original tissue is more difficult. The calculation of assay data using both the extract of the original gland shipment as well as that of a fresher gland shipment presents a dilemma.

In the assay of the other anterior pituitary principles, the old shipment compared favourably with the fresher one. In this case, there is a discrepancy in the calculated values for yield of activity. Since the methods of extraction employed in the reference extracts and the extract which was subsequently fractionated are the same, one can assume that some deterioration of growth activity must have taken place in the original tissue before reference extracts were prepared from it. If this is the case, then the growth activity of the fresher tissue would resemble that of the fractionated material more closely. The lability of the growth hormone is recognized and the conditions of storage of the original tissue could very likely cause some loss of activity. For these reasons the data calculated in terms of the fresher gland shipment are accepted.

The total yield of growth activity in terms of original tissue is, therefore, 10.5 per cent. Fraction R_1 contains 8 per cent and R_2 2.5 per cent.

Before proceeding to a discussion on the appearance of activity in these fractions it should be mentioned that the yield of activity in terms of the original tissue is poor. Aside from the fact that the extraction procedure may be inadequate, nevertheless it was the same in the crude extract and the extract which was fractionated. This gives a relative 100 per cent value to the reference material and the total yield of 10.5 per cent in the lyophilized fraction represents a great loss.

The third question concerns the value of the Cohn procedure in effecting a separation of growth activity from the other active principles of the anterior pituitary extract. It is evident that the procedure has not led to the isolation of growth activity in a single fraction, although the greater portion is in R_1.

The various reports on the chemical nature of the growth hormone as well as the recent finding of Li and Evans (203) that the isoelectric point of the pure hormone is pH6.8 - 6.9 would lead one to expect its appearance in the precipitate resulting from addition of 10% ethanol at the neutral point. Fraction R_2 resulted from a concentration of 25 per cent ethanol and a similar pH.

The loss of activity hardly seems due to rise in temperature. Although all precautions were observed to maintain temperatures at zero C or below, the rise of a few degrees in handling would not have resulted in this loss. Evans et al carry out their procedure at 2 to 3°C. Similarly the concentration of alcohol employed cannot be the cause of such a loss. Investigators have used higher concentration without serious loss.

Ruling out conditions of temperature and alcohol concentration as contributory to the loss, one can only conclude that the drying process was responsible. This cannot be explained since the operation was carried out carefully, but some condition must have resulted in destruction of a great amount of the growth activity.

ASSAY OF THYROTROPHIC PRINCIPLE

Method

The method of Rowlands and Parkes was employed in assaying the content of thyrotrophic activity in the various fractions obtained by the application of the Cohn Plasma Method to anterior pituitary tissue. The weight increase of the guinea pig thyroid possessed certain advantages over other methods for estimating the thyrotrophic activity. Guinea pigs were easily available as test animals whereas the use of day-old chicks presented difficulties. Methods based on histological study alone allowed for a great deal of subjective error and required an experienced investigator. The fractions obtained by the Cohn method were not sufficiently pure to warrant study of the iodine content by Stimmel's method.

All guinea pigs were obtained from the same dealer two weeks or so prior to the assay period. They were placed on a standard diet of dry hay to which ascorbic acid had been added. Their weight on arrival was such that they reached the required 200 gram weight by the end of the two week standard feeding period. The living conditions of the animals were standardized as closely as possible, but it should be mentioned that the room in which they were kept was

subject to temperature fluctuation.

As with the other fractions it was necessary to perform spot tests to ascertain the distribution of the active principle and then conduct assays in finer detail. Due to the inability to obtain and quarter large numbers of these animals it was necessary to conduct numerous assays so that all the results with one particular fraction or dose were not obtained at the same period of time. Such a procedure should not introduce too great an error since all the animals were of the same strain and received the same diet and it has been shown that there is not a great deal of variation in the thyroid weights of normal guinea pigs.

Procedure

200 gm. female guinea pigs were injected subcutaneously twice daily for five days and killed on the sixth. The thyroid gland was dissected and the fresh weight recorded. The glands were then fixed in Bouin's solution for histological examination according to the method of Heyl and Laqueur.

Rowlands and Parkes define a unit as the total amount of material which produces in six days a 100 per cent increase in thyroid weight. Thirty milligrams is considered the normal thyroid weight of a 200 gram female guinea pig. Weight changes during the assay period are corrected by the

TABLE VII

THYROTROPHIC ASSAY

Thyrotrophic Assay of Crude Saline Extract of Calf A.P.

Daily Dose Crude Saline Ant.Pit.	Average % Increase in Thyroid Weight		Range	Body Weight Gms. Average Before	After	Histological Examination Degree of Stimulation
20 Mgs.	35	(4)*	0 - 88	214	224	Slight
30 "	78	(4)	50 - 131	226	227	"
40 "	75	(3)	59 - 97	208	212	"
50 "	85.2	(4)	56.5 - 112	223	219	Fair
60 "	105.2	(4)	65.5 - 162	218	207	"
80 "	103.6	(3)	87 - 124	206	208	"
Normal Controls	Actual Thyroid Wts. Mgs. 30.3	(9)	20 - 36	209	220	No Stimulation

* Number of Guinea Pigs.

addition of 1 mg. to the thyroid weight for each 6 gms. increase in body weight. The percentage increase is calculated from the actual and computed weights of the thyroid at autopsy. In studying the histological picture of the thyroid gland that portion of the gland described by Heyl and Laqueur as the middle zone was routinely used. From the histological appearance the activity of the gland was scored and correlated with the weight increase.

The amount of stimulation induced in the gland was judged by the height of the acinar epithelium, the presence or absence of colloid and the general shape of the various acinii. Maximum stimulation results in the absence of colloid, and the presence of high columnar epithelium with papillary folds in the acinar lumen.

REFERENCE DOSE

A crude saline extract of dissected anterior lobes from the original batch of glands, at pH 8.5, was used as a standard reference dose. All the results which were obtained on injection of the various fractions were referred to this preparation from original tissue. Table VII shows the results of injection with the crude saline extract. These results which have been expressed as percentage increase in thyroid weight have been plotted graphically against the logarithm of the dose of crude saline extract in milligrams (Graph II)

From this regression line which was drawn by inspection it was possible to calculate the percentage yield of activity in terms of the original pituitary tissue.

From Table VII it will be observed that only a small number of guinea pigs were available for each dose of the crude preparation. The range in the percentage increase in thyroid weight is rather large and would appear to introduce a large deviation in the results. This value would have been diminished to a great extent if larger groups of animals had been employed.

Wherever the extract was effective in causing noticeable thyroid stimulation the animals as a group failed to gain weight and in some cases there was a weight loss. Metabolic studies were not conducted on these animals, but the lack of weight increment can probably be attributed to an increase in metabolic rate. It will be noted that untreated controls gain on the average two grams per day during the six day period.

The histological picture of the thyroid, even when the dose of extract was effective in causing a 100 per cent increase in gland weight, was not indicative of maximum stimulation. The constancy of the histological picture in animals treated with the same dose of extract, but over a period of months, was surprising. The middle zone of the gland was routinely used in inspection as recommended by Heyl and Laqueur,

but the classification of stimulation was rather subjective.

Table VIII shows the results of injection with the lyophilized pituitary fractions. The results are expressed in the same manner as those obtained on treatment with the crude extract.

As a group the animals failed to show a weight increase where stimulation of the gland had been produced.

The range of the percentage increase in thyroid weight is large, and here again one must remark that the use of a greater number of guinea pigs per group would have reduced the deviation. It is evident that within the administered dose range, 40 mgs. daily of the dried powders of fractions R_4 and R_5, the thyroid glands did not show any weight increase indicative of stimulation, and the histological picture confirmed this observation. One may therefore conclude that these fractions are devoid of thyrotrophic activity to some degree.

From a consideration of Table VIII one observes that thyrotrophic activity is not associated with a particular fraction, but is spread over fractions R_1, R_2 and R_3. However, the activity is not present to the same extent in each fraction.

The effects produced by high dosage of fraction R_1 and R_3 are less than those obtained with a smaller dose. It would appear that some toxic or deleterious results are produced by overdosage.

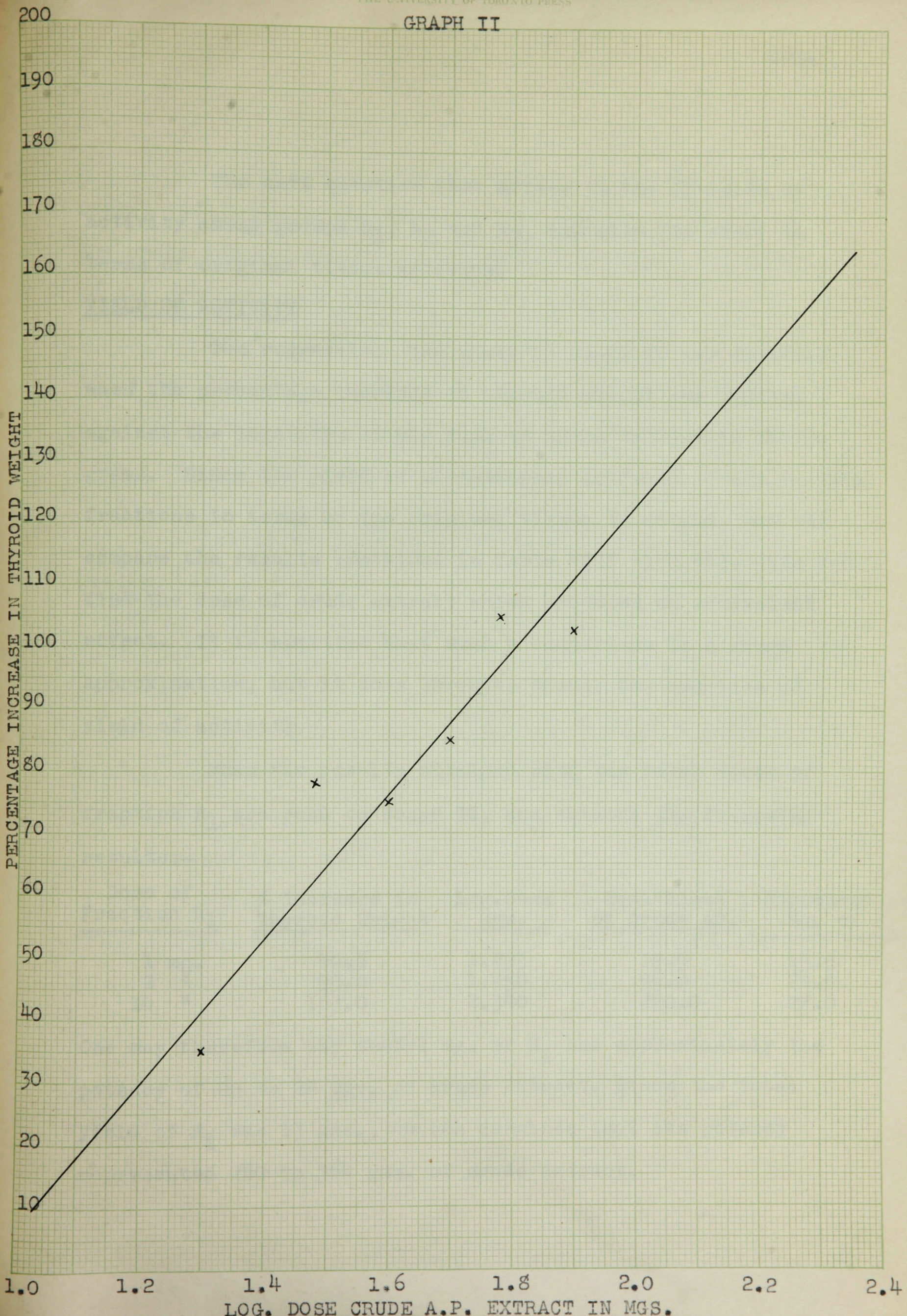
THYROTROPHIC ASSAY OF ANTERIOR PITUITARY FRACTIONS

Daily Dose Mgs. Fraction		Average % Increase in Thyroid Weight	Range	Body Weight Gms. Average Before	After	Histological Exami Degree of Stimula
R_1	1 Mg.	40.8 (3)*	15.5 - 67	205	210	Slight
"	2 "	93.0 (6)	67.0 - 140	200	207	Fair
"	10 "	163.0 (5)	81 - 287	205	207	Good
"	20 "	135.0 (2)	112 - 158	213	212	Fair
"	40 "	150.0 (2)	107 - 196	190.5	181.5	Fair
R_2	1 "	65 (3)	48 - 73	216	211	Slight
"	2 "	79 (7)	23 - 167	209	228	Fair
"	10 "	119.7 (5)	48.5 - 240	213	223	Good
R_3	2 "	35.4 (4)	0 - 64.5	208	227	Slight
"	10 "	123.0 (5)	67 - 170	203	207	Fair
"	40 "	106.0 (2)	93.5 - 120	188	200	Good
R_4	2 "	0 (2)		199	216	No Stimulation
"	10 "	0 (3)		202	213	" "
"	40 "	58 (4)	48 - 67	196	209.7	Slight
R_5	2 "	3.0 (2)	0 - 7	208	226	No Stimulation
"	10 "	8.5 (2)	0 - 17	190	200	" "
"	40 "	3.0 (2)	0 - 6	182.5	183	" "

* NUMBER OF GUINEA PIGS.

TABLE VIII

GRAPH II



The next question that arises is the division of activity among groups R_1, R_2 and R_3, and also its yield in terms of original tissue activity.

YIELD OF ACTIVITY

The regression line shown in Graph II was obtained when the percentage increase in thyroid weight was plotted against the logarithm of the dose of crude extract in milligrams. Since the yield of thyrotrophic activity of the various fractions in terms of the original tissue is sought, one can compare the results contained in Table VIII with this line and find the dose of crude extract which produces an equivalent effect. It is admitted that such a comparison is a rough approximation, but it does serve to answer the question of yield of activity.

When the results obtained with the lower doses of fraction R_1 are read on Graph II, one obtains the following results:-

Dose of Fraction R_1	% Increase in Thyroid Weight	Log. Dose Mgs.	Equivalent of Crude AP.	Expressed per Mg. of R_1
1 mg.	40.8	1.295	19.5	19.5
2 "	93.0	1.750	56.0	28.0
10 "	163.0	2.340	220.0	22.0

One may therefore say that 1 mg. of R_1 has approximately the potency of 20 to 28 mgs. of crude extract. Since the total yield of R_1 was 13 gms., one can conclude that its potency represented 260 to 364 gms. of crude extract.

Taking the average of the results above, it would appear that 1 mg. of R_1 possesses the potency of 25 mgs. of the crude extract and the total yield of R_1 has the potency of 325 gms. of crude extract.

The total yield of R_1, 13 gms., was obtained from 1235 gms. fresh AP. tissue, and the yield of thyrotrophic activity possessed by R_1 in terms of this original tissue is

$\frac{325}{1235} \times 100 = 26$ per cent.

Taking the results obtained with fraction R_2 and reading them in the same way one obtains the following results:-

Dose of Fraction R_2	% Increase in Thyroid Weight	Log.Dose Mgs.	Equivalent of Crude AP.	Expressed per Mg. of R_2
1 mg.	65	1.505	32.0	32
2 "	79	1.628	42.0	21
10 "	120	1.975	94.5	9.5

The agreement of results produced with R_2 is not as good as that of R_1; however, one may take 21 mgs. of crude extract as equivalent to 1 mg. of R_2.

And since the total amount of R_2 obtained from 1235 gms. of fresh tissue was 11.5, one can calculate the yield of thyrotrophic activity as $\frac{241.5}{1235} \times 100$ or 19.5 per cent.

Similarly with R_3.

Dose of Fraction R_3	% Increase in Thyroid Weight	Log.Dose Mgs.	Equivalent of Crude AP.	Expressed per Mg. of R_3
2 mg.	35.5	1.255	18	9
10 "	123.0	2.000	100	10

The average may be expressed 9.5 mgs. of crude extract equivalent to 1 mg. of R_3.

The total yield of R_3 was 13 gms. which is equivalent to 123.5 gms. of crude extract and the per cent yield is therefore 10.

The total yield of thyrotrophic activity possessed by fractions R_1, R_2 and R_3 may be roughly totalled to give 55 per cent.

Since the total weight of R_1, R_2 and R_3 is roughly 38 grams, the degree of purification is about 32 times.

DISCUSSION

The question of what fraction or fractions of the processed anterior pituitary extract are associated with thyrotrophic activity as well as the percentage yield of this activity in terms of original tissue have been adequately answered in the previous section.

The total yield of thyrotrophic activity in terms of original tissue is 55 per cent. This represents a rather good yield and is the largest of any activity associated with the Cohn fractions. It does not compare with the hundred per cent recoveries of investigators who have isolated the hormone in the pure state, but it should be remembered that their isolation and purification technique is specific. The yield obtained here is the result of a

general fractionation procedure, and in the light of this, the yield is good.

The next question which is of concern is the effectiveness of the Cohn procedure in separating thyrotrophic activity from the other anterior lobe principles. The method is not as efficient in this respect as one could desire. The activity is spread over the first three fractions with the greatest portion in R_1. Apart from the spread of activity over these three fractions it does not exist alone but is combined with growth, gonadotrophic and corticotrophic activities. The only other activity spread over three of the lyophilized fractions is the gonadotrophic activity. This is rather interesting since the two tend to be closely associated, and this has led to the postulation of the identity of the thyrotrophic and ICSH principles.

The reported water solubility of the thyrotrophic hormone and its solubility in low concentrations of alcohol would not lead one to expect its precipitation in R_1 or R_2. However, the globulin nature of this principle resulted in its precipitation under the conditions set forth by Cohn. It is unfortunate that the fractionation procedure did not result in a more definite association of thyrotrophic activity with a particular fraction.

One can conclude that the Cohn procedure as here applied is ineffective in confining thyrotrophic activity to a particular fraction.

The loss of some activity in terms of that possessed by the original tissue leads one to suspect the drying process; otherwise fractions R_4 and R_5 would have possessed some activity.

Whatever the condition causing loss of activity, the thyrotrophic activity was the least affected of all the principles investigated.

ASSAY OF PROLACTIN

The prolactin assays were conducted on the lyophilized fractions to determine the yield of activity of this principle in terms of original tissue and to ascertain with which fraction the greatest portion of activity was associated. Since there was no knowledge of what would constitute a dose of extract sufficient to bring about crop sac stimulation it was necessary to do a number of spot tests on rather high dosage.

METHOD

The crop sac stimulation method was employed in the prolactin assays. Pigeons were injected intrapectorally on alternative sides for 4 days and were killed on the fifth day. Great care was taken in the dissection of the crop sac to standardize its removal as much as possible. The fatty tissue adhering to the outer wall was removed, and the sac washed free of its contents and weighed. The interior wall in the region of the crop gland was examined for signs of stimulation and results were recorded when evident.

The pigeons used for assay were obtained from a dealer, and while they appeared to be of the same strain there was not any guarantee of this. It was also impossible to know the age of the birds. The race of pigeon as well as

TABLE IX

PROLACTIN ASSAY
PIGEON CROP SAC STIMULATION METHOD
INTRAPECTORAL INJECTIONS FOR FOUR DAYS - AUTOPSY ON FIFTH

Material Daily Dose Mgs.	Number of Pigeons	Equivalent Dose in Terms of Original	Crop Sac Wt. Mgs./100 Gms. Body Wt. Average	Range	Evident Stimulation
Normal Controls	4		499	460 - 585	No
Crude Saline					
A.P. Extract	3	50	910	730 - 1040	Yes
"	3	75	963	690 - 1200	Yes
"	3	100	1100	800 - 1300	Yes
R_1 2	1		540		No
"1 10	1		720		No
" 20	2		710	690 - 740	No
R_2 2	1		600		No
"2 10	1		790		No
" 20	2		560		No
R_3 2	1		750		No
"3 10	1		600		No
" 20	2		605	595 - 615	No
R_4 2	1		770		No
"4 10	1		870		No
R_5 2	1		760		No
"5 10	1		670		No
" 20	3		683	658 - 720	No

the age is recognized as an important factor in the response to the active principle, and this was a definite disadvantage in conducting the assay. The response of various breeds of pigeon has been standardized by Riddle and other workers in this field so that their assay results have a definite meaning. Considering all this an assay conducted on pigeons of unknown strain and age can be of a qualitative nature only. The variation in the body weight of the pigeons was found to be large, and it was decided to express the crop gland weight as mgs. per 100 gms. body weight.

REFERENCE MATERIAL

In the prolactin assays the crude alkaline saline extract of original tissue was used as reference material. The results are contained in Table IX . The number of animals on each dose was necessarily small so that the spread of results appeared large. The necessity of employing such high dosage of the original tissue to produce noticeable stimulation was rather surprising. Although the range of results on any dose was large there nevertheless seemed to be some correlation of crop sac weight and dosage employed. Examination of the crop wall showed a definite thickening of the secretory epithelium, and in the case of the largest dose, some crop milk.

LYOPHILIZED MATERIAL

In the preliminary assay studies the lyophilized material was administered in high dosage to insure a definite stimulating effect. Table IX does not show any great stimulating effect with high dosage and there was not any evidence of crop milk secretion in the injected pigeons. The crop sac weights do not differ greatly from those of normal control pigeons; and only in one instance did it even approach that produced by a dose equivalent to 50 mgs. of original tissue. As a result of these preliminary assays on the lyophilized fractions it was decided to discontinue the prolactin investigation. The doses that had been administered were sufficiently large to indicate the presence of prolactin activity to some extent, and when these failed to do so it was worthless to try the effects of higher dosage. The yield of prolactin activity if determined in terms of original material would certainly have been negligible.

DISCUSSION

For all practical purposes the lyophilized fractions obtained by application of Cohn's method to anterior pituitary extracts are devoid of any stimulating effect upon the crop sac of pigeons. The chemical properties of prolactin resemble very closely those of the corticotrophic principle, and one

would expect them to be rather closely associated in this fractionation procedure. Corticotrophic activity is found in fractions R_1 and R_2, the greater portion being present in R_1. Neither of these fractions appears to possess any prolactin activity.

From the results of preliminary assay of the lyophilized fractions, it would appear that some activity may have been present in R_4. This fraction resulted when the pH of the supernatant containing 40% alcohol was lowered from 5.75 to 4.70, by the addition of a buffer mixture . The total yield of this particular fraction was so small that it was used sparingly in assay work. However, the effects produced by a 10 mg. dose of R_4 were not as great as those resulting from a 50 mg. dose of the crude alkaline extract, and there was no evidence of stimulation.

It is rather difficult to account for the loss of prolactin activity. The extraction procedure should have been effective. Investigators formerly used aqueous acid and alkaline media for extraction of this principle.

The fractionation procedure is not drastic and it is inconceivable that it should have resulted in a complete loss of prolactin activity.

A solution to the loss of prolactin activity cannot be offered.

ASSAY OF THE CORTICOTROPHIC PRINCIPLE

The corticotrophic activity of the various lyophilized powders was assayed by the weight restoration of the atrophic adrenals of hypophysectomized rats. When this method proved to be unsatisfactory a weight maintenance method was then used. The assay procedures will be described.

RESTORATION METHOD

The left adrenal was removed from hypophysectomized rats two weeks post-operative and subcutaneous injections of the various fractions were given twice daily for a period of ten days. The right adrenal was removed and weighed on the eleventh day. The percentage increase in weight of the right adrenal over the left was then calculated. Both male and female rats were used in this assay since each animal served as its own control. Strictly speaking, animals of one sex only should be employed for any assay. The adrenal cortex of hypophysectomized animals of either sex responds to the active principle and it is felt that the use of both sexes did not introduce any discrepancy in the results. The control and stimulated gland were examined histologically in a few instances. It was considered that

the weight increase would give sufficient indication of stimulation.

MAINTENANCE METHOD

Male rats weighing around 125 gms. were hypophysectomized and the injections commenced the following day. The fractions to be assayed were given once daily for two weeks. At the end of this time the animals were killed and the weight of both adrenals recorded. A group of operated animals which had not received treatment were killed at the same time. Due to the variation in body weight at the end of assay it was decided to express the adrenal weight in terms of 100 gms. body weight. The initial rather than the final body weight was used in this calculation. Such a procedure may be criticized, but some fractions, because of their growth activity, brought about weight increase while others were ineffective in this respect. When the adrenal weights were compared with those of normal controls they were found to be lower, so that 100 per cent maintenance was not obtained by the administered fractions. Despite the loss in adrenal weight the animals gained in body weight and it was felt that the more accurate picture could be obtained by calculations involving the initial body weight.

TABLE X

CORTICOTROPHIC ASSAY
RESTORATION OF ADRENAL WEIGHT OF HYPOPHYSECTOMIZED RATS
TWO WEEKS POST-OPERATIVE - TWO DAILY INJECTIONS FOR TEN DAYS

Daily Dose Mgs. Material	Average % Increase of Right Adrenal Over Left	Range	Number of Animals	Remarks
Crude Saline A.P. 5.0	47	36 - 60	5	
R_1 5.0	20.9	5 - 50	4	
" 10.0	62.8	40 - 83	3	
" 50.0	97.0	82 - 113	2	
R_2 2.5	25.0	18 - 33	2	
" 5.0	28.8	5 - 60	3	
" 7.5	15.4	0 - 35	3	
" 10.0	98.7	20 - 166	4	
R_3 5.0	15.7	11.5 - 20.0	2	
" 10.0	76.0	67.0 - 84.0	3	
" 50.0	43.0	43.0 -	1	Two animals died.
R_4 10.0	65.0	50 - 80	2	Two animals died.
R_5 5.0	-	-	-	Three animals died.
" 10.0	16.0	7 - 25	2	One animal died.
" 50.0	43.0	43	1	

TABLE XI

CORTICOTROPHIC ASSAY
MAINTENANCE OF ADRENAL WEIGHT OF HYPOPHYSECTOMIZED MALE RATS

Daily Dose - Mgs. Material	Average Adrenal Wt. Mgs./100 Gms. Body Weight	Range	Number of Animals	Average Weight of Testes Gms.	Body Weight Gms. Before	After	Average Gain
Crude Saline A.P.							
5.0	9.16	7.6 - 10.3	7	1.411	130.5	146	16
10.0	12.10	10.9 - 14.8	6	1.679	121.0	124	3
15.0	13.10	11.6 - 14.6	4	1.625	124.0	131	7
R_1 1.5	14.60	14.2 - 15.4	3	2.060	129.6	166	36
" 3.0	15.70	13.2 - 17.4	4	2.281	116.2	152.9	37
" 5.0	15.80	14.4 - 19.8	5	1.350	116.0	179.7	64
R_2 3.0	12.40	11.3 - 13.6	3	1.462	118.0	126.0	8
" 5.0	14.90	12.6 - 16.5	4	1.137	121.2	153.5	32.5
R_3 5.0	9.20	8.7 - 9.7	5	1.375	124.0	132.1	8.1
Operated Controls	9.34	7.75 - 10.4	6	.954	128.2	119.2	Loss 9.0
Normal Controls	22.6	21.6 - 23.4	4	2.675	125.0	162.0	Gain 37.0

It should be mentioned that in both these methods the completeness of hypophysectomy was checked by binocular examination of the sella for pituitary remnants. The assay results were discarded if pituitary tissue was present.

REFERENCE MATERIAL

The crude alkaline extract of original pituitary tissue was used as the reference material in both the assay procedures. In the weight restoration method it was decided to use a 50 per cent increase in weight as the standard response as suggested by Collip. The dose of extract equivalent to 5.0 mgs. of original tissue was found to produce such an effect. TABLE X

In the weight maintenance method it was necessary to determine the dose of this extract that would be capable of maintaining the adrenal weight. The results of this assay are contained in Table XI . Since a dose equivalent to 5. mgs. of original tissue had proved effective in producing a 50 per cent increase in weight it was thought that a similar dose would serve to maintain the weight of an adrenal that had not suffered atrophy. The results of such a dose showed failure of the extract to maintain weight. In fact the weight of these adrenals was

not any greater than the adrenal weight of operated controls. Doses equivalent to 10 and 15 mgs. of original tissue were therefore given. There was not a great deal of difference in the effects produced by the two doses, nor were they particularly striking.

The question then arose whether storage of the original glands in the laboratory had resulted in loss of corticotrophic activity. When a fresh shipment of calf pituitaries was received from the same source, doses equivalent to 5, 10 and 15 mgs. were assayed. Of the 4 hypophysectomized animals injected with the 15 mgs. dose only 1 survived the assay. The other 3 probably died from toxic effects of the extract. It will be remembered that hypophysectomized animals treated with similar doses of this extract in the growth assay also succumbed. Two of the four animals on the 10 mg. dose also failed to survive. The 5 mg. dose did not result in the death of the animals. In all the surviving animals at the end of the assay period the adrenal weights calculated per 100 gms. initial weight failed to show any difference from those resulting from the original gland extract.

The original glands did not contain any less corticotrophic activity than the new shipment. Either

deterioration had not taken place in the laboratory stored glands or else it had proceeded to a similar extent in the more recently received glands. Due to the small number of animals that survived injection with the new extract and the similarity of response it was decided to combine the results.

The effects produced by the dose equivalent to 5 mgs. of original tissue in the two assay procedures is puzzling. In the weight restoration method this dose was effective in producing a 50 per cent increase in adrenal weight. Under its influence the cortical tissue was partially repaired. When the same dose was injected into an animal immediately after hypophysectomy, when atrophy of the cortex had not taken place, it was ineffective in maintaining adrenal weight. It would seem that the latter method would require a smaller dose of material since cortical repair does not have to be produced in the normal gland. No logical reason can be offered for this paradoxical effect.

LYOPHILIZED FRACTIONS

From Table I it will be seen that the results of the assay of the five fractions for corticotrophic activity were far from satisfactory. The wide range of the

results on any given dose was extremely high as is evidenced by the range. In order to overcome this to some extent it would have been necessary to employ large groups of animals on each dose. The number of hypophysectomized animals available for assay was limited, so a more satisfactory method of assay was sought. The large variation is hard to explain. It is true that the animals were of both sexes, but it hardly seems possible that this could have been the sole contributing factor.

When the weight maintenance method was adopted it necessitated that all animals be of the same sex, and male rats were employed. The results of this assay procedure using the lyophilized fractions are contained in Table XI. From a consideration of the effects produced by these fractions in the weight restoration assay method it was decided that the corticotrophic activity was contained in the first three fractions. Assays were therefore conducted only on fractions R_1, R_2 and R_3. Since in all these assays the results were compared with a crude extract of original tissue in order to determine the percentage yield of activity it was necessary to produce a degree of weight maintenance that corresponded with that resulting from the crude extract. The higher doses of the crude alkaline extract had failed to maintain the adrenal

LOG. DOSE MGS. FRACTIONS R_1, R_2, R_3.

PER CENT INCREASE RIGHT ADRENAL/LEFT ADRENAL

100 75 50 25

0.25 0.5 0.75 1.0 1.25 1.5 1.75

Level of response to 5 mgs. of crude

Corticotrophic Assay
Restoration Method

× = R_1
⊙ = R_2
↙ = R_3

GRAPH III

ADRENAL WT. PER 100 GMS. BODY WEIGHT

16 15 14 13 12 11 10 9

R_1

R_2

CRUDE

Corticotrophic Assay
Maintenance Method

0.5 1.0 1.5

LOG. DOSE MGS.

weight above thirteen grams and it was necessary to find a dose of the lyophilized fractions that would produce a similar effect.

LYOPHILIZED FRACTIONS

From a consideration of Table XI, it is obvious that fraction R_3 was devoid of any corticotrophic activity. The adrenal weights after treatment with this fraction were equal to those of operated controls whose adrenals had been allowed to atrophy. Fractions R_1 and R_2 produced some maintenance of adrenal weight. It is interesting to note that fraction R_1 produced the greatest body weight increase during the period of assay and that R_2 produced weight increase, but to a lesser extent. These effects on body weight bear out the findings of the growth assay on the lyophilized fractions. Fraction R_1 was also successful in maintaining the testes at a weight very close to normal, while fraction R_2 was not quite so effective.

YIELD OF ACTIVITY

Although the adrenal weight maintenance method gave more satisfactory assay results, it is rather interesting to calculate the yield of activity by both assay methods.

In the weight restoration method, which was finally abandoned, we can plot the percentage increase in adrenal weight against the log. dose in mgs. of the various fractions.

These results are shown in Graph III.

R_1 gives a good log. dose/response relation. The points of R_2 are rather scattered and the response lines of both R_2 and R_3 are somewhat steeper.

If one intersects the R_1 response line at the level of response of the 5 mg. dose of crude extract, then 5 mgs. of crude material is about equivalent to 10 mgs. of R_1.

This represents a yield of activity in terms of original tissue of 0.5 per cent.

Although fractions R_2 and R_3 possess slightly more activity, the total yield for R_1, R_2 and R_3 in terms of original tissue cannot be much more than 2 per cent.

In the maintenance assay which is also included in Graph III, the results have been calculated differently.

The adrenal weight per 100 gms. body weight was plotted graphically against the log. dose in mgs. of crude extract, and the resulting straight line was graphically extrapolated. This allows a comparison with the response lines of R_1 and R_2 which have been similarly plotted.

By comparing the lowest point of the R_1 response line with a corresponding point on the crude extract line, one finds that 1.5 mgs. of R_1 is equivalent to 21 mgs. of crude. In other words, the total amount of R_1, 13 gms., is equal to 182 gms. of crude material.

The yield of activity in terms of original tissue is, therefore, $\frac{182}{1235}$ x 100 or 15 per cent.

By comparing a point on the response line R_2 with the crude extract line, one finds 3.3 mgs. of R_2 equivalent to 14 mgs. of original material.

The yield of activity of R_2 in terms of original tissue is, therefore, 4 per cent.

The total yield of corticotrophic activity calculated from the results of adrenal weight maintenance assay is represented in fractions R_1 and R_2 and equals 19 per cent.

DISCUSSION

The Cohn fractionation procedure was not successful in isolating corticotrophic activity in a particular fraction of the anterior pituitary extract. Although the greater yield of activity is in R_1 , a small amount is present in R_2.

The total yield of activity, 19 per cent, is not as great as that obtained with some of the other principles. The extraction procedure, 0.9% NaCl at pH8.7, should have removed all corticotrophic activity. Early investigators successfully extracted this principle in combination with prolactin by using an aqueous alkaline medium.

If the extraction procedure did succeed in extracting all the activity present in the tissue, then one wonders what subsequent step in the fractionation procedure was

responsible for the loss of activity. In the adrenal weight maintenance assay, R_3 was devoid of activity in the dosage employed. In view of the results obtained in the restoration assay, it hardly seems likely that R_4 and R_5 would have proved effective.

The Cohn fractionation procedure can hardly be called drastic. The concentration of alcohol and range of pH are moderate. Temperature changes during handling were reduced to a minimum. Unless denaturation resulted during the addition of ethanol in the fractionation process, the only suggestion that can be offered for the loss of activity is denaturation during the drying process. All precautions were taken to prevent thawing. It hardly seems possible that if slight thawing were produced in one fraction it could have occurred consistently without observation.

GONADOTROPHIC ASSAY

Studies on the gonadotrophic principles of anterior pituitary tissue are complicated by the close relationship of the two factors, the so-called follicle stimulating hormone (FSH) and the luteinizing (LH) or interstitial cell stimulating hormone (ICSH). No other pituitary principle can be studied by such a variety of methods. Animals of either sex and of numerous species can be used in demonstrating the presence of these principles. Since the fractions used in the present investigation did not possess specific activities but showed contamination with other pituitary fractions no attempt was made to assay for FSH and ICSH separately.

METHOD

The standard maturity test using the twenty-one day old female rat was employed in assaying the gonadotrophic activity present in the fractions obtained from pituitary extract by application of the Cohn method. The animals were injected subcutaneously three times daily for three days and killed on the fourth day, seventy-two hours after starting the test. The subcutaneous route of injection is an important condition for the success of the assay.

Vaginal smears were taken before killing the animals.

The smears were examined for the typical oestrus signs - the absence of leucocytes and presence of cornified squamous cells. The results were subjective and were scored by a 1 to 4 plus classification. Smears were taken when the vaginal orifice was not patent. The fresh weights of ovaries and uterus were recorded. All weights have been expressed as mgs. per 100 gms. body weight because of the great variation in the body weight of immature rats in our colony.

As with the other assays it was necessary to perform a few spot tests to obtain information about the fractions that possessed activity, and the effective dose. The five fractions were tried in this manner, and then more detailed assays were conducted on the fractions showing gonadotrophic activity.

REFERENCE DOSE

A crude saline extract was used as the reference dose material. It was prepared as previously described and administered in various dilutions. Although it was realized that bovine anterior pituitary is not particularly potent in gonadotrophic activity it was rather surprising to note the high dosage that had to be employed before noticeable stimulation could be produced. The first effective daily dose was 50 mgs. of crude saline extract. Higher dosage was not particularly effective in producing greater

TABLE XII

GONADOTROPHIC ASSAY OF CRUDE SALINE EXTRACT OF ORIGINAL A.P.

REFERENCE DOSE ASSAY

Daily Dose Crude Saline A.P. - Mgs.	Number of Animals	Average Wt. Ovaries Mgs./100 Gms. Body Weight	Range	Average Wt. Uterus Mgs./100 Gms. Body Weight	Range	Vaginal Examinatio	
						Orifice	Average Smear
2.5	4	31.5	21.6 - 35.0	53.5	50.0 - 63.0	Al. Patent	1 Plus
5.0	3	39.8	34.3 - 47.8	71.0	63.0 - 84.0	Al. Patent	1 Plus
10.0	4	39.6	35.3 - 47.2	74.7	49.2 - 101.0	Not Patent	1 Plus
20.0	4	38.8	27.8 - 50.0	56.5	52.0 - 90.0	Not Patent	2 Plus
50.0	4	57.5	47.8 - 57.6	75.3	58.6 - 105.0	Not Patent	1 Plus
50.0✱	4	40.5	31.3 - 42.9	59.9	52.8 - 70.5	Not Patent	1 Plus
100.0	4	66.3	59.2 - 77.0	107.4	81.2 - 143.0	Some Patent	2 Plus
100.0 ✱	4	48.0	42.8 - 54.8	72.0	65.5 - 79.9	Some Patent	2 Plus
150.0	5	54.1	45.6 - 67.7	96.8	76.5 - 125.0	Not Patent	1 Plus
200.0	5	58.1	38.0 - 78.1	139.8	77.8 - 246.0	Not Patent	1 Plus
Normal Controls	6	34.9	28.3 - 45.8	57.6	36.4 - 96.7		

✱ FRESH CALF A.P. USED.

TABLE XIII

GONADOTROPHIC ASSAY OF LYOPHILIZED PITUITARY FRACTIONS

CONDUCTED ON 21-DAY OLD FEMALE RATS

Daily Dose Mgs. Fraction	Number of Animals	Wt. Ovaries Mgs./100 Gms. Body Weight	Range	Wt. Uterus Mgs./100 Gms. Body Weight	Range	Vaginal Examination Orifice	Vaginal Examination Average Smear
R_1 1.0	2	30.4	25.4 - 35.5	67.1	61.2 - 73.0	Not Patent	Neg.
" 2.5	2	42.1	41.1 - 43.1	60.6	57.8 - 63.4	Not Patent	Neg.
" 5.0	2	47.5	39.5 - 55.0	73.2	63.1 - 83.3	Al. Patent	1 Plus
" 10.0	2	41.7	39.4 - 44.0	76.8	79.6 - 74.0	Al. Patent	1 Plus
" 20.0	3	59.1	46.2 - 77.8	79.8	66.6 - 101.0	Al. Patent	1 Plus
R_2 1.0	2	50.3	42.0 - 58.6	83.8	78.0 - 89.6	Not Patent	1 Plus
" 2.5	2	50.7	44.2 - 57.2	77.0	77.0 - 77.0	Not Patent	1 Plus
" 5.0	7	48.1	32.6 - 75.0	91.8	69.5 - 122.0	Al. Patent	1 Plus
" 10.0	5	95.6	72.0 - 133.0	180.5	149.0 - 212.0	Patent	4 Plus
" 20.0	6	143.0	42.0 - 223.0	231.7	114.0 - 365.0	Patent	4 Plus
R_3 3.5	2	40.7	32.9 - 48.6	68.1	59.5 - 76.7	Al. Patent	
" 5.0	5	50.2	41.0 - 55.3	101.8	82.5 - 129.0	Al. Patent	
" 10.0	5	71.2	48.2 - 81.3	132.3	68.5 - 182.0	Patent	4 Plus
" 20.0	5	101.6	51.3 - 152.0	154.7	80.7 - 274.0	Patent	4 Plus
R4 2.5	3	53.9	51.7 - 57.9	78.3	72.1 - 89.5	Not Patent	Neg.
" 5.0	3	43.4	39.4 - 46.0	84.5	80.5 - 88.0	Not Patent	Neg.
R_5 2.5	2	36.1	33.8 - 38.9	66.4	57.8 - 75.0	Not Patent	Neg.
" 5.0	2	34.3	30.6 - 38.1	91.5	52.5 - 130.5	Not Patent	Neg.
" 20.0	2	34.2	34.2 - 34.2	146.2	137.5 - 155.0	Not Patent	Neg.

stimulation. Table XII

It was thought that the necessity of such high dosage might have been the result of deterioration of gonadotrophic activity in the stored glands. To answer this objection fresh calf anterior pituitary extract was employed in the same dosage. The assays using new and old glands were carried on simultaneously. The results are indicated in Table XII . It was rather surprising to note that the original stored glands produced more stimulation than the fresh glands. It is generally accepted that a variation in any specific activity can exist between two separate shipments of pituitary glands. This is the only reason that can be offered for the lower results obtained with the fresh pituitaries.

In normal controls the ovarian weight is 34.9 ± 6 mgs per 100 gms. body weight. In all daily doses below 50 mgs. the crude saline extract failed to produce ovaries above normal weight. The doses of 50 and 100 mgs. of original glandular tissue showed stimulation. In the latter case the ovarian weight was increased 100 per cent. The failure of doses of 150 and 200 mgs. to produce at least an effect equivalent to a 100 mg. dose is unexplainable. The high dosage assay was performed about three weeks later, but it is inconceivable that such a change could take place in the glands during that period, particularly when they were kept

under the same conditions of storage. The greatest ovarian responses were produced by daily doses of 50 and 100 mgs.

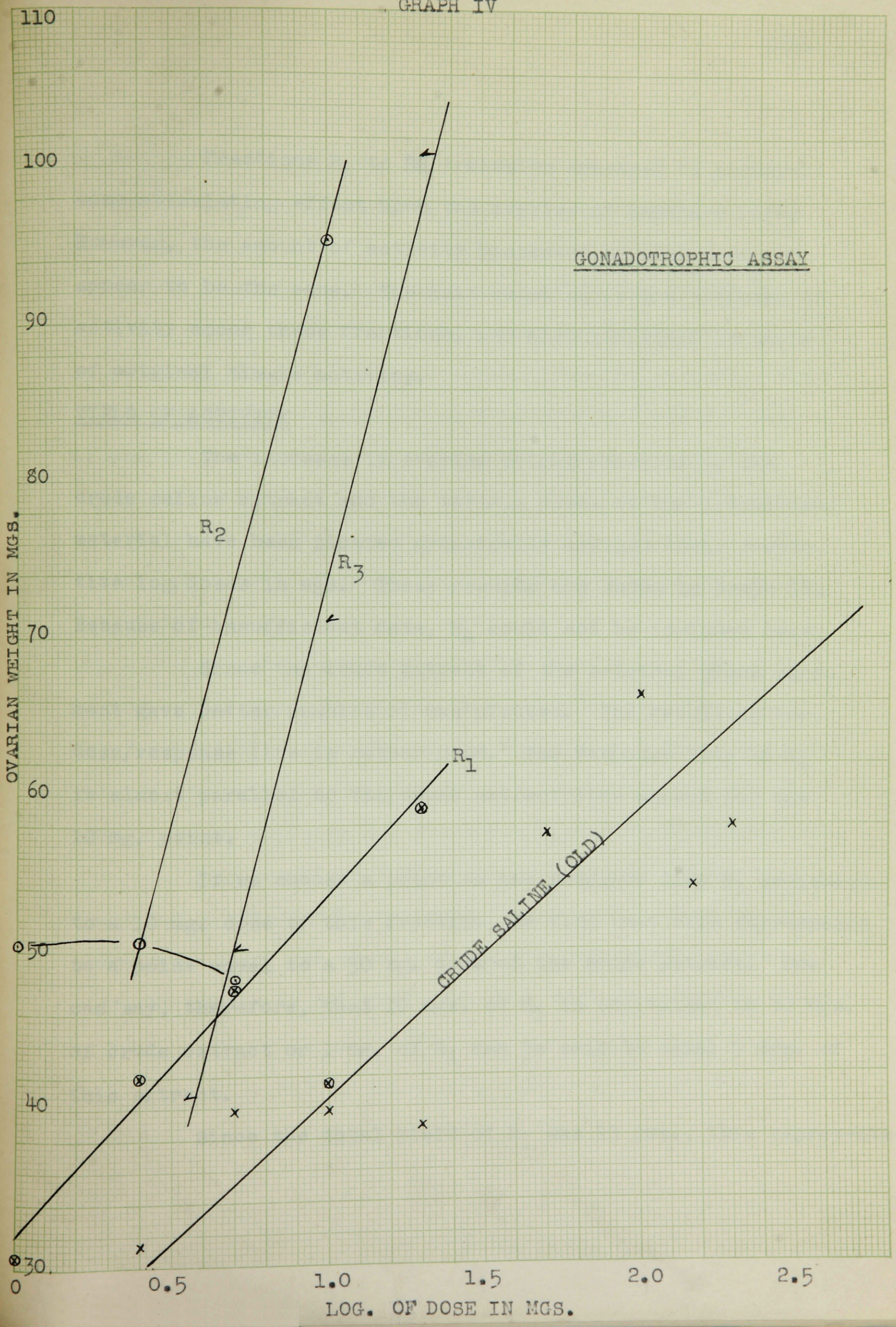
The ovarian response is more satisfactory than the uterine weight response. In the case of the uterus there did not appear to be any correlation between dosage used and effect produced. In addition the individual variations were larger than those for ovarian weight. One rather strange finding was the absence of a full oestrus vaginal smear even on the highest dosage.

LYOPHILIZED FRACTIONS

Table XIII shows the ovarian and uterine weight response produced by the lyophilized anterior pituitary fractions. The number of female immature rats available at any time did not allow one to conduct concurrent assays of the various fractions nor on all doses of a particular fraction.

A consideration of these results indicates that fraction R_5 failed to produce any stimulation of ovarian weight in the dosage employed. The ovarian weights on each dose failed to exceed those of normal control rats. In each case the vaginal smear was not typical of oestrus and the orifice was not patent. As far as the uterine weights are concerned, there appeared to be an increase in weight with higher dosage. The variation was so large in all the uterine weights that these changes could not be considered of any significance.

GRAPH IV



Fractions R_1 to R_4 inclusive appeared to produce some stimulating effect upon the ovaries of immature rats. However, the amount of activity possessed by each does not appear to be the same. The next question is the division of activity among these fractions, and also its yield in terms of original tissue activity.

YIELD OF ACTIVITY

The increase in ovarian weight produced by the crude saline extract and the various doses of the lyophilized material have been plotted graphically against their respective log. doses in mgs. These results are shown in Graph IV. Because of insufficient data, R_4 could not be included here.

Since the crude extract of the original gland shipment gave better results it was plotted. The resulting log. dose/response line is rather good. The response line of R_1 is almost parallel to the crude extract line except for the 10 mg. point.

If we choose a point on the response line R_1 equivalent to a 10 mg. dose of this fraction, we find that this corresponds to a point equal to a 50 mg. dose of the crude extract. One can say, therefore, that 10 mgs. of R_1 is equivalent to 50 mgs. of crude extract or 1 mg. of R_1 can be said to equal 5 mgs. of this extract.

Since the total yield of R_1 was 13 gms., this represents

65 gms. of crude extract. The total yield of gonadotrophic activity of R_1 is, therefore, $\frac{65}{1235} \times 100$, or slightly over 5 per cent.

Fraction R_2 assayed poorly. The first three doses produced almost similar ovarian response. While the response produced by the 20 mg. dose extends beyond this graph, nevertheless an almost straight line relationship exists between it and the responses to the 10 and 2.5 mg. doses of this fraction. This line is much steeper than those given by R_1 and the crude extract. However, the steep line of R_2 is almost parallel to the excellent response line given by the four points of R_3.

If one chooses conservative points on lines R_2 and R_3 and compares them with the crude extract response line, then the yield of activity can be calculated. From this one obtains the rather conservative estimates of 17 and 9.5 per cent yield of activity for fractions R_2 and R_3 respectively.

The total yield of gonadotrophic activity for fractions R_1, R_2 and R_3 in terms of original tissue is, therefore, 31.75 per cent. Fraction R_4 obviously possesses a slight amount of activity, but this has not been calculated.

ASSAY FOR ICSH ACTIVITY

It was previously stated that an attempt was not made to differentiate the yields of activity of the two pituitary gonadotrophic factors since the fractionation procedure had not led to a definite separation of the other active pituitary principles. The synergistic action of the F S H and I C S H on the immature rat ovary is generally recognized, and it was felt that such an assay would give sufficient indication of the yield of activity. However, the L H or I C S H content of bovine pituitary is greater than the F S H content and it was decided to conduct at least a preliminary assay for this activity by using the seminal vesicle stimulating effect.

METHOD

Twenty-one day old male rats were injected subcutaneously twice daily for five days and killed on the sixth. The fresh weights of seminal vesicles and testes were recorded. When the seminal vesicles are greatly stimulated and contain fluid it is customary to fix them in Bouin's solution prior to weighing. The degree of stimulation produced in this assay did not demand such a procedure. From a consideration of the results produced in immature female rats it was decided to use only those lyophilized fractions that exhibited some gonadotrophic activity. Similarly high

TABLE XIV

ICSH OR LH ASSAY

21-Day Old Male Rats Injected Subcutaneously
Twice Daily for 5 Days - Autopsied on Sixth Day

Daily Dose Mgs. Material	Weight Seminal Vesicles per 100 Gms. Body Weight		Weight Testes per 100 Gms. Body Weight	
	Average	Range	Average	Range
Crude Alk. Ant. Pit.				
50	33.5 (4)*	31.2 - 34.8	701 (4)	600 - 850
100	32.2 (4)	23.7 - 39.7	740 (4)	681 - 873
R_1 10	30.5 (4)	27.3 - 32.4	775 (4)	735 - 792
R_2 5	35.2 (4)	31.2 - 46.8	865 (4)	725 - 1050
" 10	43.7 (4)	39.0 - 52.1	888 (4)	836 - 915
R_3 5	34.6 (4)	31.0 - 35.7	998 (4)	871 - 1050
" 10	31.5 (4)	27.0 - 35.0	961 (4)	885 - 1040
Normal Controls	26.1 (3)	24.3 - 28.2	739	686 - 781

* Number of Animals.

doses of crude extract of original tissue were employed. The results were expressed as mgs. per 100 gms. of body weight because of the wide range of body weight in these animals. Table XIV shows the results of I C S H assay. The seminal vesicle weight produced by injection of high doses of the reference material did not differ greatly from that of normal controls. Injection of the lyophilized fractions did not produce any striking effects, but it was observed that the fractions most effective in producing ovarian stimulation also showed greatest activity in stimulating seminal vesicle weight.

YIELD OF ACTIVITY

From the small amount of data available it is impossible to calculate the yield of activity by construction of a log. dose/ response curve. The results produced with 50 and 100 mg. doses of the crude saline extract are about the same and do not differ from the normal range to any great extent.

From the values obtained with the lyophilized material it is evident that R_2 and R_3 possess about equal activity. Fraction R_1 contains less activity than R_2 or R_3.

The results of this preliminary assay showed that no advantage would be gained by conducting a separate assay for I C S H. Gonadotrophic activity as assayed by immature

female rats answered the question quite adequately since the Cohn fractionation procedure was not effective in separating the two gonadotrophic principles.

DISCUSSION

The total yield of gonadotrophic activity is roughly 32 per cent. This activity is not confined to a particular fraction, but is spread over R_1, R_2 and R_3.

From a consideration of Table XIII and Graph IV, we observe the striking difference in activity between the crude extract and R_1 on the one hand and R_2 and R_3 on the other. R_1, which contains the lowest yield of activity, parallels the log. dose/response line given by the crude extract. The two fractions which have greater activity, R_2 and R_3, give lines which are parallel to each other, but much steeper than the line for R_1. It will be remembered that conservative estimates of the yield of activity of R_2 and R_3 were made. The lowest region of each line was used for comparison with the crude extract.

If either R_2 or R_3 gave a response line which was strikingly steeper, it would seem fortuitous, but the similarly steep parallel relationship of the two response lines leads one to seek an explanation. The slope differs so from the crude extract and fraction R_1 that it leads one to speculate. It would appear that the removal of some

substance present in the crude extract and R_1 is effected by the Cohn fractionation procedure. This substance probably plays an inhibitory role and prevents maximum ovarian stimulation. Whatever it is, a concentration of 10% ethanol at the neutral point is successful in removing it, since it is absent from R_2 , which precipitated at a concentration of 25 per cent ethanol and a similar pH.

One could say that the increased response to R_2 and R_3 was due to the presence of I C S H which acted synergistically with F S H to produce larger ovaries. However, the failure to produce similar results with the crude saline extract vitiates this theory. The preliminary assay for I C S H also failed to show any striking content of this principle in R_2 and R_3.

This "inhibitor" substance is probably a protein and one cannot add more than this concerning its nature. If such a substance does play a physiological role in the pituitary-gonadal relationship, nothing is known of its mode of action. It would be interesting to determine if this substance is also found in the pituitary tissue of mature animals.

The total yield of gonadotrophic activity is not too poor when one considers that general extraction and fractionation procedures were employed. Bovine anterior

pituitary is admittedly a poor source of the gonadotrophic hormones. The method of extraction, 0.9% NaCl at pH8.7 should have been adequate. Fevold finds that pH6 does not give a poor yield, but recommends a more alkaline medium. The gonadotrophic principles are globulin in nature and are extremely water soluble. It is difficult to effect a clear-cut separation of the two principles due to their similar chemical properties and the tendency of their solubilities in any medium to overlap.

From the assay results presented, one can conclude that the application of the Cohn fractionation procedure is ineffective in separating F S H and I C S H activity and gonadotrophic activity is not confined to a particular fraction.

SUMMARY AND CONCLUSIONS

The present investigation was undertaken with a three-fold purpose. It was undertaken to determine the activity associated with the various fractions obtained by the application of Cohn's blood plasma method to an alkaline saline extract of calf anterior lobe tissue. In conjunction with this, the percentage recovery of the activity of each specific principle in terms of that possessed by the original tissue was also sought. And finally, it was undertaken to evaluate the effectiveness of the Cohn method as a procedure in fractionating anterior lobe extracts.

Permission to adapt the Cohn blood plasma method to an anterior pituitary extract was obtained. Cohn urges careful regulation of temperature, control of pH and concentration of alcohol when fractionating protein solutions. Recommendations similar to those presented in his Monograph can be found in earlier published work (204). The importance of adhering to these experimental conditions was recognized, and all care was taken to observe the necessary precautions as closely as the laboratory facilities would permit.

The application of the Cohn fractionation procedure to an alkaline extract of anterior lobe tissue is described. The resulting fractions, which have been named R_1 to R_5 inclusive, form the basis for the present investigation.

TABLE XV

Distribution of Activity in Lyophilized Fractions

Percentage Yield of Activity in Terms of Original Tissue

ACTIVITY	R_1	R_2	R_3	R_4	R_5	Total Yield	Degree of Purification
Growth	8.0	2.8	-	-	-	10.80	50 times
Thyrotrophic	26.0	19.5	10.0	-	-	55.00	32 times
Prolactin	-	-	-	-	-	-	-
Corticotrophic	15.0	4.0	-	-	-	19.00	50 times
Gonadotrophic	5.25	17.0	9.5	-	-	31.75	32 times

Each fraction was dried under high vacuum when in the frozen state, and the yield of the various powders from the original crude extract is recorded. The extremely small yield of R_4 necessitated its exclusion from some preliminary studies which were conducted, so that sufficient would remain for the assay work.

The nitrogen content of the various fractions was studied. Slight contamination of R_5 with the oxytocic principle of the posterior pituitary is shown, and the presence of some sensitizing substance in all the fractions studied is evidenced by the few results obtained on guinea pigs.

Before assays for the various recognized pituitary principles were undertaken, it was necessary to decide upon a reference material so that the percentage yield of activity in terms of original tissue could be determined. A crude alkaline saline extract of calf anterior pituitary from the original shipment was used throughout as this reference standard. It was decided to use this material as a standard when a similarly prepared, but subsequently lyophilized fraction, showed some loss of activity.

The reference extract was freshly prepared every three days from the undissected glands which were preserved in the frozen state. The use of such a reference standard

may be subject to criticism since deterioration of activity could probably take place in the whole glands during the storage period. Deterioration was suspected on several occasions and led to assay difficulties; however, on investigation, the stored glands exhibited as much activity in the majority of instances as a fresher gland shipment.

Any investigator of the anterior pituitary principles is confronted by a variety of assay procedures. In the present investigation the methods used in the estimation of the growth, thyrotrophic, prolactin, corticotrophic and gonadotrophic content of the various lyophilized fractions were the standard methods recommended by numerous investigators. The chosen methods have a trustworthy reputation, and it was felt they would yield the best results.

All assays were carefully conducted. The necessity of using such small groups of animals on various doses greatly handicapped assay procedure and the results of assay. It was impossible to obtain a sufficiently large number of animals of one age and sex from our rat colony at any one time. Coupled with the small number of available animals, the rather difficult technique of hypophysectomy and the dearth of such suitably prepared animals was a further handicap. Rather than perform all the tests on a certain dose at the same period of time, it was sometimes necessary to spread

the results out over a period of months. However, all attempts were made to standardize the experimental conditions as closely as possible, and the results checked rather well even after a few months' interval.

Hypophysectomized rats of either sex were used in the investigation of growth activity in the various fractions. Injections were started ten days after operation and continued daily for twenty days. The weight increase obtained with various doses of the lyophilized material was compared with the results produced with the crude alkaline extract of original tissue.

Guinea pigs were employed in the thyrotrophic assay. The percentage increase in thyroid weight produced by dosage with the crude extract or the various lyophilized fractions above that of normal untreated guinea pigs was the basis of assay. These results were correlated with histological evidence of thyroid activity.

The futility of the prolactin assay which was based upon crop sac weight increase was soon evident. Only a negligible amount of lactogenic activity was present in the lyophilized material. The prolactin assays could not have given more than a qualitative result under the circumstances. The necessity of using a single strain of pigeon of a particular age is stressed by various workers.

Hypophysectomized rats were also employed for the corticotrophic assay of the lyophilized material. A weight restoration method proved unsatisfactory and finally an adrenal weight maintenance method was adopted. The results of assay were compared with the degree of weight maintenance produced by the crude alkaline extract of original tissue.

In the investigation of the gonadotrophic activity of the various fractions immature female rats were employed. The use of hypophysectomized rats is recommended by those working with purer hormone fractions. The degree of separation of the two gonadotrophic factors did not warrant the use of operated animals in conducting the assay. The ICSH activity was investigated by the effects on seminal vesicle weight of immature male rats.

The results of all these assays are contained in detail in the corresponding tables. In Table XV a summary of the results with the five lyophilized fractions is given. This answers the question of division of activity among the various fractions as well as the percentage yield of activity in terms of original tissue.

The percentage yield of activity was obtained by calculation from the results of the various assays which were plotted graphically in terms of a log. dose response line. Details of calculation are included in the experimental section.

Growth activity is found in fractions R_1 and R_2, the greater portion being in R_1. Deterioration of the growth activity of the original stored tissue led to the use of a reference extract prepared from a fresh gland shipment. In the light of the yield of activity of some of the other principles, the total yield of growth activity, 10.8 per cent, is low.

The thyrotrophic activity is represented by fractions R_1, R_2 and R_3, the greatest activity being in R_1. The total yield of activity, 55 per cent, is the largest for the principles investigated.

Corticotrophic activity, as assayed by the adrenal weight maintenance method, is present in the first two fractions. Although the degree of purification is about fifty times, the yield of activity, 19 per cent, is not high.

Gonadotrophic activity is present in the first three fractions, but the greatest yield is in R_2. About one-third the activity of the original tissue is recovered.

It is interesting to note that all the principles investigated are present in the first two fractions. Only in the case of the thyrotrophic and gonadotrophic principles does R_3 possess significant activity. The almost complete absence of activity from R_4, and its definite absence in R_5 is rather puzzling, particularly when the yield of

activity in the other fractions does not approach that of the crude extract.

Fraction R_4 precipitated when the supernatant containing 41 per cent ethanol at pH5.75 was further acidified by a buffer mixture to pH4.7. In fraction R_5, which represents the residue of the protein which was extracted from the anterior pituitary, one would expect to find any active material which had not been precipitated by the other steps of the Cohn fractionation procedure.

It should be mentioned that the volume of supernatant, before concentration under high vacuum, was 6 litres. This was reduced to 300 ccs. in a water bath whose temperature did not rise above 40°C. The evaporation of such a large volume took many hours, and the temperature within the flask may have risen above the required 17°C, thus producing loss of activity. Dialysis was not carried out prior to evaporation so that the salt concentration increased considerably when the volume was reduced to one-twentieth of the original. Prolonged heating in such a medium could very likely result in destruction of activity. However, if R_4 possessed activity and R_5 did not, one would attribute its absence to destruction during the concentration process, but the negligible amount of activity possessed by R_4 leads one to question the truth of this speculation.

The presence of so many principles in the first three fractions, and the lack of a clear-cut separation of these principles, was rather disappointing. It was hoped that a particular fraction might contain a certain principle to the exclusion of others. The number of active proteins present in the pituitary, as well as their close chemical relationship, added to the difficulty of fractionation.

The successful fractionation of any protein solution is attended with difficulties. By slight alteration of salt content, temperature and pH, the nature and yield of a fraction can be altered. This is evident when one considers Cohn's published work on the preparation and properties of serum and plasma proteins by an ethanol-water fractionation method which is essentially the same as that employed in the present investigation (204).

Apart from conditions that may have led to denaturation of the protein fractions and consequent loss of activity (addition of ethanol and rise in temperature), it should be stressed that the conditions of temperature, alcohol concentration, pH and ionic strength set forth by Cohn relate specifically to the components of serum and plasma. The number of pituitary proteins as well as their chemical properties probably require slight variation of the conditions suggested by Cohn.

From the results of investigation of the Cohn method applied to the fractionation of an anterior pituitary extract one can conclude that certain modifications must be introduced before it can prove of definite value in this respect. By careful control of temperature and the observance of precautions to maintain low temperatures, as well as the avoidance of denaturation when adding the ethanol, the yield of activity may possibly be greatly improved. Similarly, better fractionation of the various active protein principles may be obtained by modification in the ethanol-water fractionation procedure.

In conclusion the author wishes to state that although the method of protein fractionation employed is not original, nevertheless, its application to an extract of anterior pituitary constitutes an original investigation. While low temperature alcohol precipitation methods have been employed by Mellanby (205), Hardy and Gardiner (206), and many subsequent investigators, some of whom were concerned with the preparation of antibodies, and while it has been successfully applied to the preparation of serum and plasma proteins, the literature does not contain any reference to its application in the preparation of anterior pituitary hormone fractions.

The three-fold purpose of the investigation has

been fulfilled, and while the results obtained are not of a startling nature, they offer promise that, with modifications, the method may prove highly successful.

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