THE CONCENTRATION OF ACTH AND GROWTH HORMONE IN BOVINE PLASMA

by

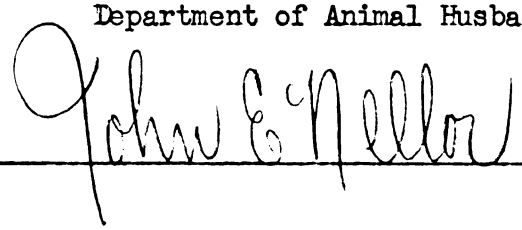
DAVID DENIS SUTTON

AN ABSTRACT

Submitted to the College of Agriculture, Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Husbandry

Approved 

ABSTRACT DAVID DENIS SUTTON

Blood plasma from a 14 year old and a 2 year old bull was fractionated into eight different blood fractions by a modification of the method of Cohn et al. (1950). Each fraction was assayed for growth hormone activity by the tibia assay procedure (Greenspan et al., 1949) and ACTH activity by Sayers et al. (1950). A separation of the ACTH and growth hormone activities was accomplished into two different blood fractions. Blood fraction IV+V contained the ACTH activity, and the growth hormone activity was isolated in the blood fraction II. An attempt to quantitate the various activities was undertaken without success due to the extreme variation in the assay animals. Even with the great variation obtained in the assay animals there did appear to be a higher ACTH activity in the plasma of the 14 year old bull.

The ACTH activity present in blood fraction IV+V was readily dialyzable. Growth hormone activity did not appear to be dialyzed to any measurable extent.

A substance was detected in blood fraction VI which caused an increase in the weight of the adrenal in one hour after its injection. This factor was not dialyzable and was not thought to be analogous to the ACTH activity.

Blood fraction III-O contained an anti-ACTH activity which increased the adrenal ascorbic acid in one hour after its injection. The anti-ACTH factor was not found to be dialyzable.

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INTRODUCTION

Galen (200 A.D.) and other early anatomists were aware of the location of the pituitary gland, but they had no correct knowledge of its function. The term "pituitary", introduced by Vesalius, perpetuated an erroneous idea which was held for many years. It was supposed that the pituitary gland served as a filtration apparatus for wastes from the brain. The filtrate was thought to pass into the nasopharynx through minute pores in the cribriform plate of the ethmoid bone and to function as a lubricant for the nose and throat. During the seventeenth century it was shown that no channels were present in the ethmoid bone, and, since no other function was known, many came to regard the pituitary gland as merely a vestigial relic of no particular importance. It was not until the early nineteen hundreds that the hormonal function of the pituitary gland was actually brought to light. Within the past twenty five years six hormones have been isolated from the anterior lobe of this gland. Each of these hormones has been prepared in either a pure or a relatively pure form. There has been considerable debate as to the number of hormones secreted by the gland. Adequate evidence has established the fact that the six hormones which are identifiable as individual substances in extracts of anterior pituitary tissue are follicle-stimulating, interstitial-cell stimulating (luteinizing), lactogenic, thyrotropic, adrenocorticotropic and growth promoting hormones. Although there may exist in the secretions of the anterior

pituitary gland additional hormonal principles whose physiological effects are as yet undescribed, the specific chemical substances which have been isolated account for most of the recognized hormonal roles assigned to the anterior pituitary gland.

The elucidation of these hormonal functions has been from observations of (1), the ameliorative effects resulting from injection of the anterior pituitary tissue extracts into hypophysectomized animals; (2), the physiological effects of extirpation of the anterior pituitary gland and (3), the results produced by injecting an excess of the gland's secretions in the form of extracts, into the normal animal.

The application of these research methods has disclosed the diverse types of physiological activity which are under hormonal control of the anterior pituitary gland. The isolation of the six chemical substances has resulted from these physiological studies. It has been demonstrated that each of the isolated products will restore in the hypophysectomized animal at least one of the physiological processes whose rate was greatly retarded as a consequence of hypophysectomy.

All the hormones obtained from the anterior pituitary gland have been protein in nature. In 1937, White et al. announced the crystallization of the lactogenic hormone, but in 1942 the crystalline protein was shown to be a homogeneous substance and identified as the hormone (White et al., 1942). Since this hormone was the first to be indubitably isolated (Li et al., 1940; Bonsnes et al., 1942) its chemistry has been the most extensively explored. One of the most interesting facts established by this exploration is that the hormone isolated from glands of

different mammalian species is not an identical substance. For instance, the tyrosine content of ox and swine lactogenic hormone is considerably higher than that found in the hormone obtained from sheep pituitary glands (Li et al., 1943). The concentration of tyrosine in the porcine glands appears to be higher than in the other two (Sayers et al., 1943). It should be kept in mind that the hormones thus far identified are derived from extracts of the pituitary gland. The question as to the actual number of hormones secreted by the gland is still far from clear as well as the actual chemical structures. Differences in the adrenocorticotropic potency of the fresh and stored gland have been interpreted as indicating a difference in the extracted and secreted hormones (Fraenkel-Conrat et al., 1940). A final verification of the difference will have to wait upon the isolation and characterization of the hormones actually encountered in the blood stream.

It is well known that many metabolic processes are attributed to the effects of anterior pituitary hormones (Long, 1943), particularly the growth and adrenocorticotropic hormones. Biological studies suggest that the metabolic changes resulting from the administration of the growth hormone may involve an entirely different process from that provoked by the adrenocorticotropic hormones which produce opposite and opposing reactions towards each other. Thus, the antagonistic effect between the two hormones necessitates their separation before any accurate estimation of their content in a biological system can be made.

The predominance of research in the past has been concerned with glandular hormone concentration. More recently interest has turned

towards the systemic hormone concentration. The purpose of this thesis was to attempt a more realistic approach to the understanding of hormonal mechanisms by measuring the hormone content of plasma which has been previously fractionated. The purpose of the fractionation was to obtain a potency increase of the respective hormones as well as to separate those materials which will have either a synergistic or antagonistic effect on the bioassays involved.

(4) The posterior portion of the gland, which is connected to the brain by a stalk (the infundibulum), and is variously designated as the posterior lobe of the hypophysis (or pituitary), the neuro-hypophysis, pars nervosa or neuralis, infundibular body, or neural lobe.

The posterior pituitary gland is also designated as the processus infundibuli. The posterior pituitary gland, along with the pars intermedia of the adenohypophysis, is separated in many mammals from the rest of the adenohypophysis by the residual lumen of Rathke's pouch. This lumen forms a natural line of cleavage dividing the entire gland into an anterior portion (consisting of pars distalis and pars tuberalis) and a posterior lobe (consisting of the pars intermedia and neural lobe). The neural stalk, or infundibulum, comprises: (1) a stem (pediculus infundibularis); (2) a bulb (bulbus infundibularis); and (3) a rim (labrum infundibularis). The neural stalk with a portion of the lubus glandularis is usually designated as the hypophyseal stalk (Grollman, 1941).

The anterior pituitary gland was shown by Smith and Smith in 1923 to be composed of three main types of cells, chromophobes, basophiles, and eosinophiles. These investigators showed that the central zone is composed of chromophobes and basophiles, the outer area of eosinophils and chromophobes among which are scattered basophils. An injection of a saline extract of the two zones into tadpoles resulted in different physiological reactions. The tadpoles receiving the central part grew more slowly and advanced towards metamorphosis more rapidly than those receiving the outer portion. The thyroid gland was usually hyperplastic and the specimens exhibited some of the characteristics

of thyroid treated tadpoles. The tadpoles receiving the outer portion grew unusually large but had a normal body-leg proportion. However, their thyroid glands were not hyperplastic.

The actual cellular site of the origin of the anterior pituitary hormones continues to be a controversial topic, particularly with regard to the source of ACTH. One difficulty in this connection arises from the fact that ACTH appears to be a simple protein and may not be differentially detected by histochemical means. The application of the periodic acid-Schiff (PAS) technique to the glycoprotein pituitary hormones, FSH, LH, and TSH, has led to considerable advances in pituitary gland cytology. Purves and Griesbach (1951) reported that the "gonadotrophs" tend to be clustered around the terminal branches of the portal vessels on the lower surface of the rat pituitary gland, while "thyrotrophs" occupy the more central position in the gland. Both cell types were described as PAS positive basophiles. More recent work by Purves and Griesbach in 1955 has indicated that the FSH-secreting cells are situated peripherally while the LH-producing cells are more centrally located in the gland. Support for a basophilic cell origin of gonadotrophins and TSH comes from the studies of Jubb and McEntee (1955) on the bovine gland. The cytological changes in the rat anterior pituitary gland from birth to maturity were examined by Siperstein et al., (1954), which reported that degranulation of basophilic gonadotrophs occurred at puberty and that aldehyde-fuchsin staining thyrotrophs could be recognized from birth onwards while ACTH secreting cells could not be identified.

Koneff et al. (1948) reported that the most consistent changes in the anterior pituitary glands of rats injected chronically with pure growth hormone were in the acidophilic cells. They were decreased in number and size with a conspicuously decreased gramular content. This was opposed by an increase in number of the chromophobes. The function of endocrine organs is frequently depressed by the presence of an excess of their own hormonal principle. Koneff et al., using this principle, postulated that the acidophils were the source of growth hormone. Thus, the main histological change in the pituitary gland subsequent to chronic injection of growth hormone may be interpreted as a depression in the functional activity of the cells producing growth hormone, in this case the acidophils.

Halmi (1950) distinguished two types of basophils (beta and delta cells) in the pituitary gland of the rat by means of a combined aldehyde-fuchsin staining technique with a modified azan method. This method showed characteristic differences in the number, distribution, and cytological appearance of the cell when the animal was subjected to castration, thyroidectomy, hemeadrenalectomy and stress. The delta cells were shown to be less numerous in the female and became progressively hyperplastic and vacuolated after castration and thyroidectomy. Higher delta cell counts were found in the rats subjected to hemeadrenalectomy and subsequent exposure to cold, acute stress and prolonged treatment with desoxycorticosterone acetate (DOCA). The delta cells were shown to be equally numerous in both sexes, remained unaffected by castration, and tended to disappear after thyroidectomy. These cells were

hyperplastic, enlarged and partially degranulated in hemeadrenalectomized-cold exposed group. A high number of fully granulated beta cells was encountered in the DOCA treated rats. It was concluded that the delta cells were the most likely source of follicle-stimulating hormone and Thyrotrophin, and that the role of the beta cells in the formation of ACTH still was not definite.

The fundamental difficulties in evaluating the histophysiological significance of morphological findings in the pituitary gland have not yet been surmounted, regarding the site of ACTH formation. No cogent difference between the changes in the secretion of this hormone and the behavior of the three cell types has been established. Nevertheless, the beta cells remain as a possible source of ACTH. Their existence should be taken into consideration in further studies which attempt to elucidate this problem.

Purvis and Griesbach (1954), by utilizing testosterone injections, caused the disappearance of the luteinizing hormone and were able to further identify the basophils. The peripherally situated gonadotrophs were found to exhibit coarse granulation due to glycoproteins and are considered to be responsible for FSH production. The more centrally located gonadotrophs had finer granules and are considered responsible for secretion of the luteinizing hormone. In 1955, the same researchers reported on the changes in gonadotrophs after gonadectomy. Barrnett, Ladman, McAllaster and Siperstein (1956) reported that 2.5% trichloracetic acid precipitated FSH and TSH and left LH in solution which they assayed in the hypophysectomized rats. In contradiction

to Purves and Griesbach (1955), they found LH to be scattered throughout the cells. They also presented evidence that FSH and LH may be produced by the same cells.

Smelser (1944) reported that thyrotropic, adrenotropic and gonadotropic hormones are found in greater concentrations in extracts of tissue taken from the basophilic central zone, than in the more peripheral and predominantly acidophilic and chromophobic cortical portion of the beef anterior pituitary gland. It was also reported that although the ratio of basophilic material contained in preparation of central and peripheral tissue was fixed, the concentration ratios of these hormones in center:cortex was not the same. The following are examples of the ratios: gonadotropic 2:1; adrenotropic 6:1 or 8:1; thyrotropic 16:1 or 32:1. The thyrotropic, adrenotropic and gonadotropic hormones of the beef pituitary gland were presumed to be produced or stored by several distinct cell types which have different spatial distribution in the gland.

The occurrence of higher concentration of some of the anterior lobe hormones in the basophil rich central zone, and of the other hormones in the acidophilic peripheral portion of the gland has been taken as evidence that growth hormone and prolactin are products of the acidophils. Similarly, the gonadrotropic, thyrotropic, but not the adrenotropic hormone may be assumed to be derived from the basophils.

Each of the several preparations of central and peripheral anterior pituitary tissues tested should have contained a definite proportion of material derived from basophils. The concentration of the

hormones tested should be directly proportional to the amount of basophilic material contained in the extract. Therefore, the center:cortex ratio of the hormones found should be essentially the same for each location. The work of Smelser (1944) did not justify this assumption. The evidence is fairly clear, however, that each distinctive cell type of the anterior pituitary is capable of producing several different hormones.

B. Growth Hormones

a. Biological Characteristics of Growth Hormone

The work of Crowe et al. (1910), Smith (1916), Allen (1916), Evans and Long (1921), and Smith (1930) provided a very convincing cumulative argument to the effect that the anterior pituitary gland secretes a hormone possessing growth promoting activity. The most outstanding contribution on the relationship between the anterior pituitary and growth was reported by Smith (1926, 1930) subsequent to removal of the pituitary gland (hypophysectomy) in the growing rat. Hypophysectomy in the immature rat resulted in a discontinuation of growth and dwarfism. The final proof for the existence of growth hormone was furnished when a highly purified growth promoting principle was isolated (Li et al., 1945; Wilhelmi et al., 1948) from alkaline extracts of bovine pituitary glands.

1) Effect of growth hormone on body growth

The most readily observed result of the injection of growth hormone into either normal or hypophysectomized animals is the effect on

body weight, a phenomenon which represents a selective activity of growth hormone (Li and Evans, 1947). The name "growth hormone" further implies that it selectively increases the body growth, independent of its effect on other endocrine organs. It was found that growth hormone extracts were equally active in adrenalectomized animals (Simpson et al., 1944) and in animals which were completely thyroidectomized (Scow and Marx, 1945). It has also been observed that the gonadal hormones are not required for the action of growth hormone (van Wagenen, 1928; Evans and Simpson, 1931). Growth hormone, therefore, presumably has an anabolic action independent of the participation of other endocrine tissues.

There has been considerable discussion on the ability of growth hormone to cause continuous growth in normal or in hypophysectomized rats. Long (1943) stated in regard to this developed resistance that

> In the first experiments reported by Evans and Long the rats were injected daily for as long as 8-13 months with a crude alkaline extract. Growth was not as rapid in the later period as at first. Nevertheless, it was continuous throughout the period of injections. Later attempts to repeat this experiment, even in the same laboratory, showed that, after an initial period of brisk growth, the animals became refractory to the extract and even lost some of their gained weight. The same results were also obtained in hypophysectomized animals but even more discouraging was the fact that partial purification of the extract did not correct this decreased responsiveness.

Since practically all work prior to Long's research were carried out with only partially purified growth-promoting extracts, it is naturally difficult to decide if the results were due to growth hormone itself or to other contaminating proteins.

Evans et al. (1946) injected daily increasing dosages of .4 mg to 2.0 mg of highly purified growth promoting preparations into normal adult female rats for 435 days. Growth continued during the whole period with the experiment terminating due to the advanced age of the animals. The greatest weight attained was 662 gms. The range of final weights in the experimental control groups did not overlap; the smallest experimental rats weighed 410 gms and the largest control animal was 353 gms. The average gain of the eight experimental rats was 293 gms, while that for the control animals was 57 gms. The liver, heart, kidneys, stomach and intestine increased in weight in proportion to body weight. The thymus gland was not hypertrophied as occurs upon acute administration of growth hormone. The other endocrine organs were not increased proportionally to body weight, as might be anticipated by purified growth hormone preparations.

A similar experiment with hypophysectomized female rats, 26-28 days of age, and 12-14 days postoperative, also indicate that the pure growth hormone is capable of inducing continuous growth. There was no evidence of refractoriness with over 400 days of injection.

2) Growth hormone's effect on nitrogen retention

True growth is generally interpreted as the accumulation of intracellular proteins. It is therefore reasonable to expect that an important anabolic function of growth hormone is to retain protein nitrogen which in turn increases the protein content of the body tissues. Earlier reports (Teel and Watkins, 1929; Gaebler, 1933; Harrison

and Long, 1940) have shown that growth-promoting pituitary extracts cause a reduction in both the blood non-protein nitrogen and urinary nitrogen. Later experiments (Marx et al., 1942; Fraenkel-Conrat et al., 1943) with partially purified growth hormone have confirmed these conclusions.

The changes in body composition of animals after treatment with pituitary extracts have been studied by several workers (Downs, 1930; Wasehn, 1932; Bierring and Nielsen, 1932; Lee and Schaffer, 1934). The results of these investigations indicate that the weight gain of treated animals is due to an increase in protein and water content and a decrease in the fat constituents of the body. Kleiber and Cole (1939), however, did not find significant differences in the ash, fat or protein content of injected and control rats. The same investigators have also studied the metabolic rate in rats made gigantic by chronic administration of growth-promoting extract. The metabolic rate per unit body weight was reported to be less in injected animals than in the controls. The metabolic rate per unit dry weight of the giant rat diaphragms in vitro was also lower than that of the controls. These changes in the metabolic rate make their reported lack of alteration in water or protein content of the animals all the more surprising.

Although all the experiments cited were performed with crude or partially purified preparations, it appears likely that the growth-promoting activity of pituitary extracts is accompanied by a reduction of urinary nitrogen and an increase of body proteins. These changes

may be interpreted as being caused by either an increase of protein anabolism, a decrease of protein catabolism, or both. The experiments of Fraenkel-Conrat et al. (1943) showed that purified growth hormone causes a decrease in the liver arginase content indicating that the purified hormone could inhibit protein catabolism.

Recent studies with the pure growth hormone have shown that the hormone markedly reduces the urinary nitrogen under various conditions. In some cases, the lowering of urinary nitrogen corresponds almost quantitatively to the gain in body weight (Li et al., 1954). In the normal rats with a constant diet containing 24% casein, the injection of growth hormone induced a significant lowering of the urinary nitrogen within 24 hours. It will be seen that rats made diabetic by alloxan also retain nitrogen after growth hormone treatment. The nitrogen-retaining effect of growth hormone has also been demonstrated in rats with bilateral fracture of the femur (Bennett et al., 1946). Normal and injured rats given growth hormone showed approximately the same decrease in nitrogen excretion when compared with their respective controls.

3) The effect of growth hormone on the epiphyseal cartilage

It has been mentioned previously that growth hormone brings about a specific stimulation of the epiphyseal cartilages in hypophysectomized rats. The first change observed in animals after hypophysectomy (Becks et al., 1945) is a marked thinning of the epiphyseal cartilage plate, due to a decrease in the number and particularly the size of the cartilage cells. Becks et al. (1946) showed that administration of

growth hormone to hypophysectomized rats, even after postoperative intervals of a year or longer, was able to reawaken chondrogenic and osteogenic processes in the epiphyseal cartilage of the tibia to an extent comparable in normal, young, growing rats. It was noted that animals receiving growth hormone had enlarged cartilage cells in the juxtamedullary zone and in a wide zone of newly-formed, delicate trabecular bone. There are numerous and active osteoblasts at the margin of the cartilage and along the surface of the trabeculae. These workers stated there could be little doubt that growth hormone has a direct influence on bone growth.

The relation of phosphatase to bone regeneration was demonstrated by Wilkens and Regen (1935). In the growth of normal rats, a rise in plasma phosphatase activity from birth to maturity was observed by Weil (1941). One could, therefore, expect that growth hormone would increase the phosphatase content in the tissues of normal or hypophysectomized rats. Results of Mathies and Gaebler (1949a, 1949b) indicate that the plasma phosphatase content of hypophysectomized male rats is more than double the content of control rats, after treatment with as small a dose as .05 mg of growth hormone daily for 14 days. This increment in the phosphatase content suggests some relation of the enzyme to the process of bone formation.

b. Chemical and Physical Characteristics of Growth Hormone

A growth hormone preparation, from hypophyseal tissue and of high purity, was first made by Li, Evans and Simpson in 1945. An improved method, yielding much larger quantities of a crystalline product

having a similar order of purity, was described in 1948 by Wilhelmi, Fishman and Russell. Ox pituitary glands were the source material for both growth hormone preparations which have been studied most extensively and used widely in work on the biological activity of growth hormone. During the past few years many attempts have been made to purify further the products isolated by the published procedures of Li and Evans (1945).

The bovine growth hormone is a protein with a molecular weight of 45,757. The value was calculated by the acid paper chromatography method of the dinitrophenyl derivatives of the amino acids (Li and Chung, 1956). The molecular weight determination differs somewhat from that found by osmotic pressure determinations (Li et al., 1945) where the molecular weight was estimated to be 44,250. From sedimentation data, as well as from the diffusion coefficients which were determined in a Spinco electrophoresis-diffusion apparatus, and from values for partial specific volume which were computed from preliminary amino acid data (Li, 1956), the molecular weights for human and monkey growth hormone were calculated to be 27,100 and 25,400 respectively. Li et al., (1945) found the isoelectric point for bovine growth hormone to be 6.85. This value was obtained from electrophoresis measurements. Li and Chung showed that the protein nitrogen could be accounted for completely by its amino acid and amide content.

Li (1956) reported that growth hormone consists of a branched polypeptide chain with two N-terminal residues (phenylalanine and alanine) and only one C-terminal residue (phenylalanine), and a total of 396 amino acids. The N-terminal residues were determined by means

of both the fluorodinitrobenzene and phenylisothiocyanate procedures, and the N-terminal amino acid sequence was established by the isolation and identification of DNP-peptides from partial acid hydrolysates of DNP-somatotropin. The C-terminal amino acid sequence was elucidated by submitting the hormone to the action of carboxypeptidase (Marris et al., 1954).

According to Li (1957) cleavage of the -S-S bridges should not give rise to two fragments because the branched chain is probably derived from a peptide linkage involving the amino group of a lysine residue. Li stated that studies of the product obtained by the performic acid oxidation of the protein hormone were in agreement with the proposed structure.

When the bovine growth hormone, from which two residues of phenylalanine had been removed by carboxypeptidase, was assayed for biological activity, it was found that the C-terminal phenylalanine was not essential for the biological function of the hormone (Harris et al., 1954).

It was found by Li et al. (1956) that when the hormone was subjected to chymotriptic hydrolysis to approximately 25% no inactivation occurred, but longer digestion did abolish the biological potency. It was noted that if an active digest is desired, the degree of hydrolysis must not be permitted to exceed 30%. The non-protein nitrogen was separated from the whole digest either by dialysis or by treatment with a 5% solution of trichloroacetic acid. It was demonstrated by biological

assay that the growth-promoting activity resides in the remaining core, which was non-dialyzable and insoluble in the trichloroacetic acid solution. When the results of a multiple-dose assay of the core were compared with those of a similar assay of the undigested hormone, statistical analysis of the data showed that there was no significant difference between the core and the untreated material with respect to growth-promoting activity.

Boundary electrophoresis of the core has given no indication of a component which behaves like the untreated bovine hormone. Analysis of the N-terminal residues of the core revealed a number of new residues (threonine, serine, tyrosine, lysine, and etc.), in addition to the phenylalanine and alanine. If the terminal phenylalanine and alanine residues have come from the undigested bovine hormone, it can be estimated that the native hormone in the core amounts to less than 20%, a percentage certainly not sufficient to account for the biological activity of the core. From the results of Li (1956) it may be concluded that the activity does not depend upon the integrity of the bovine protein, and that the growth-promoting activity resides in a center (or centers) of activity in the molecule.

Many attempts have been made to find the effectiveness of bovine growth hormone in man but they have met with disappointment. One of the obvious explanations for this failure is that the bovine growth hormone is chemically different from the primate hormone. Indeed, it has been shown that the growth hormone prepared from fish

pituitary glands is active in fish (Pickford, 1954) but not in rats; and monkeys are not responsive to bovine growth hormone, whereas they are responsive to growth hormone prepared from pituitary glands of their own species (Knobil et al., 1956 and Knobil et al., 1957). Recent investigations with monkey and human pituitary glands indicate that the human and monkey growth hormones are similar in structure and properties, but that they both differ completely from the hormone molecule isolated from the pituitary glands of cattle. Thus, from all indications, growth hormone appears to be specific in its chemical structure for each species as well as varying in physical characteristics.

c. Bio-assay Techniques Used in Assaying Growth Hormone

For years the literature was deficient in material relating to the bio-assay of growth hormone, due to the lack of a suitable purified material for standardization. With the isolation and purification of pituitary growth hormone by Li et al. (1944, 1945) a standard preparation became available which permitted the development of various bio-assay techniques. The bio-assay techniques were based upon anabolic responses of the animal, either at the tissue level or involving demonstrative changes in body weight or conformation.

The acceptable biological assay techniques for growth hormone based on the increase in body structure are performed on normal plateaued rats (Marx et al., 1942), hypophysectomized rats (Marx et al., 1942) or dwarf mice (Dobbs et al., 1936). The assays used in the detection of growth hormone involving changes in bone structure are the increase in

the tail length of hypophysectomized rats (Dingemanse et al., 1946, 1948) and the increase in width of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats (Greenspan et al., 1942).

There have been several body changes observed following the injection of growth hormone which involve chemical and enzymic changes in specific tissues. Various assay methods have been suggested for the bio-assay of growth hormone based upon the tissue's reactions brought about by growth hormone. The suggested methods involve an increase in either liver weight or body weight of stilbestrol-treated rats or the incorporation of labeled amino acids into body protein (Fridberg and Greenberg, 1947). Other methods involved changes in serum phosphorus and phosphatase (Li et al., 1947) or in nitrogenous constituents of the blood (Gaebler, 1933), or any change in the nitrogen or phosphorus balance.

The problem of the selection of "most satisfactory" assay procedure for growth hormone is difficult. Each of the established procedures has its advantages and limitations, its proponents and opponents. In general it has been found that the tibia test in the hypophysectomized rat is the most sensitive index of growth hormone activity. It has the obvious disadvantage in that the assay animals require an operative procedure resulting in fatality of a large number of animals. On the other hand, the plateaued rat weight assay is equally as accurate, but large amounts of the hormone are required due to the long period of injections of the material to be assayed. The plateaued rat weight assay has the advantage in that the animal requires no operative procedure.

The assay procedures involving the increase in weight and the increase in tail length of hypophysectomized rats have the disadvantage in requiring the use of an animal which has first undergone an operative procedure. The operative procedure requires considerable time to develop and a large number of fatalities is always experienced among the animals. The limitations of the weight change assay in the dwarf mouse are primarily concerned with the difficulty in maintaining the strain of animals over long periods of time due to their delicacy. In addition, the order of accuracy of the method is low when short term injection periods are used.

The tibia assay method for the quantitative determination of growth hormone is the most sensitive, requires the shortest period of injection and has the highest degree of specificity. The tibia method was used in this thesis and therefore warrants a more detailed discussion.

1) Bio-assay of growth hormone by the increase in width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats (tibia test)

Hypophysectomy causes regressive changes at the proximal end of the tibia in the immature rat. Within a few days, the thickness of the cartilage plate decreases significantly, a consequence of a reduction in cartilage formation and its active destruction. Administration of growth hormone reverses these regressive changes. The initial effects of growth hormone treatment consist predominantly of chondrogenesis. Activation of esteogenesis follows if treatment is prolonged. The width

of the cartilage disc increases during the first 6-8 days of treatment with growth hormone until the normal equilibrium between chondrogenesis and osteogenesis is re-established. During the initial period of growth hormone administration the resulting increase in width of the cartilage is, within certain limits of dosage, proportional to the quantity of growth hormone injected.

Based on this phenomenon, a method was developed (Greenspan et al., 1943) for a bio-assay of growth hormone which consists of the following procedure: Immature female rats are hypophysectomized when 26 days old. After a postoperative interval of 12 days, the preparation to be assayed is dissolved in saline and injected intraperitoneally once daily for four days. Twenty-four hours after the last injection the animals are autopsied, the right tibia taken, freed from soft tissue, split at the proximal end in a sagittal plane, and fixed in formalin, or stained immediately, after thorough washing with water and acetone, with silver nitrate. The silver nitrate stains all the calcified areas of the tibia a dark brown, leaving the epiphyseal cartilage a light color. The epiphyseal cartilage is then measured under a low powered microscope using a calibrated eyepiece.

It was reported that a value of 10 micra represented a statistically significant difference and twice this value was selected as the basis for the minimal significant response. Therefore, the minimal effective dose (MED) of a growth hormone preparation is defined as the amount given under the conditions cited which causes an increase in width of the proliferating zone of the cartilage over the control width

of 20 micra. One growth hormone unit is defined for this test method as the equivalent of the quantity of growth hormone causing this same effect.

d. Synergistic and Antagonistic Effect of Other Hormones on the Bio-assay of Growth Hormone

The value of a bio-assay must be considered in terms of the factors influencing the bio-assay and therefore the interaction of a number of hormones. On the other hand, if the conditions of the bio-assays of growth hormone are maintained, growth hormone is the only substance which will cause continuous increase in tissue growth or demonstrative changes in body weight or conformation. Among the hormonal substances which will cause a transitory or slight increase in body weight of the assay animals are testosterone, lactogenic hormone and thyroxine. In addition, several hormones will synergize or antagonize the activity of growth hormones. Thyroxine and growth hormone will induce a larger increment in growth than growth hormone alone (Smith, 1933; Evans et al., 1939). Adrenocorticotrophic hormone will antagonize the effect of growth hormone (Evans et al., 1943; Li and Evans, 1947). It is important therefore, in the bio-assay of growth factors to determine the degree of contamination of the other hormones in order to assess the possibility of synergism or antagonism in the resultant physiological action.

C. ACTH

a. Source of ACTH

The content of ACTH in the pituitary glands of various species differs considerably. There appears a decrease in the glandular content

of the hormone according to the following order: (1) pig, (2) man (Burns et al., 1949), (3) sheep (Li et al., 1951), (4) whale (Holtermann, 1951), (5) rat (Gemzell et al., 1951), (6) ox (Dedman et al., 1952), and (7) fish (Rinefret and Hane, 1955). Pig pituitaries have a potency of 200 IU per gram of dry weight, while sheep pituitaries are only one-fourth as potent. Pig and sheep pituitary glands are commonly used for the preparation of concentrated ACTH for experimental and chemical use. They may be desiccated with acetone or by lyophilization which produces a storable dry powder. There is no loss in ACTH activity in desiccated pituitary preparations stored for periods up to three years.

ACTH activity has been assayed in human serum and placenta as well as in the plasma of rats and dogs. It has been reported that human plasma possesses an ACTH potency as high as .2 IU per 100 ml (Bornstein and Trewhalla, 1950). Human placenta has been estimated to have an ACTH activity of 3 IU per 100 grams of dried chorionic villi (Assali and Hamermesz, 1954). No ACTH activity was detectable in the plasma of normal rats, but three weeks after adrenalectomy the ACTH content in 100 ml of plasma was found to be .2 IU (Gemzell et al., 1951).

Nellor (1958) detected the presence of ACTH activity in as little as 30 cc of bovine plasma.

b. Biological Characteristics of ACTH

It now seems well established that the adrenal cortex secretes a mixture of biologically active steroids, and that secretion of these

steroids is under the control of higher brain centers. In all species studied, by either in vivo or in vitro techniques, the rate of secretion of corticosteroids from the adrenal gland is greatly increased in the presence of ACTH. The first direct evidence of the ability of ACTH to stimulate corticosteroidogenesis under in vitro conditions was demonstrated in the isolated cow gland perfused with citrated blood (Hechter et al., 1951) and later was observed in sliced adrenals of rats (Saffran and Bayliss, 1953). By means of these techniques Hechter and Pincus demonstrated in 1954 that, as its major in vitro effect, ACTH stimulated corticosteroidogenesis. Rauschkolb et al. (1954) produced a significant increase in the rate of secretion of 17-hydroxycorticosterone within two minutes after the intravenous injection of ACTH into hypophysectomized dogs.

ACTH is also capable of altering the pattern of corticosteroid secretions. This is thought to be caused by an increase in the synthesis of enzymes which are involved in the biosynthetic process of corticosteroid formation. Hechter and his collaborators (Kass et al., 1954) reported that the initial administration of ACTH causes an increase in the output of corticosterone from the adrenal vein whereas, after a few weeks of injections with ACTH, the chief secretory product changes to 17-hydroxycorticosterone. This work was done on rabbits and implies that chronic treatment with ACTH increases the activity of the 17-hydroxylating enzyme in the adrenal gland. Since it is well known that ACTH is the specific agent for the growth and development of the adrenal cortex, and since this growth process involves protein synthesis,

it should not be surprising that the concentration of a specific enzyme should likewise be affected by prolonged stimulation with the hormone. In this connection, Ganis et al., (1955) showed that ACTH promotes protein synthesis in the adrenal gland when isolated cow adrenals were perfused with homologous blood containing radioactive lysine and other amino acids with an added ACTH preparation.

In addition to corticosterone and hydrocortisone, the secretion of other biological steroids from the adrenal gland is also stimulated by ACTH. In dogs, the concentration of 11-deoxycorticosterone in adrenal venous blood is markedly increased following intravenous injection with ACTH (Farrell et al., 1954). An increase of aldosterone secretion of ACTH stimulated adrenal glands of hypophysectomized rats has been reported (Stack-Dunne and Singer, 1954). By indirect evidence (Lyons et al., 1953) it has been demonstrated that secretion of progestins from hypertrophied adrenals of hypophysectomized rats is elicited by treatment with ACTH, although no such steroid has been encountered in the nonstimulated gland. There is also evidence (Li, 1956) that the secretion of androgens by the adrenal cortex in experimental animals is stimulated by the action of ACTH.

The basic mechanism whereby ACTH promotes biosynthesis of corticosteroids is unknown. It has been suggested that the influence of ACTH upon corticosteroid biosynthesis may be concerned with increasing the transfer of cholesterol through the mitochondrial membrane, to make cholesterol available for the enzymatic apparatus inside this organelle (Hechter, 1955). This mechanism implies the assumption that

ACTH is involved in a single step (Cholesterol-pregnanolone) in the sequence of corticosteroidogenesis. Although the hypothesis is an attractive one, no experimental data to the writer's knowledge has been reported to support or disprove this view.

The structural and functional effects elicited by ACTH in the reproductive system of male rats is variable and, obviously, involves factors which are not understood at the present time. With doses ranging from 1 to 3 mg daily, administered by intermittent subcutaneous injection, there occurs a rather consistent involution of the seminal vesicles concomitant with a reduction in number and size of the Leydig cells in the testicular interstitial tissue. Since the Leydig cells are considered to be the source of androgen, it is assumed that in these experiments ACTH retards the synthesis, or secretion, of the pituitary gland's interstitial-cell-stimulating hormone or antagonizes the action of this hormone at the end organ (Baker et al., 1950). In contrast, no significant interference with the production of spermatozoa in the seminiferous tubules was observed following ACTH administration.

Subsequent studies revealed that higher doses of ACTH fail to intensify the involution of the seminal vesicles as logically might be expected. In fact, under these circumstances, the response of the testicular Leydig cells and seminal vesicles is much more irregular. Administration of 6 mg of ACTH daily by continuous injection to some animals fails to cause atrophy of the seminal vesicles. Likewise, the Leydig cells may remain prominent and in some cases appear more numerous than those of the controls. However, this treatment did induce a

profound degeneration of the germinal epithelium of the seminiferous tubules. This response varied greatly from rat to rat and also in different tubules of the same animal. Alongside the involuted tubules were other tubules in which production of spermatozoa was maintained. Thus, some seminiferous tubules, or portions of the tubules, are more resistant to the damaging effects of ACTH (Baker et al., 1950).

This variability of the male reproductive system in response to ACTH injection is carried over to its use in hypophysectomized animals. In some rats the involution of the testes which follows hypophysectomy is intensified, whereas the seminal vesicles of other animals are stimulated sufficiently to maintain their normal histology. A possible contamination of the ACTH extract with gonadotrophic hormones could account for the unpredictable effect elicited by ACTH. The demonstration of traces of interstitial-cell-stimulating hormone in some ACTH preparations lends evidence to this belief (Asling et al., 1951). However, the varying responsiveness of animals must be an important factor, since a single preparation does not always have the same effect.

c. Chemical and Physical Characteristics of ACTH

Early in this century, Evans (1923), working with growth-promoting substances, observed that these substances caused adrenal hypertrophy when injected into animals. Smith (1926) reported that hypophysectomy of the rat produced a marked atrophy of the adrenal cortex. It was found that this degenerative change could be prevented, or the normal condition restored, by intramuscular implantation of fresh rat pituitary glands.

This work led to the discovery of a distinct hormonal secretion of the pituitary gland with a trophic influence on the adrenal cortex. By 1933, the specificity of pituitary-adrenal interrelationship was firmly established.

It was a decade later, however, before the techniques of protein fractionation had advanced sufficiently to permit much progress toward the preparation of active concentrates of adrenocorticotrophin (ACTH). Li, Evans, and Simpson (1943) and Sayers, White, and Long reported in 1943 the isolation of what appeared to be pure proteins having ACTH activity. The former group used sheep pituitary glands whereas the latter group used hog pituitaries. Both preparations appeared to have similar physical and chemical characteristics. It was noted that several years before these reports on the homogenous protein, Anselmino (1944), Collip (1937), and later Astwood and Tyslowtiz (1942), Cooke et al. (1948), and Cortis-Jones et al. (1950) had reported the preparation of physiologically active extracts by an ultrafiltration technique which normally would yield only polypeptides or proteins of low molecular weight. Li (1948) reported that 50% of his protein could be digested with pepsin without loss of activity, and in 1947 he reported acid hydrolysis of the protein material yielded an active material having a molecular weight of approximately 1200. Morris and Morris (1950) and Lesh et al. (1950) reported polypeptides having from 10 to 120 times the potency of the earlier protein products. The latter group felt that the molecular weight was between 2500 and 5000.

Brink et al. in 1952 announced the isolation from pepsin-hydrolyzed sources of what appeared to be a pure polypeptide having approximately 300 units of activity per mg. The steps used in the isolation of this protein consisted of oxycellulose adsorption, pepsin digestion for 24 hours at pH 2.5 and 37° C, trichloroacetic acid precipitation, and a 200 transfer counter-current distribution in the S-butanol/0.5% aqueous trichloroacetic acid system. The end product was converted to the acetate by means of Amberlite IRA - 400 resin. The resulting substance was designated corticotrophin-B since its solubility behavior in the counter-current distribution system was different from that of the purified unhydrolyzed corticotrophin as reported by Astwood et al. (1951). A more detailed report of this isolation was given by Kuehl et al. (1955), in which it was noted that inactive corticotrophin had the same distribution pattern as did the highly potent material. The similarity in behavior between active and degraded preparations had been previously reported in 1951 by White.

In 1953 White announced the isolation of an apparently pure product from unhydrolyzed materials and designated it corticotrophin-A, since it appeared that corticotrophin-B may be derived from it. The steps used in the isolation were: oxycellulose adsorption, chromotography on XE-97 resin and counter-current distribution in a system of 2-butanol/0.2% aqueous trichloroacetic acid.

With respect to molecular weight, little is known as yet concerning ultracentrifuge value for corticotrophin-A. However, the molecular weight of corticotrophin-B is reported (Brink et al., 1953) as being 5200

by direct measurement and 6000 to 7000 when calculated from molar amino acid ratios. It should be mentioned that perhaps the pH at which the molecular weight was determined experimentally may have considerable influence upon the results obtained. In this regard, the dialyzability of corticotrophin concentrates at low pH and lack of the same ability at higher pH was described by Morris and Morris (1950) and independently noted elsewhere (Hays and White). This suggests that there is formation of a polymeric form at high pH.

When intermolecular bonding was eliminated the value corresponding to the molecular weight was approximately 2300 (Hays and White).

While complete electrophoretic data for corticotrophin-B has not been reported, it has been stated by Richter et al. (1952) that in one run on a purified preparation, there appeared two components with isoelectric points above pH 4.5. Using paper electrophoresis the isoelectric point appears to be in the range of pH 7.8 for corticotrophin-A. By using the same technique corticotrophin-B appears to have an isoelectric point near pH 10.

Both of the corticotrophins show the characteristic ultraviolet absorption spectra of proteins containing aromatic amino acids. Assuming that none of the tyrosine or tryptophane is lost in the conversion of corticotrophin-A to corticotrophin-B the relative absorption values indicate a reduction of 25% in the molecular size.

When the apparently pure corticotrophin-A became available it was subjected to a modification (Landman et al., 1954) of the thiohydantoin method of N-terminal amino acid analysis. It was found that only one

thiohydantoin was present which corresponded to serine. An application of the process to the residual peptide gave the thiohydantoin corresponding to tyrosine. This gave indication of the sequence: -Ser.tyr.-- Continuing on into the chain it was felt that the next two positions were occupied by histidine and phenylalanine. Using carbosypeptidase to determine the C-terminus of corticotrophin-A, phenylalanine was released first, followed by glutamic acid and leucine. Thus, the sequence --Pro.Leu.Glu.Phe.--. Additional evidence for this sequence has been obtained by the isolation of the tetrapeptide: Pro.Leu.Glu.Phe.-- from the products of the peptic digestion of corticotrophin-A.

Because of the specific requirements of carboxypeptidase, it was not possible to conclude from this work that corticotrophin-A is made up of a single unbranched chain. Such evidence must come from the application of other techniques.

d. Assay Methods of ACTH

1) The repair test

It is well known that the pituitary gland influences the size and function of the adrenal cortex. The first evidence for the occurrence of an adrenocorticotropic substance in pituitary extracts was given by Smith (1930), and it appears that, although hypophysectomy decreases the size of the adrenal cortex, it is without effect on the medulla. The isolation of the adrenocorticotropic hormone in pure form has been achieved independently by two laboratories (Li et al., 1942b; 1943; Sayers et al., 1943).

The adrenal weight in rats or chicks has been proposed for the estimation of ACTH potency. Collip and co-workers (1933) suggested the removal of one adrenal from the hypophysectomized rat as the control, the weight of which was then compared with the weight of the remaining adrenal after the animal had received an ACTH extract. Moon (1937a) employed the 21-day-old male rat as the experimental animal, injected ACTH intraperitoneally once daily for three days and the weight of the adrenals on the fourth day was compared with that of uninjected controls. Later, Moon (1940) reported the use of 4-day-old suckling rats; the method seems to be more sensitive but it has a disadvantage in that it is difficult to inject crude extracts or those containing toxic substances into young animals. Bates et al. (1940) used the increment in adrenal weights in 2-day-old chicks for assaying ACTH preparations. This method has been found to be unsatisfactory.

The repair (Simpson et al., 1943) or maintenance (Simpson et al., 1943; Astwood and Tyslowitz, 1942) of the adrenal glands of hypophysectomized rats has been used for the standardization of ACTH.

2) Histological assay

The histological change in the adrenal cortex of rats after hypophysectomy is characterized by the appearance of a specific sudanophobe zone (Reiss et al., 1936). This is due mainly to the change in distribution of lipids; the lipids also become large and more irregular in size in the hypophysectomized animals. The repair test is thus based on the ability of ACTH to amend these changes. Female rats, 26-28 days of age are hypophysectomized and the adrenal glands allowed to regress

for 14 days, by which time injections were instituted and continued for four days, once daily intraperitoneally, followed by autopsy 96 hours after the first injection. The adrenals were fixed in formalin, cut as frozen sections, and stained with Sudan Orange. The slightest observable effect of pure ACTH causes a beginning redistribution of the lipids. This minimal effective dose lies somewhere between .01 and .025 mg.

3) The maintenance test

This assay is based on maintenance of the weight of adrenals by the institution of ACTH injection immediately after hypophysectomy. Male rats 40 days old are hypophysectomized and injected intraperitoneally daily (except Sunday) from the day of operation for 15 days (14 injections). The adrenal weight of uninjected hypophysectomized animals regressed during this period from 26 mg to a constant weight of 12 mg. The amount of pure hormone which maintains the adrenal at 26 mg is about .2 mg daily dose. It must be emphasized that this value was obtained from single daily injections. It is known that multiple daily injections of ACTH produces a better adrenal response than from a single dose. It has been shown that the sensitivity of the method is influenced by the strain of rats employed. When the adrenal size is expressed per 100 gms of body weight, assays in different laboratories may be satisfactorily compared (Emmens, 1950).

4) Ascorbic acid and cholesterol depletion as a bio-assay

Sayers et al. (1944, 1946) reported that a single dose of ACTH causes a decrease of cholesterol in the adrenal gland of normal

rats and guinea pigs within a period of a few hours. The cholesterol level tends to return to normal 24 hours after hormone treatment. In hypophysectomized rats, similar effects on adrenal cholesterol occur if ACTH injection is started three days after the operation; the decrease in adrenal cholesterol content cannot be observed after a longer postoperative interval. It is significant that the fall of cholesterol is accompanied by a rise in liver glycogen.

Similar studies have been made on the ascorbic acid content of the adrenal gland (Sayers et al., 1946). It was found that the injection of ACTH into rats and guinea pigs produces a prompt fall in adrenal ascorbic acid. The return of adrenal ascorbic acid in the rat to a normal level is quite rapid but the level in the guinea pig remains subnormal even 24 hours after the injection.

Based on the above phenomenon Sayers, Sayers and Woodbury (1948) established an assay procedure based on the depletion of ascorbic acid from male hypophysectomized rats. These workers showed that this action is highly sensitive to the presence of ACTH. The depletion is expressed as the difference between the concentration of the ascorbic acid in the left adrenal, removed immediately before hormone injection, and the concentration of ascorbic acid in the right adrenal, removed one hour after the intravenous injection. A rectilinear relation exists between this depletion and the logarithm of the dose over the range of .15 to 2.5 mg of a highly purified preparation of ACTH.

e. Antagonistic Effect of Growth Hormone on the Bio-assay of ACTH

Using purified ACTH extract, Moon (1937b) found that it caused a retardation of the somatic growth of young castrated male rats. Evans et al., (1943) using a pure ACTH preparation observed a similar effect on the growth of normal as well as gonadectomized male rats. It was further shown that this effect disappeared if adrenalectomized rats were used. These results are in harmony with the findings of Ingel et al. (1938) who have reported that certain adrenocortical substances adversely affect growth, and that ACTH increases the output of adrenocortical substances.

When ACTH is injected simultaneously with growth hormone into hypophysectomized rats a counteraction exists between these two substances (Marx et al., 1943). It was found that the growth promoting activity is antagonized by the action of ACTH. This antagonism may be demonstrated both by the measurement of body weight increase and by the degree of proliferation of the proximal epiphyseal cartilage of the tibia.

The treatment of normal rats with ACTH results in a retardation chondrogenesis and osteogenesis in the region of the proximal epiphysis of the tibia (Becks et al., 1944a). Comparisons have been made of the proximal epiphyseal regions of the tibia of hypophysectomized rats when injected with ACTH, with growth hormone and with the combination (Becks et al., 1944b). ACTH administered alone can further modify the inactive condition of the epiphysis of hypophysectomized animals,

whereas growth hormone always causes a resumption of activity. When ACTH was administered concordantly with a known effective dose of growth hormone, the following effects were observed (Li and Evans, 1947):

(a) The proximal epiphyseal cartilage of the tibia was greatly decreased in width when compared with the width after growth hormone was added.

(b) Endochondral bone formation was significantly retarded.

(c) Osteoblastic as well as osteoclastic activity was greatly decreased, perhaps accounting for the irregular arrangement of bony trabeculae.

(d) The cartilage columns in the erosion zone were also more irregular.

Adrenocorticotropic hormone, therefore, may be regarded as a specific growth-inhibiting substance. A complete explanation of its action is not available, but there is no doubt that ACTH adversely influences certain metabolic reactions that promote growth. For example, Gordan et al., (1946b) have found that ACTH causes an increase in urinary nitrogen excretion with proportionate loss of body weight in the normal rat. The effect was manifested on the second day of the hormone administration and persisted for 24 hours after the injections were terminated. Fraenkel-Conrat et al. (1943) showed that liver arginase is increased by the administration of ACTH as well as by certain adrenal cortical substances, whereas growth hormone induces a decrease of the original activity.

Li et al. (1946) reported that ACTH reduces the alkaline phosphatase content in the plasma of both hypophysectomized and normal rats and the effect is neutralized by growth hormone injections (Li and Evans, 1947).

METHODS USED IN THIS STUDY

A. Fractionation Procedure

Several preliminary blood fractionations were conducted on approximately 300 cc samples of bull blood in order to perfect fractionation methods. Preliminary studies were considered completed when fractions were obtained from split samples of blood of comparable protein concentration, ionic strength and electrophoretic similarity.

Approximately 12 liters of blood were drawn from each of two bulls at slaughter, one Holstein bull 14 years and the other a 20-month old Holstein bull. The blood was obtained in buckets which had been previously oxalated. It was then placed in a Servall model S4 centrifuge and spun at 4-5000 r.p.m. for 20 minutes to remove the blood cells. The plasma was drawn off and fractionated in 300 ml aliquets.

The fractionating procedure was a modification of that used by Cohn et al. (1950). It consisted of bringing all components to a relatively inert solid state as rapidly as possible and maintaining them insoluble at about 2° C until separation from each other and from the enzymes for which they were the substrate. Separation of a pure component, or group of components, by fractional extraction of a precipitate has the advantage in that the material which remains insoluble is protected from the various changes, chemical and enzymatic, which occur rapidly in solution. The greater stability of proteins in the solid state has long been recognized. The system used was devised so that many

separations, accomplished by fractional extraction, followed the initial precipitation. In this way each component remained continuously in the solid state during procedures undertaken to dissolve a particular class of proteins.

Fractional extraction can be carried out more rapidly than fractional precipitation since a shorter time of equilibration is found adequate. Since the ethanol concentration need not be initially lowered in order to permit complete solution of a precipitate, less concentrated ethanol may be added to achieve the final condition. Equally important, the use of less concentrated ethanol allows a very high rate of mixing of the suspended precipitate with the precooled reagent, both because temporary exposure to higher concentrations of ethanol is avoided, and because very little heat of mixing need be dissipated.

Three hundred ml of plasma was placed into a 2-liter flask and the flask itself placed in an alcohol bath at -5° C. This was followed by the plasma being separated into two main fractions by the addition of an alcohol solution. Each fraction was then subjected to further subfractionation to yield highly purified components. In this initial step 1200 cc of a precooled solution containing 250 cc of 95% ethanol and 2.5 cc of .8 $\Gamma/2$, pH 4 acetate buffer per liter, were added through a 14 gauge needle while the plasma was being continuously stirred with a magnetic stirrer. Some of the albumins, together with most of the gamma globulins and $beta_2$ globulins were thus separated from the other components of the plasma by taking advantage of the solubility of their sodium salts which were formed in this solution. The pH of the final

solution was 5.8± .2 and the ethanol concentration was 20%. This first precipitate was designated as fraction I+II+III.

The albumins, together with certain alpha and $beta_1$ globulins which remained in solution following the first precipitation from plasma, were precipitated by the addition of zinc to the solution but without any change in pH, temperature, or ethanol concentration. This was accomplished by the addition of 120 cc of a freshly prepared, precooled solution containing 200 cc of ethanol and 54.8 grams of zinc acetate per liter of solution. This fraction was equivalent to approximately 30% by weight of all the total proteins present in the plasma. This fraction was designated as precipitate IV+V. The supernatant which resulted from the above precipitate was designated as solution IV and was composed of less than 1% of all the total proteins. Electrophoretically, fraction VI appeared to be composed of gamma globulins only.

The fractions obtained were brought to the dry state by lyophilizing and then stored at 2° C.

Further subfractionation of precipitate IV+V involved removal of the serum albumins by taking advantage of the insolubility of the barium and zinc salts of the alpha and $beta_1$ globulins at a pH of 5.5 and an ethanol concentration of 15.2%. This solution was composed of the following per liter of solution: 160 cc of 95% ethanol, 4.6 gms of barium acetate, 20 cc of 1M sodium acetate and 7.3 cc of 1M acetic acid. Two thousand one hundred cc of this solution were used to extract the serum albumins (solution V).

The precipitate remaining following removal of the serum albumins was labeled precipitate VI. This fraction containing albumins and alpha-globulins was further subfractionated into two fractions by raising the pH to 6.2 and maintaining 15.2% ethanol concentration. This was accomplished by adding 300 cc of solution containing 160 cc of 95% ethanol, 50 cc of 1M sodium acetate and .1 gm of zinc per liter. The fractions obtained were designated as precipitate IV-1 and solution IV-6+7. Precipitate IV-1 was composed mainly of albumins and alpha globulins. Solution IV-6+7 contained only alpha globulins.

Precipitate I+II+III which contained the $beta_2$ and gamma globulins was further subfractionated into four different fractions. This fraction II, containing only gamma globulins, was obtained by suspending the precipitate I+II+III in a solution containing glycine (which was used to break the protein complex by raising the dielectric constant of the solution) and lowering the pH to 5.5. The ethanol concentration of the resulting solution was 14.3%. The solution used in obtaining this environment contained 150 cc of 95% ethanol, 2 cc of 1M sodium acetate, 1.4 cc of 1M acetic acid and 45 gms of glycine per liter of solution. Six hundred cc of this solution were used to extract solution II. The resulting precipitate is referred to as fraction I+III.

Precipitate I+III was suspended in a solution containing 160 cc of 95% ethanol, 45 gms of glycine, 2.5 cc of sodium glycinate buffer, 3.2 cc of .5M sodium hydrogen phosphate and 2.4 cc of .5M sodium dihydrogen phosphate per liter of solution. The pH of this solution was 6.8 and the ethanol concentration was 15.2%. One thousand two hundred cc of this solution were used. The resulting solution is termed III-0 and

consisted of additional gamma globulins. The remaining precipitate is called I+III-1,2,3. The subfractionation of this precipitate resulted in two fractions, precipitate I+III-3 and solution III-1,2. These two fractions were obtained by suspending the original precipitate, I+III-1,2,3, into 300 cc of a solution containing 160 cc 95% ethanol, 1.2 cc of 1M citric acid and 120 cc of 1M trisodium citrate per liter of solution. The ethanol concentration of the final mixture was 15.2% and the pH 7.2. Precipitate III-1,2 contained fibrinogen and plasminogen while fraction I+III-3 contained predominantly the $beta_2$ globulins.

In each case where the precipitate was suspended into a solution in order to dissolve a fraction, it was first worked into a smooth paste with a little of the solution. This was followed by stirring the paste into the remaining volume of the solution and its continuous stirring for one hour. This suspension was then centrifuged at about 9000 r.p.m. for 30 minutes. Only in the first two cases was the material kept in an alcohol bath at -5°. (This was done in obtaining precipitates I+II+III and IV+V). The other solutions were added in the cold room at a temperature of 2° C. All centrifugation was also carried out in the same environment at 2° C.

B. Electrophoresis

The protein components of the various blood fractions were separated by paper electrophoresis. A model R, single cell Spinco apparatus was used with a veronal buffer. The buffer had a pH of 8.6 and an ionic strength of .075. Five tenths ma of current was used for an interval of 16 hours.

Upon completion of an electrophoretic run the strips were dyed by the method of Jencks et al. (1955) using bromphenol blue as a dye.

C. Assay Method

The presence of ACTH activity was determined by the method of Sayers et al. (1948). Sprague-Dawley male rats were used and maintained in an environmental temperature of 72° F. for at least 5 days prior to the assay. Rats weighing between 120 and 160 gms were hypophysectomized. Twenty-one to 27 hours after hypophysectomy the rats were anesthetized with sodium pentobarbital (4 mg per 100 gm of body weight by intraperitoneal injection). The left adrenal gland was removed for ascorbic acid analysis. The solution to be assayed was then injected intraperitoneally. One hour later the right adrenal gland was removed and prepared for ascorbic acid analysis. The response was expressed as the difference in concentration of ascorbic acid between the left and right adrenal glands.

The excised adrenals were transferred to filter paper, and the external fat and connective tissue removed with the aid of a fine pair of scissors. The capsule was kept intact, and care was taken to leave no trace of extra-adrenal tissue which could introduce an error in the quantitative analysis of the gland. During this procedure the gland continuously occupied the spot on the filter paper which had previously been moistened. After the extraneous tissue was removed, the gland was placed on a piece of tin foil and transferred to an analytical balance and weighed to the nearest .1 mg. The

adrenal tissue was then placed in a 12 ml conical centrifuge tube containing 2 cc of a 5% meta phosphoric acid solution and a small quantity of sand. The tissue was finely ground with the aid of a glass rod. Three additional cc of the meta phosphoric acid solution were then added, followed by 1 cc of a citric acid solution and 4 cc of distilled water. The total volume was 10 cc. At this time Norite was added to the tube. This suspension was shaken and filtered through Whatman No. 1 filter paper. Duplicate 4 cc samples of the filtrate were placed into each of two test tubes. One drop of thiourea solution was added to each sample. One tube was held as a blank and 1 cc of an acid 2,4, dinitrophenyl hydrazine solution was added to the duplicate sample. This tube was placed in a water bath at 58° C. for 45 minutes. At the end of this period both tubes were placed in an ice bath and 5 cc of 85% sulfuric acid were added, followed by 1 cc of the 2,4 dinitrophenyl hydrazine solution to the tube which was previously held as a blank. The tubes were allowed to set for about one-half hour and then read at a wave length of 540 mu in a Beckman Colorometer. The ascorbic acid was expressed as its concentration per one hundred grams of adrenal tissue.

Growth promoting activity was assayed by the method of Greenspan et al. (1949) utilizing 21- to 28-day-old immature hypophysectomized female rats as assay animals. Samples to be assayed for growth promoting activity were injected intraperitoneally into the assay animals 14 days following hypophysectomy, for four days, and the rats were autopsied 24 hours after the last injection. The right tibia was removed and freed

from soft tissue, split at the proximal end in a sagittal plane, and fixed in neutralized 10% formalin. Previous to staining, the bone halves were washed thoroughly in water, immersed in acetone for at least one hour and washed again. They were then immersed for about 1 1/2 minutes in a 2% solution of silver nitrate and exposed to strong light while under water, until the calcified parts appeared dark brown. Fixation of the tibias was accomplished by placing them in a 10% solution of sodium thiosulfate for about 1/2 minute followed by thorough washing under running water. The epiphyseal disc was measured under a microscope using low power and a micrometer eyepiece with light coming from beneath and above. The magnification was adjusted so that one ocular unit equalled 14 micra.

RESULTS

Table I contains data obtained from the bio-assay of the blood fractions for ACTH. When the equivalent of 50 cc of fractions II, III-1,2, III-0, V, and I+III-3 were injected into hypophysectomized rats weighing between 120-160 gms, death resulted in less than one hour. The toxic factor did not appear associated with protein concentration, for the injection of a plasma equivalent of 8 cc also resulted in death of all animals in one hour. If the blood fractions were dialyzed for 24 hours against distilled water the toxic factor apparently was eliminated or at least considerably reduced. In the routine process of dialysis of fractions containing toxic materials there was an increase in volume of solution contained within the boundaries of the membrane, that is, the material to be assayed. The membrane was placed in a concentrated solution of glucose in order to reduce the volume to one desirable for assay procedures. The dialyzed blood fractions were then injected into the assay animals in the amounts equivalent to 50 cc, and the animals lived throughout the duration of the assay.

Biological assay of the various blood fractions demonstrated that ACTH activity was limited, within the sensitivity of the assay, to fraction IV+V. The decrease in adrenal ascorbic acid is noted in assay animals 1, 2 and 3 of Table I where there is a difference in the concentration of ascorbic acid of 295, 69 and 278 mgs, respectively, between the left and right adrenals. Fraction IV+V, obtained from a 14

year old bull, was consistent in its ability to decrease the adrenal ascorbic acid in the hypophysectomized assay animals. A positive response for ACTH activity was also obtained in fraction IV+V from another 14 year old bull and from a 2 year old bull (Tables II and IIa).

An attempt was made to quantitate the ACTH activities in the plasma of a young bull and of an old bull. Thirty-six, 45 and 54 cc plasma equivalents of fractions IV+V from each bull were injected into hypophysectomized assay animals (Tables II and IIa). A considerable variation in adrenal ascorbic acid response was obtained, both from the duplicate samples assayed and between graded samples assayed. The variability in response was expressed as a 355 mg difference in adrenal ascorbic acid content between the largest and smallest response from the group receiving the equivalent of 54 cc of plasma in the form of fraction IV+V from the 14 year old bull. The other levels of plasma equivalent from the 14 year old bull also showed a variability to this same extent (268 mgs for the group receiving the equivalent of 36 cc of plasma and 271 mgs for the group receiving the equivalent of 45 cc of plasma). Considerable variation in ascorbic acid depletion also existed when duplicate samples from the 2 year old bull were assayed for ACTH. The greatest difference in response was in the assay rats which received the equivalent of 45 cc of blood plasma in the form of blood fraction IV+V. The difference between the largest and smallest response in this group was 208 mgs of ascorbic acid. A decrease of 253 mgs of adrenal ascorbic acid was also observed for the group receiving 36 cc of plasma equivalent in the form of blood fraction IV+V from the young bull. With

the number of animals used for each sample assayed and with the variability obtained, the statistical analysis showed that the averages of the various groups, both from the old bull and young bull, were not statistically significant. The 14 year old bull, even with this large variation between groups and within groups, appeared to have a higher concentration of ACTH per unit volume of blood as compared to the younger animal. The difference in ACTH activity is noted in the groups receiving the equivalent of 45 cc of plasma in the form of fraction IV+V. At this level the older animals blood fraction IV+V produced an average decrease in the ascorbic acid between the two adrenals of 147 mgs while the younger animal had a decrease of only 57 mgs. A difference in ACTH activity is noted again (Table II and IIa) following the injection of an equivalent of 45 cc of plasma in the form of fraction IV+V. The assay rats receiving the fraction from the 14 year old animal consistently showed a decrease in the concentration of ascorbic acid in the two adrenals. Despite the considerable variability in response, the injection of fraction IV+V consistently resulted in a decrease in ascorbic acid in the assay animals. The injection of this same level of plasma equivalent from a young bull did not show this consistency in the animals receiving 45 cc of plasma equivalent (Table II). Two animals increased the concentration of ascorbic acid after the injection of the fraction to the extent of 5 and 29 mgs. A concentration difference in ACTH per unit volume of blood between the two animals cannot be shown due to the extreme variation obtained in a group of assay animals receiving the graded doses of the blood fraction.

In order to determine whether the variations in adrenal ascorbic acid depletion were inherent within the assay or whether the proteins in fraction IV+V contained a component which caused this variation, an experiment was designed with purified ACTH. The results are tabulated in Table VI. The purified ACTH preparation was injected at levels of .02, .03 and .04 IU into different groups of assay animals. Again there appeared a large variation in response on duplicate ACTH samples. The variation was to the extent of a 224 mg difference of ascorbic acid depletion between the largest and smallest response in the group receiving .02 IU, and 197 and 75 mgs for the groups receiving .03 and .04 IU of purified ACTH, respectively. The differences in average responses of these groups were not significant, except the .04 IU level, due to the extreme variation contained within the groups. Taking into account the responses of the purified ACTH as well as the blood fraction IV+V, the variations obtained in each case appeared to be inherent in the assay itself and were not due to any component contained within the blood fraction. In order to have an average which would be significant but at the same time contain the same degree of variation within an injected level of plasma equivalent as was experienced above, it would be necessary to inject over 30 animals per level of plasma equivalent.

If blood fraction IV+V was dialyzed for 24 hours in the manner previously described and then injected at a level equivalent to 35 cc of plasma, the difference in concentration of ascorbic acid in the right adrenal compared to that of the left adrenal taken one hour previously

was -16, +47 and +29 mgs (Table VII) in three animals. The injection of this same plasma equivalent of undialyzed material resulted in a decrease in the ascorbic acid concentration of the right adrenal of 5 animals (compared to the left adrenal taken one hour previously). The decrease between the five animals ranged from 25 to 293 mgs of adrenal ascorbic acid. The above figures are represented in Table VII. It is apparent that the ACTH activity contained in blood fraction IV+V from the old bull is dialyzable or that the activity is destroyed by dialysis. A decrease in ACTH activity was encountered when blood fraction IV+V was subjected to subfractionation. The subfractions appear in Table I in the form of fractions V, IV-I and IV-6+7. There appeared no consistent decrease in adrenal ascorbic acid in the groups receiving injections of the various subfractions.

When blood fraction VI was injected into the assay animals at a plasma equivalent level of 50 cc an apparent decrease in the adrenal ascorbic acid content of 123 and 358 mgs was obtained in two animals when the concentration in the right adrenal was compared to the left adrenal's concentration taken one hour previously. There was also an increase in the weight of the right adrenal gland after the injection of this blood fraction. The increase in adrenal weight resulted in this false conclusion, since the ascorbic acid content is based on its concentration per 100 gms of adrenal tissue. If one used the expected adrenal weight of 100 mgs for the right adrenal of animal number 3, and 110 mgs for the right adrenal of animal number 4, no significant decrease in the adrenal ascorbic acid would be observed. This same blood

fraction produced a dark blue discoloration of the entire intraperitoneal cavity. The increase in adrenal weight and the discoloration of the intraperitoneal cavity caused by the injection of fraction VI were not eliminated by dialysis.

Blood fraction III-0 contained a component which caused the opposite effect on the adrenal ascorbic acid content when the results were compared with those obtained from ACTH. Blood fraction III-0 caused an increase in the ascorbic acid of the right adrenal as compared to that of the left adrenal taken one hour previously, to the extent of +32, +147 and +140 mgs in three animals. Each animal received an equivalent of 50 cc of plasma in the form of this blood fraction. The results are tabulated in animal numbers 31, 32 and 33 on Table I. It was necessary to dialyze this blood fraction for 24 hours previous to its injection due to the large concentration of glycine. The glycine was present in the solution used to extract blood fraction III-0 from the main precipitate I+II+III. Blood fraction III-0 retained its anti-ACTH activity, or adrenal ascorbic acid increasing capacity, after this period of dialysis.

The injection of the equivalent of 50 cc of plasma in the form of blood fraction II into 28 day hypophysectomized female rats 14 days postoperative, produced a significant increase in the epiphyseal disc of the tibia or a positive response for growth hormone activity. The increase in the epiphyseal disc was induced in animals 3 and 4 (Table III). The response was 271 micra and 227 micra in the two animals. An attempt was made to produce a graded dose response using 35, 45 and 54 cc

plasma equivalent in the form of blood fraction II. No significant difference was observed either between the graded doses or between fractions coming from either the 14 year old bull or the 2 year old bull. The lack of significance is shown in Table V which contains the analysis of variance of the animals receiving the various graded doses. In obtaining blood fraction II, each bull's plasma was handled in an identical manner. The same solution was used to extract the blood fraction as well as its extraction at a constant temperature. It is felt that the assay animals themselves are responsible for the variability in the responses. Blood fraction II required dialysis due to the high concentration of glycine it contained from the solution used in its extraction from precipitate I+II+III.

Uterine stimulation was observed in animals 10, 11, 13 and 14 shown in Table IV. These animals received the equivalent of 35 cc of plasma in the form of fraction IV+V from the young bull. At higher levels of plasma equivalent no uterine stimulation was observed. No uterine stimulation was observed in the animals receiving fractions IV+V from the old bull, either at the 45 or 54 cc levels of plasma equivalent. The uterine stimulation is interpreted as caused by the gonadotrophic activity present in the blood fraction.

TABLES

TABLE I

Preliminary Bio-Assay of Blood Fractions for ACTH Activity[1]

Animal No.	Body Wt.	Blood Fraction Injection	Amt. of Blood Fraction Injection[2]	Wt. of Left Adrenal Gland	Wt. of Right Adrenal Gland	Conc. of AA/100 Gms L. Adrenal Tissue[3]	Conc. of AA/100 Gms R. Adrenal Tissue[4]	Diff. in Conc. of AA Between Left and Right Adrenal Tissue[5]
				.1 mg	.1 mg	mg	mg	mg
1	149	IV+V	50 cc	141	116	588.64	293.11	-295
2	135	IV+V	50 cc	106	113	514.15	455.78	- 69
3	139	IV+V	50 cc	145	134	531.07	253.74	-278
4	143	VI	50 cc	107	125	523.38	400.00	-123
5	124	VI	50 cc	121	172	541.29	183.14	-358
6	146	VI	50 cc	158	113	481.00	314.18	-167
7	142	VI	50 cc	110	132	495.46	344.71	-151
8	131	V*	50 cc	123	103	508.13	553.45	+ 45
9	135	V*	50 cc	110	101	495.46	509.98	+ 14
10	134	V*	50 cc	102	95	558.83	657.88	+ 99
11	143	IV-1*	50 cc	110	99	495.5	520.20	+ 25
12	122	IV-1*	50 cc	119	79	432.76	291.13	-141
13	146	IV-1*	50 cc	122	92	397.56	461.98	+ 64
14	156	IV-1*	50 cc	187	138	438.54	525.34	+ 88
15	143	IV-1*	50 cc	137	119	408.74	504.18	+ 96
16	155	IV-1	50 cc	124	123	568.26	508.13	- 60
17	137	IV-1	50 cc	123	104	587.80	466.33	-121
18	134	IV-6+7	50 cc	120	128	574.98	511.75	- 63
19	134	IV-6+7	50 cc	113	92	455.78	559.81	+104
20	133	IV-6+7*	50 cc	130	110	549.98	627.30	+ 78
21	144	IV-6+7*	50 cc	119	107	504.18	532.72	+ 28
22	144	IV-6+7*	50 cc	119	110	579.81	495.46	- 84
23	146	IV-6+7*	50 cc	122	91	446.74	500.00	+ 54
24	157	IV-6+7*	50 cc	141	125	464.53	496.00	+ 32

TABLE I (Continued)

Animal No.	Body Wt.	Blood Fraction Injection	Amt. of Blood Fraction Injection[2]	Wt. of Left Adrenal Gland .1 mg	Wt. of Right Adrenal Gland .1 mg	Conc. of AA/100 Gms L. Adrenal Tissue[3] mg	Conc. of AA/100 Gms R. Adrenal Tissue[4] mg	Diff. in Conc. of AA Between Left and Right Adrenal Tissue[5] mg
25	133	I+II+III	50 cc	112	105	486.63	571.44	+ 85
26	142	I+II+III	50 cc	122	110	491.82	568.19	+ 77
27	140	I+II+III	50 cc	110	101	563.06	564.36	+ 1
28	131	II*	40 cc	131	117	526.75	559.83	+ 33
29	140	II*	60 cc	124	105	576.65	571.44	- 5
30	128	II*	50 cc	115	106	447.84	650.95	+203
31	146	III-0*	40 cc	142	120	584.49	616.61	+ 32
32	143	III-0*	50 cc	122	94	491.82	638.28	+147
33	130	III-0*	50 cc	135	96	485.16	625.02	+140
34	131	III-1,2*	50 cc	139	133	597.10	255.65	-242
35	140	III-1,2*	50 cc	134	115	597.04	600.00	- 3
36	138	III-1,2*	50 cc	100	90	545.00	572.22	+ 27
37	142	I+III-3*	50 cc	118	112	411.04	459.84	+ 48
38	141	I+III-3*	50 cc	122	114	422.15	526.32	+104

*Dialized

[1]A modification of Sayers' method of assaying for ACTH was used.

[2]Equivalent of plasma injected intraperitoneally.

[3]Concentration of ascorbic acid per 100 gms of adrenal tissue.

[4]Concentration of right adrenal's ascorbic acid based on the concentration per 100 gms of adrenal tissue. The right adrenal gland was taken 1 hour after the removal of the left adrenal gland and the injection of the blood fraction.

[5]Difference in concentration of ascorbic acid per 100 gms of adrenal tissue between left and right adrenals.

TABLE II

Quantitative Estimation of ACTH Activity in Fraction IV+V From 2 Year Old Bull[1]

Animal No.	Body Wt.	Blood Fraction Injection	Amt. of Blood Fraction Injection[2]	Wt. of Left Adrenal Gland	Wt. of Right Adrenal Gland	Conc. of AA/100 Gms L. Adrenal Tissue[3]	Conc. of AA/100 Gms R. Adrenal Tissue[4]	Diff. in Conc. of AA Between Left and Right Adrenal Tissue[5]
				.1 mg	.1 mg	mg	mg	mg
1	124	IV+V	36 cc	94	91	441.48	423.06	- 18
2	131	IV+V	36 cc	111	111	513.51	427.93	- 86
3	124	IV+V	36 cc	94	110	494.67	445.46	- 49
4	152	IV+V	36 cc	138	121	481.86	210.73	-271
5	135	IV+V	36 cc	124	111	483.90	256.76	-227
6	136	IV+V	45 cc	122	94	491.82	473.39	- 18
7	145	IV+V	45 cc	163	134	503.07	238.84	-203
8	126	IV+V	45 cc	95	82	484.20	452.95	- 32
9	132	IV+V	45 cc	102	91	500.00	505.49	+ 5
10	135	IV+V	45 cc	123	112	439.02	468.77	+ 29
11	140	IV+V	45 cc	97	98	474.21	423.47	- 51

[1] A modification of Sayers' method of assaying for ACTH was used.

[2] Equivalent of plasma injected intraperitoneally.

[3] Concentration of ascorbic acid per 100 gms of adrenal tissue.

[4] Concentration of right adrenal's ascorbic acid based on the concentration per 100 gms of adrenal tissue. The right adrenal gland was taken 1 hour after the removal of the left adrenal gland and the injection of the blood fraction.

[5] Difference in concentration of ascorbic acid per 100 gms of adrenal tissue between left and right adrenals.

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TABLE IIa

Quantitative Estimation of ACTH Activity in Fraction IV+V From 14 Year Old Bull[1]

Animal No.	Body Wt.	Blood Fraction Injection	Amt. of Blood Fraction Injection[2]	Wt. of Left Adrenal Gland	Wt. of Right Adrenal Gland	Conc. of AA/100 Gms L. Adrenal Tissue[3]	Conc. of AA/100 Gms R. Adrenal Tissue[4]	Diff. in Conc. of AA Between Left and Right Adrenal Tissue[5]
				.1 mg	.1 mg	mg	mg	mg
1	138	IV+V	36 cc	94	92	648.92	510.89	-138
2	121	IV+V	36 cc	65	75	558.48	533.32	- 25
3	113	IV+V	36 cc	105	110	550.01	506.01	- 44
4	122	IV+V	36 cc	129	110	465.12	172.73	-293
5	127	IV+V	36 cc	100	100	475.00	320.00	-155
6	131	IV+V	45 cc	110	107	639.19	602.82	- 37
7	127	IV+V	45 cc	140	117	475.01	192.31	-283
8	127	IV+V	45 cc	103	100	446.61	385.00	- 61
9	123	IV+V	45 cc	110	91	495.46	456.04	- 39
10	138	IV+V	45 cc	150	126	495.69	178.58	-317
11	141	IV+V	54 cc	150	112	570.03	496.28	- 74
12	129	IV+V	54 cc	127	122	571.64	521.00	- 50
13	140	IV+V	54 cc	130	107	657.67	298.03	-359
14	129	IV+V	54 cc	110	100	554.55	550.00	- 4
15	120	IV+V	54 cc	111	100	594.59	535.00	- 59
16	115	IV+V	54 cc	107	103	542.07	490.95	- 52

[1]A modification of Sayers' method of assaying for ACTH was used.

[2]Equivalent of plasma injected intraperitoneally.

[3]Concentration of ascorbic acid per 100 gms of adrenal tissue.

[4]Concentration of right adrenal's ascorbic acid based on the concentration per 100 gms of adrenal tissue. The right adrenal gland was taken 1 hour after the removal of the left adrenal gland and the injection of the blood fraction.

[5]Difference in concentration of ascorbic acid per 100 gms of adrenal tissue between left and right adrenals.

TABLE III

Assay of the Various Fractions for Growth Hormone Activity[1,4]

Animal No.		Fraction and Blood Equivalent[2]	Epiphyseal Width in Micra[3]
1	Dialized	50 cc IV-1	176.82
2	"	50 cc IV-1	402.50
3	Dialized	50 cc II	271.32
4	"	50 cc II	227.50
5		50 cc IV-6+7	267.68
6		50 cc IV-6+7	189.00
7		50 cc IV-6+7	169.00
8	Dialized	50 cc IV-6+7	127.82
9	"	50 cc IV-6+7	134.82
10	"	50 cc IV-6+7	281.82
11	Dialized	50 cc V	280.00
12	"	50 cc V	130.00
13		50 cc IV	124.32
14		50 cc III-1,2	255.50
15		50 cc III-1,2	171.50
16		50 cc V	152.32
17		50 cc III-1,2	201.32
18		50 cc III-1,2	178.50
19		50 cc III-1,2	112.00
20		50 cc IV-6+7	287.00
21		50 cc IV-6+7	155.82
22		50 cc I+III-3	134.82
23		50 cc I+III-3	129.40
24		50 cc I+III-3	173.32

[1]Tibia assay method of Greenspan et al (1949) was used.

[2]Total volume injected in a four day period.

[3]Epiphyseal cartilage width was measured under a low power microscope with a calibrated eye piece (1 ocular unit = 14 micra).

[4]Control epiphyseal cartilage was 160 micra.

TABLE IV

Quantitative Estimation of Growth Hormone Activity[1] Contained in Blood Fractions IV+V[4]

Rat No.		Blood Equivalent[2]		Epiphyseal Width in Micra[3]
1	1	30	14 Year	126.00
2	2		Old Bull	145.25
3	3			168.00
5	4			169.75
Average				152.00
6	5	40	14 Year	134.75
8	6		Old Bull	152.25
9	7			136.50
39	8			140.00
14	9			134.75
Average				142.92
15*	10	30	2 Year	162.75
19*	11		Old Bull	117.25
29	12			150.50
23*	13			147.00
25*	14			131.25
Average				131.25
16	15	40	2 Year	131.25
17	16		Old Bull	138.25
26	17			141.75
28	18			127.75
Average				136.40

[1]Tibia assay method of Greenspan et al. (1949) was used.

[2]Total plasma equivalent used in a four day period.

[3]Epiphyseal cartilage width measured under a low power microscope with a calibrated eye piece (1 ocular unit = 14 micra).

[4]Control epiphyseal cartilage width was 140 micra.

*Uterine stimulation

TABLE V

Analysis of Variance of the Animals Receiving Graded Injections of Fraction IV+V

	Sum of Squares	d.f.	Mean Square	F Ratio
Mean	628.25	3	212.75	$F = \frac{212.75}{204.14} = 1.04$
Within	2857.928	14	204.14	$F_{.95}\ (3,17) = 3.34$
Total	3496.185	17		

TABLE VI

Standard ACTH Assay[1]

Animal No.	Body Wt.	IU of Purified ACTH Injected	Wt. of Left Adrenal Gland	Wt. of Right Adrenal Gland	Conc. of AA/100 Gms L. Adrenal Tissue[2]	Conc. of AA/100 Gms R. Adrenal Tissue[3]	Diff. in Conc. of AA Between Left and Right Adrenal Tissue[4]
			.1 mg	.1 mg	mg	mg	mg
1	122	.02 IU	116	93	474.16	452.00	- 22
2	129		115	107	378.28	341.13	- 37
3	142		98	89	525.51	337.08	-188
4	160		119	114	613.42	399.13	-214
5	133		127	119	527.56	281.50	-246
6	143	.03 IU	117	105	470.09	233.34	-237
7	125		124	110	588.75	413.64	-175
8	128		176	134	517.06	477.63	- 40
9	123		112	101	491.10	301.98	-190
10	122		106	94	518.87	425.52	- 93
11	139		102	92	553.93	434.80	-119
12	140	.04 IU	107	101	588.80	297.03	-291
13	140		148	123	604.75	378.05	-226
14	123		113	104	486.75	206.72	-280
15	149		154	134	474.06	182.84	-292
16	137		154	142	582.76	281.68	-301

[1]A modification of Sayers' method of assaying for ACTH was used.

[2]Concentration of ascorbic acid per 100 gms of adrenal tissue.

[3]Concentration of right adrenal's ascorbic acid based on the concentration per 100 gms of adrenal tissue. The right adrenal gland was taken 1 hour after the removal of the left adrenal gland and the injection of the blood fraction.

[4]Difference in concentration of ascorbic acid per 100 gms of adrenal tissue between left and right adrenals.

TABLE VII

Quantitative Estimation of ACTH Activity in the Dialized Fraction IV+V From the 14 Year Old Bull[1]

Animal No.	Body Wt.	Blood Fraction Injection	Amt. of Blood Fraction Injection[2]	Wt. of Left Adrenal Gland	Wt. of Right Adrenal Gland	Conc. of AA/100 Gms L. Adrenal Tissue[3]	Conc. of AA/100 Gms R. Adrenal Tissue[4]	Diff. in Conc. of AA Between Left and Right Adrenal Tissue[5]
				.1 mg	.1 mg	mg	mg	mg
1	138	IV+V*	35 cc	130	125	584.67	568.00	- 16
2	143	IV+V*	35 cc	130	119	511.52	558.80	+ 47
3	135	IV+V*	35 cc	119	109	613.42	642.18	+ 29

*Dialized

[1]A modification of Sayers' method of assaying for ACTH was used.

[2]Equivalent of plasma injected intraperitoneally.

[3]Concentration of ascorbic acid per 100 gms of adrenal tissue.

[4]Concentration of right adrenal's ascorbic acid based on the concentration per 100 gms of adrenal tissue. The right adrenal gland was taken 1 hour after the removal of the left adrenal gland and the injection of the blood fraction.

[5]Difference in concentration of ascorbic acid per 100 gms of adrenal tissue between left and right adrenals.

FIGURES

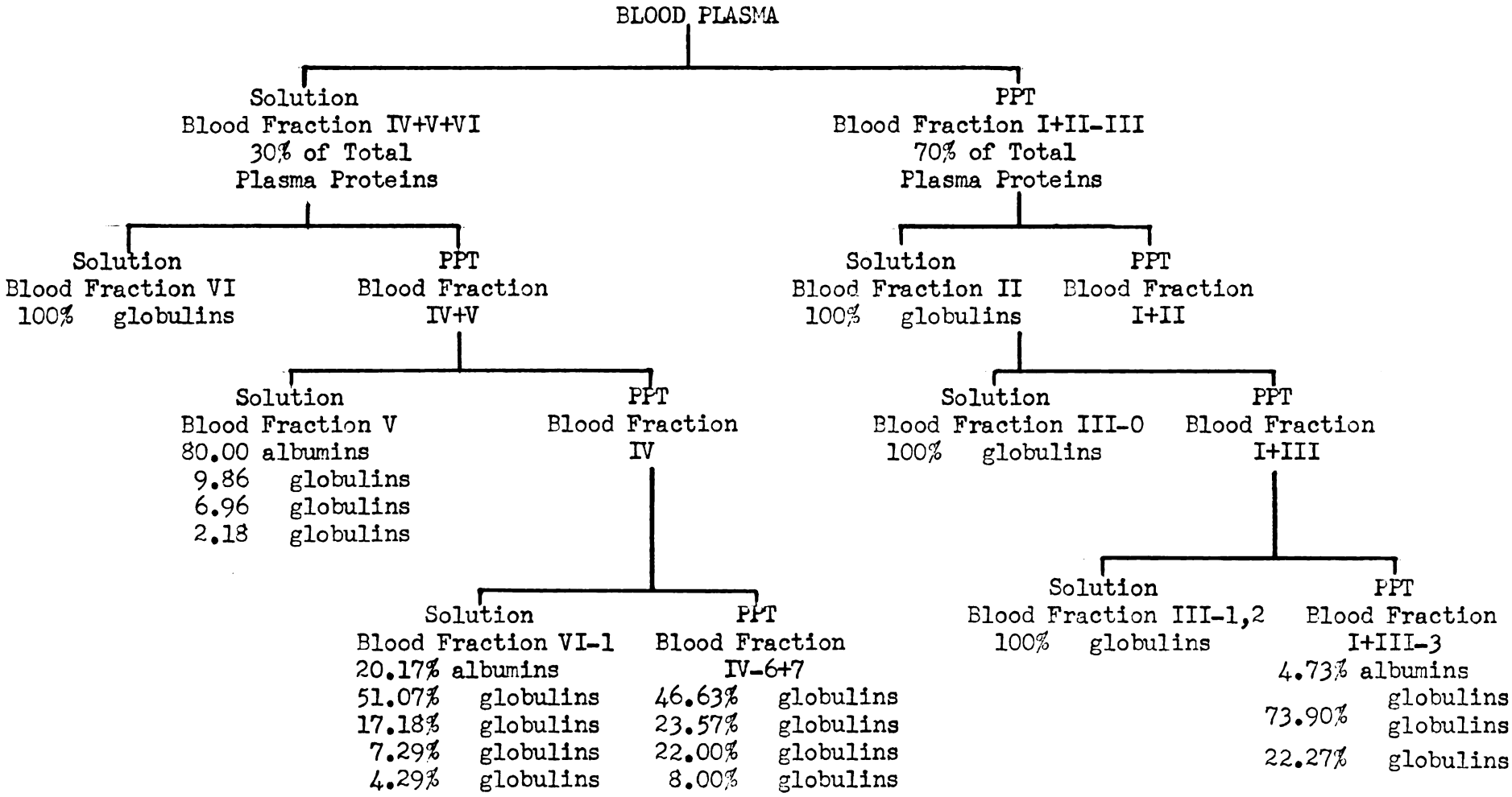


FIGURE A

Flow Scheme for Plasma Fractionation

Plasma Components	% contained in supernatant[1]
γ-globulins	100

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 1

Electrophoretic mobilities of proteins in supernatant II, the sample biologically assayed.

Plasma components	% contained in Supernatant[1]
γ-globulins	100

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 2

Electrophoretic mobilities of proteins in supernatant III-0, the sample biologically assayed.

Plasma components	% contained in Supernatant[1]
albumins	4.73
β1 globulins	73.00
β2 "	
γ "	22.27

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 3

Electrophoretic mobilities of proteins in supernatant I + III-3, the sample biologically assayed.

Plasma components	% contained in Supernatant[1]
γ-globulins	100

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 4

Electrophoretic mobilities of proteins in supernatant III-1,2, the sample biologically assayed.

Plasma components	% contained in Supernatant[1]
γ-globulins	100

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 5

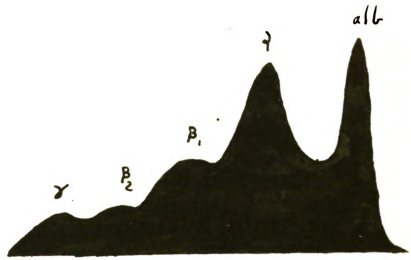
Electrophoretic mobilities of protein in supernatant VI, the sample biologically assayed.

Plasma components	% contained in Supernatant[1]
albumins	80.00
β_1-globulins	9.86
β_2- "	6.96
γ - "	2.18

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents

Figure 6

Electrophoretic mobilities of proteins in supernatant V, the sample biologically assayed.

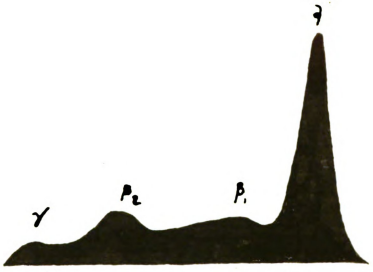


Plasma components	% contained in Supernatant[1]
albumins	20.17
α-globulins	51.07
β1- "	17.18
β2- "	7.29
γ- "	4.29

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 7

Electrophoretic mobilities of proteins in supernatant IV-1, the sample biologically assayed.



Plasma components	% contained in supernatant[1]
α-globulins	46.63
β1- "	23.57
β2 "	22.00
γ "	8.00

[1]Expressed in terms of percent of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 8

Electrophoretic mobilities of proteins in supernatant IV-6 + 7, the sample biologically assayed.

DISCUSSION

ACTH activity has been detected in the plasma of several different animal species (noted on page 26). The data reported here suggest that the plasma ACTH activity reported by other workers did not adequately represent the total activity of this hormone. There are a number of factors present in the plasma of cattle which may result in erroneous conclusions with respect to the amount of ACTH activity present. Blood fraction VI and III-O, when injected into the assay animals, as previously described, affected the adrenal glands in the following manner. Fraction IV produced a decrease of adrenal ascorbic acid concentration not by reducing the absolute amount of the vitamin present but by increasing the weight of the adrenal glands through their accumulation of water. Since ACTH activity is based on the ability of a blood fraction to decrease the ascorbic acid per 100 gms of wet adrenal tissue, any increase in weight of the adrenal gland would result in a high estimate of ACTH activity in the blood fraction assayed. An increase in adrenal weight has not been previously considered when calculating the adrenal ascorbic acid depletion caused by the injection of purified ACTH or the ACTH activity present in plasma. Blood fraction III-O also produced a fictitious value for ACTH activity but in this case the activity was diminished rather than increased. Blood fraction III-O contained a substance which caused an anti-ACTH response in the assay animals resulting in an increase

in the adrenal ascorbic acid concentration. If this fraction were combined with the fraction containing the ACTH activity (IV+V) a lower value of ACTH activity would result than is actually present in the blood fraction. Previous biological assays of plasma have not taken into consideration the activities which are reported in this study in fractions IV and III-O. The values reported by other researchers, therefore, represent the algebraic sum of all the biological activities which affect the adrenal gland or the ascorbic acid contained in the adrenal glands. If a true estimation of the ACTH activity is to be obtained it is necessary to first eliminate all other components which will influence adrenal gland activity and if this is not feasible, to develop a bio-assay technique which is specific and sensitive for ACTH. One might suggest that the algebraic sum of activities is a more valid expression of ACTH activity since the adrenal in the host animal is exposed to all of the factors. This is not necessarily true, however, since removal of plasma from an animal and chemical fractionation provides a product not necessarily of physiological nature.

When ACTH was obtained from the anterior pituitary gland in the unhydrolyzed form the molecular weight was reported to be approximately 12,000. The unhydrolyzed ACTH is given the description of ACTH-A and when subjected to a considerable amount of hydrolysis it is designated as ACTH-B and then has a molecular weight of about 5000 to 6000. A molecule which has a molecular weight of 12,000 (the size of ACTH-A) is known to be non-permeable through a cellophane dializing membrane. The work undertaken in this thesis has suggested that the molecule or

molecules which contain the ACTH activity in the blood plasma of cattle may be of smaller molecular size than the ACTH-A obtained from the anterior pituitary gland. This conclusion is drawn because of the ease of which the ACTH activity is dialized from the blood fraction IV+V. It may be concluded that the ACTH activity present in the systemic system is not the same molecule as that found in the ACTH obtained from the anterior pituitary gland or else a considerable amount of hydrolysis has taken place in the process of plasma fractionation to produce a much smaller molecule. Another explanation of this may be that the component in the plasma causing the decrease in ascorbic acid of rats is not ACTH at all but some other biological activity component acting simular to ACTH, but having a smaller molecular size.

ACTH activity was not thought to be destroyed in the process of dialysis because of its extreme resistance to dematuration. In the preparation of ACTH, commercially, all the other trophic hormones are destroyed by hydrolyzing the material with acetic acid. In the process of hydrolysis there did not seem to be any detectable loss in the ACTH activity.

A modification of the method of Sayers, Sayers and Woodbery (1948) for the detection of ACTH activity was used in this study. The modification was that the material to be assayed was injected intraperitoneally instead of intravenously as conventionally carried out in the Sayers method. The intraperitoneal injection of a solution containing ACTH activity considerably lowered the sensitivity of the assay.

The intraperitoneal injections were considered essential for it was not feasible to concentrate the protein contained in the various blood fractions to allow their injection intraveneously or subcutaneously. A total volume of -12 cc of the various blood fractions were injected intraperitoneally. Intravenous injection of this amount of foreign protein would have resulted, at any rate, in death of the assay animal. On the other hand, absorption of ACTH from a large subcutaneous protein depot would be very slow and not suitable for a one-hour assay. It was realized that the animal did not absorb the entire volume in this relatively short period of one hour. The amount of absorption which did occur should have been proportional to the volume of material injected into the assay animals. The total concentration of ACTH activity present per unit volume of the injected blood fractions was therefore not detected. In the assay procedure described it was only possible to determine the difference between any two fractions if the same volumes were injected of the two blood fractions. If a comparison is made between the two bulls' blood fraction IV+V for ACTH activity, it appears that the 14 year old animal's plasma contain a higher concentration of ACTH activity than the 2 year old animal. The variation inherent in the responses of the assay animals were too large to permit a quantitative estimation of the difference in ACTH activity contained in the two blood fractions from the two bulls.

The variability existing in the assay animals may be due to a great variety of factors. It is possible that the blood fraction, or

ACTH preparation itself, contains some factor which caused the animals to vary in their responses measured by the animal's depletion of adrenal ascorbic acid. The variations may also have been due to the environmental conditions under which the animals were shipped to the laboratory or to which the animals were subjected prior to hypophysectomy. It is very pertinent that the animals are standardized very rigorously before their use in the assay. After running some 600 animals by the modified Sayers method of assaying for ACTH, it was found that there was considerably less variation between animals run on a single day than those animals run over a period of several days. Thus it may be concluded that in order to eliminate the variations among the assay animals, they must be rigorously standardized and the assay itself run over as short a period of time as possible, preferably in a single day.

In assaying the various blood fractions for growth hormone activity, only blood fraction II produced a significant increase in the width of the epiphyseal cartilage. This increase in the epiphyseal cartilage was interpreted as being caused by growth hormone. When an attempt was made to produce a graded response using this blood fraction at a later date, the assay animals did not respond as in the preliminary assay. The blood fractions were obtained in identical manners. The lack of sensitivity in the second assay gives evidence that the animals used for the various bio-assay techniques vary considerably in their sensitivity to any bioactive substance.

In conclusion, it may be said that the quantitative estimation of either growth hormone activity or ACTH activity present in bovine blood plasma is not possible under the assay procedures outlined in this thesis. If such a quantitative estimation of activity is to be determined in the plasma of cattle, it will be necessary to eliminate all variations among the assay animals or use more sensitive and specific methods.

SUMMARY

Blood plasma was obtained from a 14 year and a 2 year old bull and fractionated into eight different blood fractions. A toxic factor was present in some blood fractions and this was eliminated by dialysis. In the process of dialysis there was an increase in the volume of solution contained within the boundaries of the membrane. This increase in volume was undesirable because of the dilution of the protein contained within the solution. The volume of solution was decreased by placing the membrane in a solution of concentrated sucrose which, having a higher osmotic pressure than the interior medium of the membrane, drew the excess water out.

In assaying the various fractions for ACTH activity by the ascorbic acid depletion method, fraction IV+V was the only blood fraction which produced a true decrease in the adrenal ascorbic acid or a significant ACTH response. It was noted that there also appeared to be a relative decrease in the concentration of ascorbic acid when fraction IV was assayed. This was not due, however, to an absolute decrease in ascorbic acid but to an increase in the weight of the right adrenal gland after the injection of fraction IV. Since ascorbic acid content is based on the concentration per 100 gms of adrenal tissue, any change in adrenal weight during the assay must be taken into consideration. If one used the expected adrenal weight, there appeared no decrease in the adrenal ascorbic acid. This same fraction

produced a dark blue discoloration of the entire intraperitoneal cavity. The biological effects cited for fraction IV were not eliminated by dialysis.

A quantitative estimation of the ACTH activity present in both young and aged animals was attempted. Graded levels of fraction IV+V were injected into the assay animals. The equivalent of 36, 45, and 54 cc of plasma was injected. The results showed extreme variability and could not be used to draw definite conclusions. The aged animal's plasma, however, appeared to contain a higher level of ACTH activity than the plasma from the younger animal.

In the attempt to make a quantitative estimation of the ACTH activity contained in the plasma, the variability in response extended throughout an assay group receiving a particular level of plasma equivalent as well as between assay groups receiving graded doses.

A standard ACTH preparation was also assayed and the results showed that the variation was inherent in the assay animals. A large number of assay animals would be required in order to obtain statistical significance in the assay for ACTH activity.

Fraction III-O contained an anti-ACTH activity, for injections of this fraction increased the ascorbic acid content when based on its concentration per 100 gms of adrenal tissue. No further manipulation of this fraction was undertaken.

Fraction II was found to have growth promoting activity. It was necessary to dialize this fraction before biological assay, due

to its high content of glycine. The glycine was a component of the solution used to extract the fraction from the main precipitate, I+II+III. An attempt was also made to quantitatively estimate the amount of growth promoting activity present. Due to the large variation which existed among the animals used in the assay, no significant difference was shown between groups when the data was analyzed statistically.

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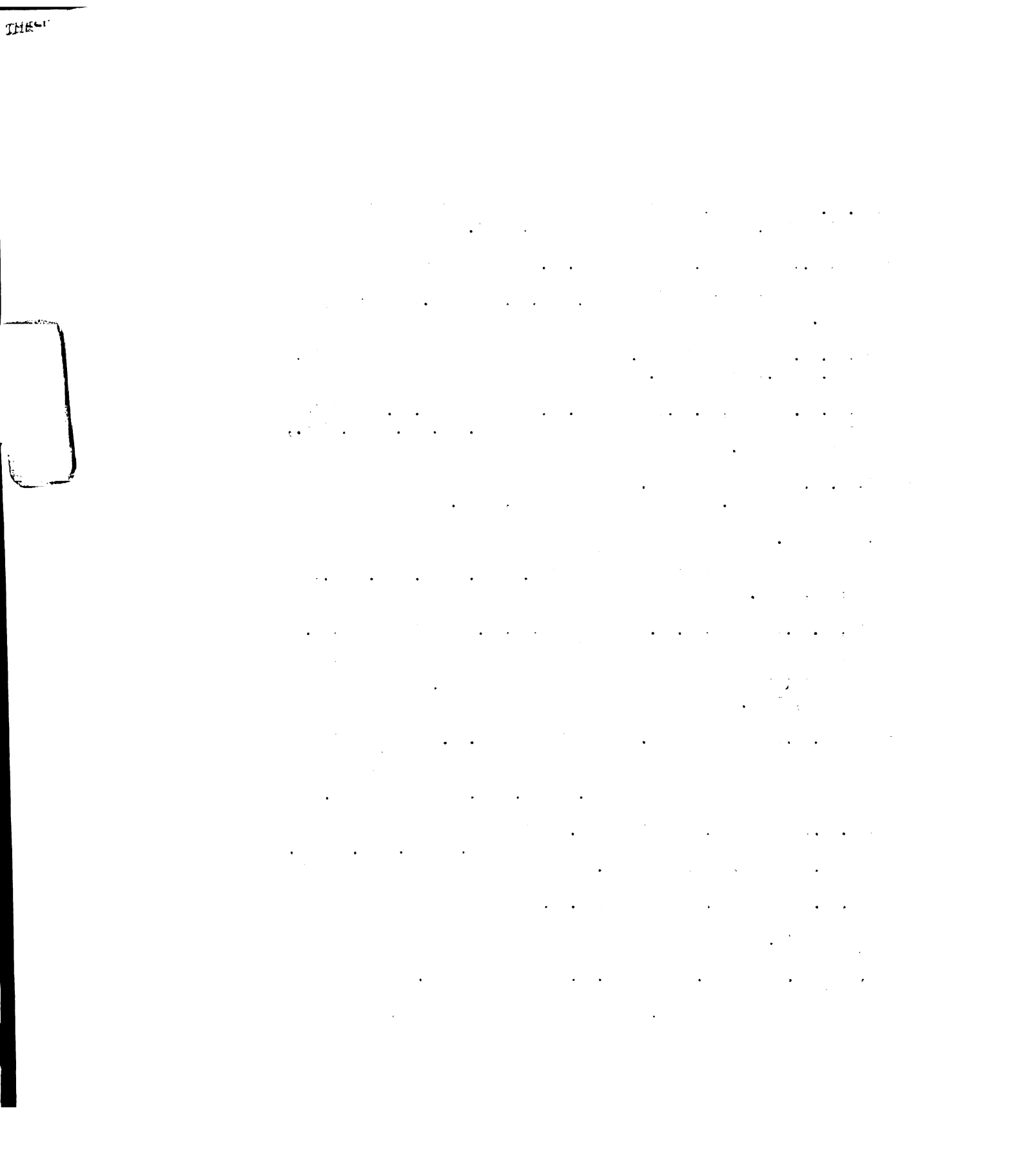

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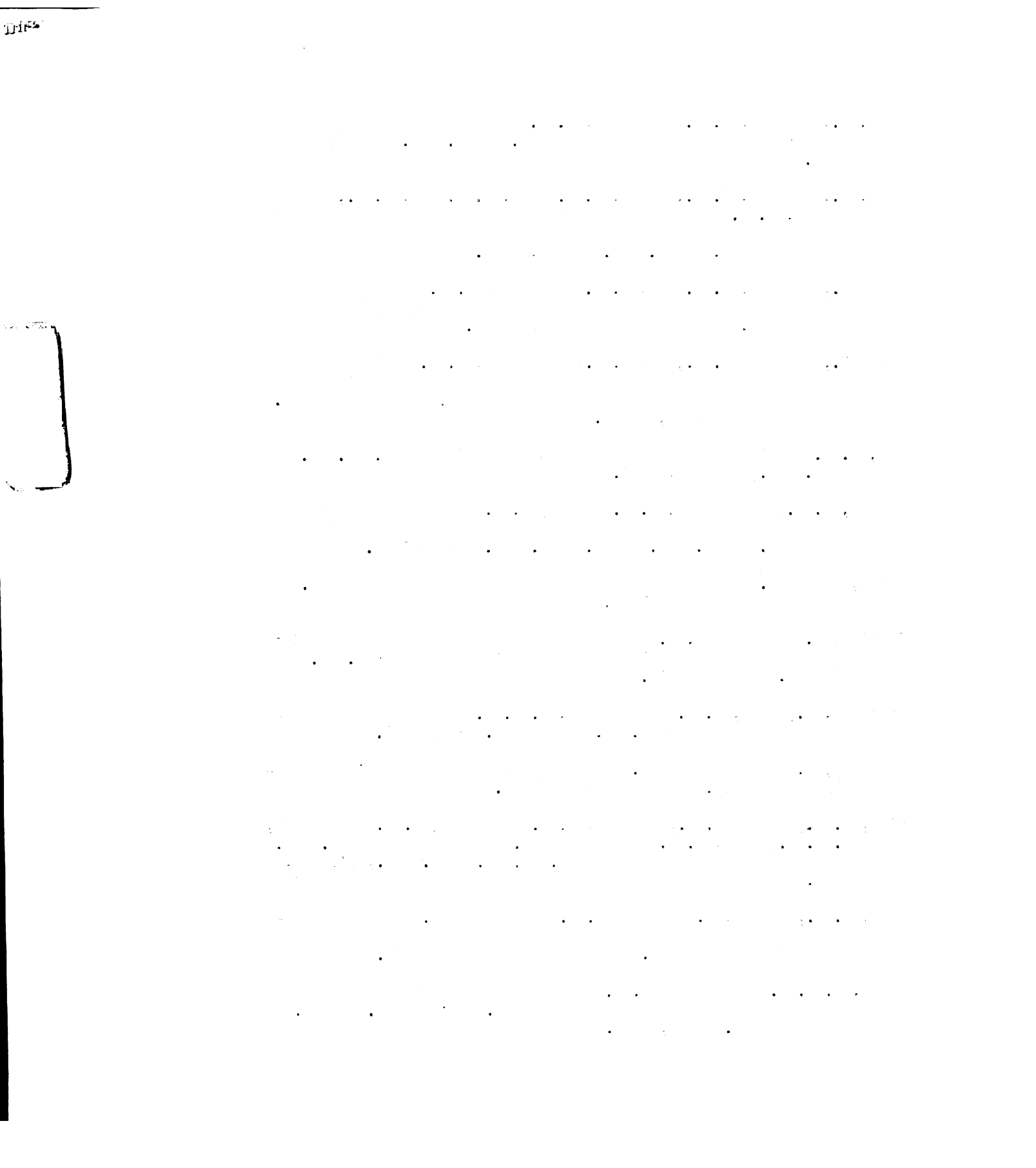

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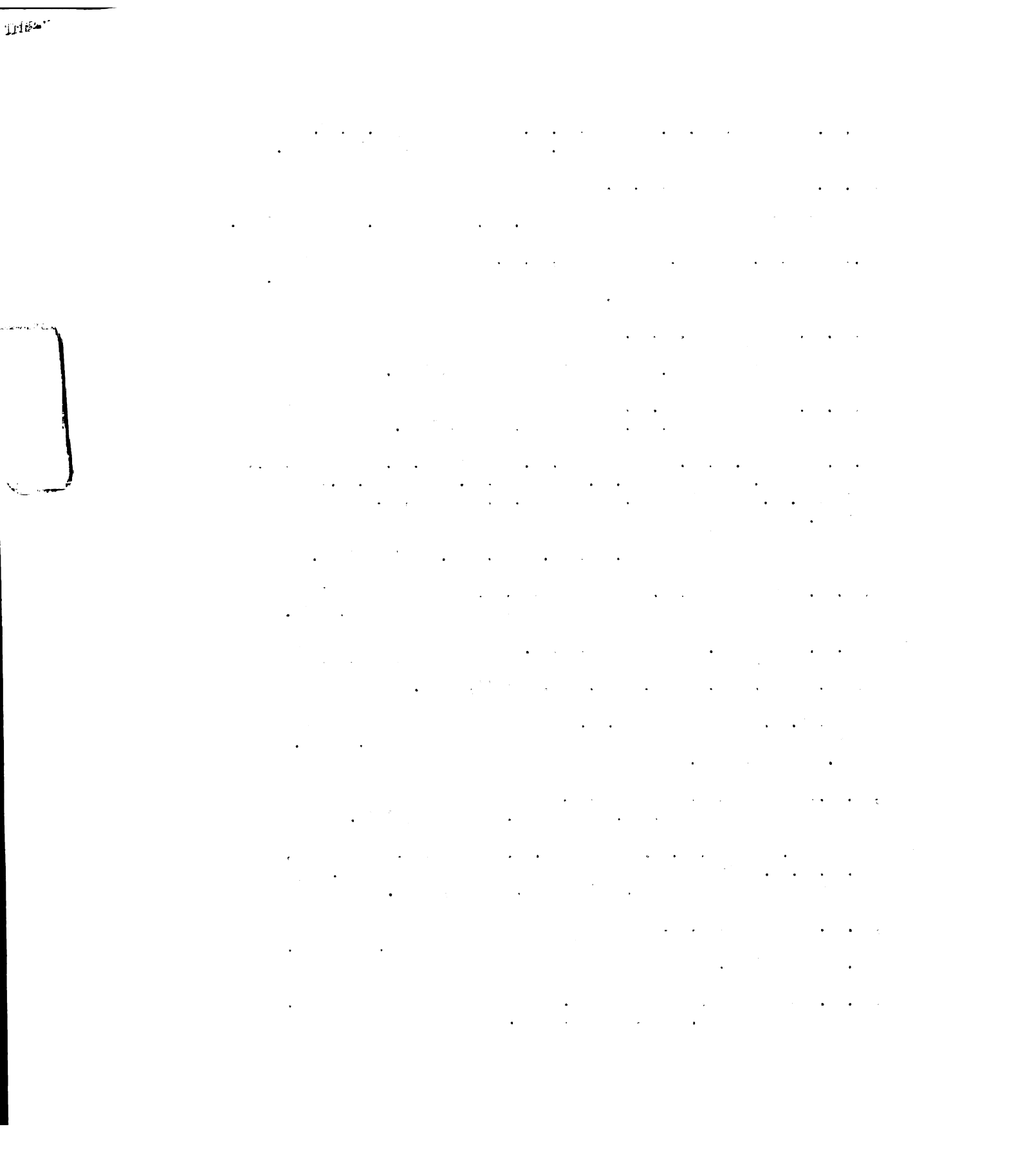

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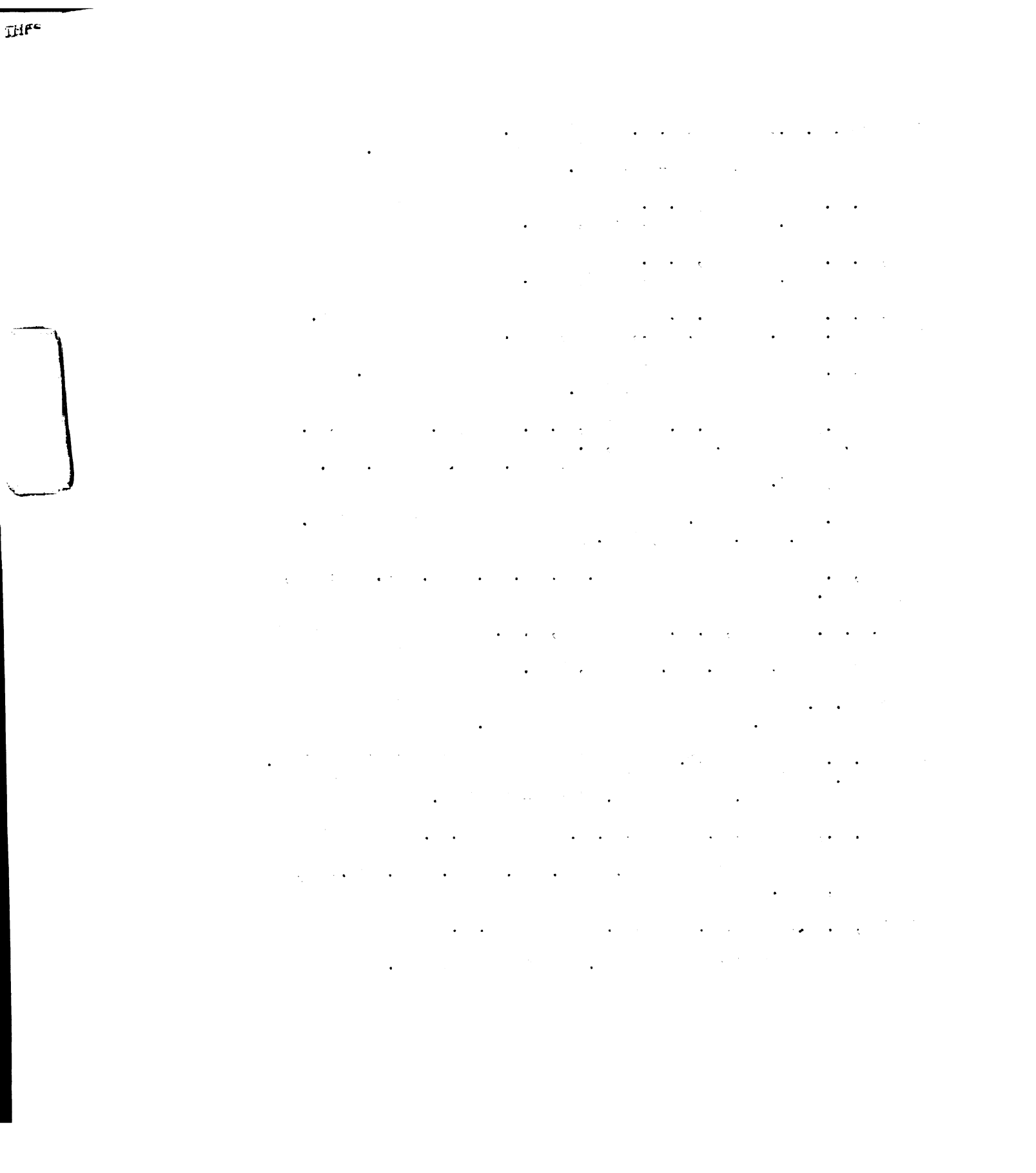

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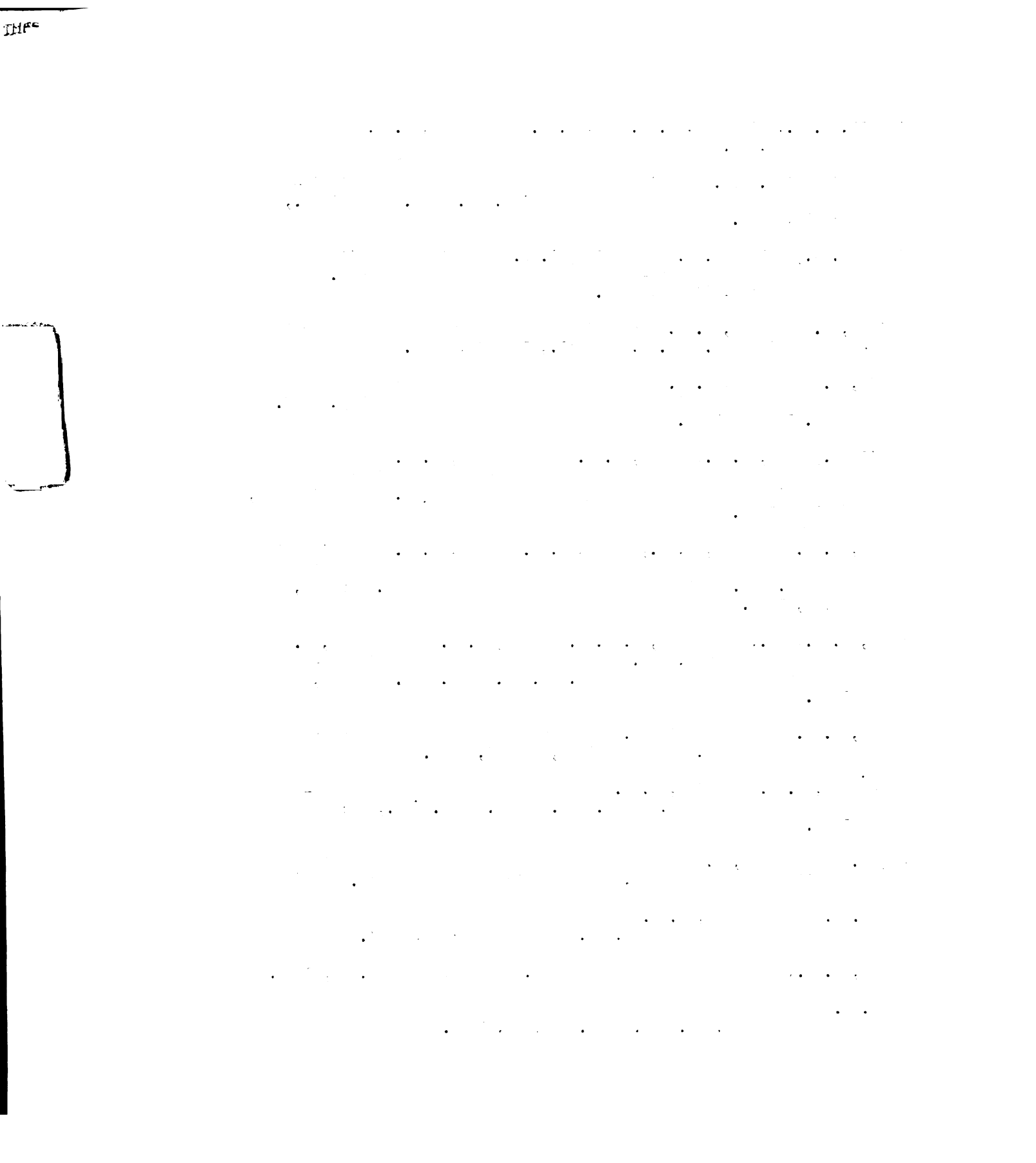

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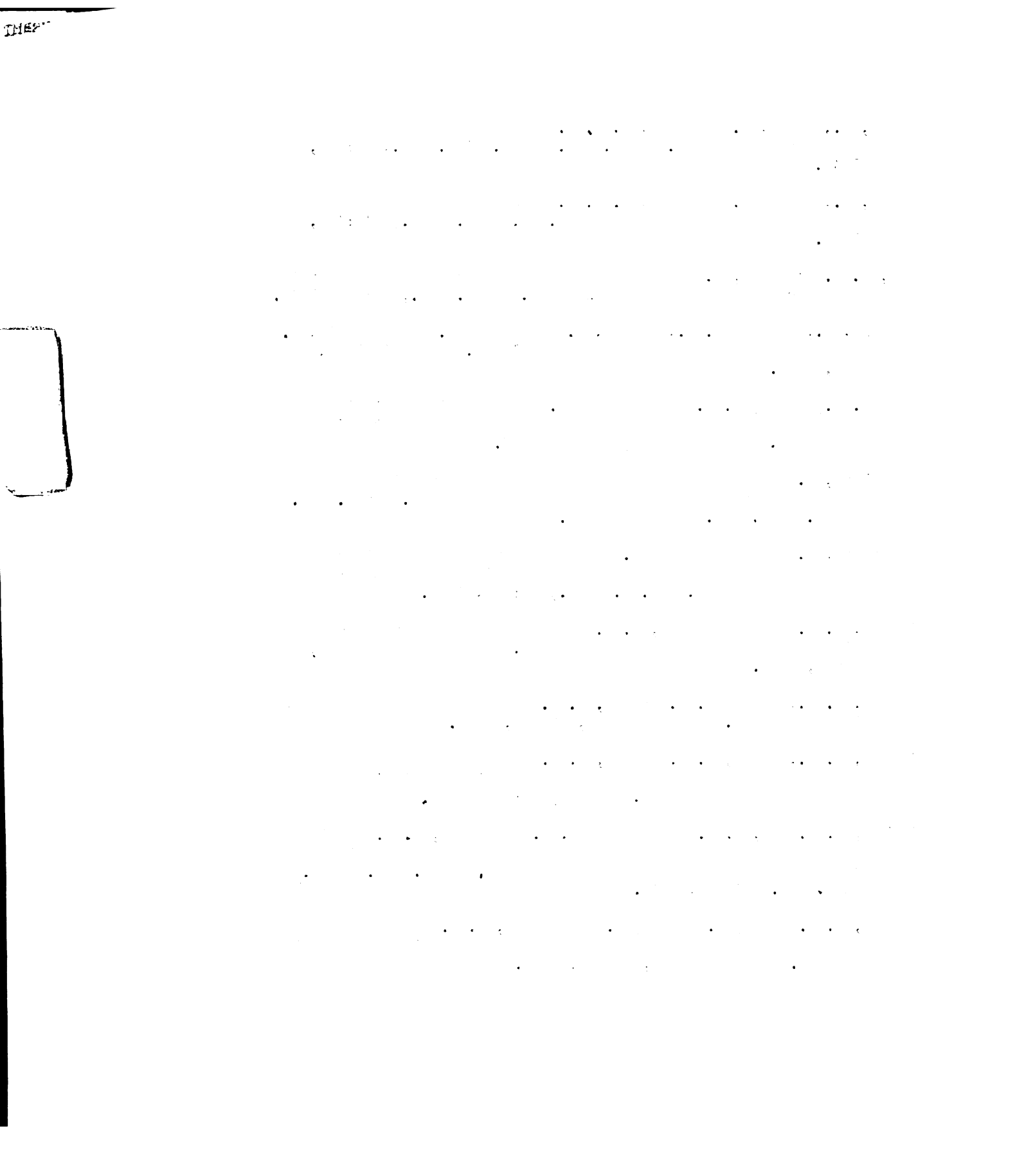

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