FURTHER STUDIES OF THE ACTION OF ANTUITRIN GROWTH ON ALBINO RATS DEFICIENT IN VITAMIN A

by

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INTRODUCTION

The factors which cause the animal to increase in size, or grow, have been objects of much speculation since the time of earliest man. The results of biological investigations have contributed much to our knowledge of the factors that control the rate, and limit the extent of those anabolic processes which result in growth. Very early it was realized that the prime requisite for growth was proper food; later it was learned that minute quantities of certain specific compounds were necessary for the utilization of food materials, and that only certain foods contained these vital compounds. These essential substances, of which little was known, were at first called vitamines, but after their composition came to be known, the error of such a term was at once apparent, and therefore, have since been called, vitamins.

In 1913 McCollum and Davis, and Osborne and Mendell, independently reported that certain natural fats were essential for growth and development of young animals. They described the symptoms which have become classic for vitamin A deficiency. Subsequent investigations have confirmed these findings, have determined the chemical structure of the vitamin A molecule (Holmes and

Corbet, 1937), and have contributed both chemical and biological methods by which the action of this substance could be studied (Carr and Price, 1926; Ellison, 1931; Baumann and Steenbock, 1932; and Aberle, 1933).

These advances made possible critical studies of the disturbances in the metabolism, and the concomitant anatomical changes in animals deficient in this vitamin. Classical conditions in avitaminosis A were found to manifest themselves in the following ways: failure to gain weight, xerophthalmia, shaggy coat and loss of hair, atrophy of intestinal mucosa, and constant cornification of the vaginal epithelium. Aberle (1934) and Sutton and co-workers (1934) reported neurological disturbances which culminated in paralysis in extreme cases, and which they explained, as some effect on the myelin sheath of the femoral and sciatic nerves by the absence of adequate amounts of vitamin A. When animals suffering from the above conditions were given food containing vitamin A before the conditions became too extreme, the animals returned rapidly to a normal condition. Baumann, Rising, and Steenbock (1934) found large quantities of vitamin A to be stored in the liver (approximately ninety-five per cent of all the stored vitamin A), while lesser amounts were present in the lungs and kidneys. Rats less than three

weeks of age, according to these workers, have only traces of stored vitamin A. However, after that age, the reserve, if obtainable from the diet, increases rapidly. Through the establishment of these facts the vitamin A molecule has been proved to be an essential substance for the growth of the animal body.

In addition to vitamins, the secretions of certain ductless glands have proved recently to be factors affecting growth. Such a substance is a secretion of the pituitary gland. Centuries before any insight as to its function was gained, anatomists ascribed many varied functions to this gland. It has been known since the time of Galen, and is thought to have been named from the Latin pituita, (which means mucus) by Vesalius because he considered it a gland which secreted mucus. Basing his conclusions on anatomical evidence, Conrad Schneider, about 1660, announced that it could not be a mucus gland and considered it a structure of vestigal origin. The first hint of the actual function of the gland came in 1864, when Verga described hypertrophy of the pituitary gland accompanying facial and digital overgrowth, a condition now known as acromegaly. The French neurologist, Pierre Marie, in 1886, was the first to determine a relationship of this disease to the pituitary gland and to show that it had some action on the

growth of the animal body. These findings were confirmed by Klebs in 1894 (Hoskins, 1933).

In 1921, Evans and Long were unsuccessful in changing the estrous cycle in rats by feeding anterior lobe of pituitary glands; they, therefore, tried in the same year, the effect of macerated anterior lobes of pituitary glands injected intraperitoneally. This proved to have very definite effects. In animals that received injections of the fresh gland, greater rate of growth was found, as well as inhibition of sexual development when compared with the untreated controls. These findings caused many workers to investigate further the cause of the action of this small gland. Evans et al in 1932, stated that the anterior pituitary gland contained a growth producing substance, that hypophysectomized animals became mature when injected with the substance but retained infantile genitalia, and that in large doses diabetes occurred. Again in 1935, Evans summarized the action of the growth hormone, reporting upon developments of its clinical use and progress in the isolation of the active growth principle. Since the anterior lobe of the pituitary gland secretes other endocrine substances, the problem of purification and separation of the active principles has been a challenge to many physiological chemists. The growth promoting substance is very

thermolabile and therefore has resisted attempts to determine its very complex chemical structure. At present workers must be content to consider it a substance of unknown composition and to study its manifestations in the animal body, rather than its structure.

Because the absence of vitamin A causes damage to many tissues and halts growth, which in many respects is similar to the condition in hypophysectomized animals; Wimmer, as early as 1931, conducted preliminary experiments on the possible relationship of vitamin A and the growth hormone. The results he obtained (unpublished) prompted him, in collaboration with Ayers, (Wimmer and Ayers 1936) to conduct further work along this line. They injected extracts known to contain the growth principle into rats in various stages of vitamin A deficiency in an attempt to bring the rate of growth up to that of normally fed animals. From these investigations they found indications that extracts of the growth promoting substance[1] of the anterior pituitary

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1. Because it is not known whether the growth hormone is a single substance or a group of active compounds, in this study reference will be made to the growth producing material of the anterior pituitary gland as a single hormone or substance. This does not imply that the active principle is considered to be a single substance, or a group of substances, but is used to simplify the wording regarding this principle.

gland, intraperitoneally administered, caused a partial return to normal condition in rats on a diet deficient in vitamin A. As an index of deficiency the vaginal smear method of Aberle (1933) was used. From these results, indications were such that possibly the growth hormone was not produced in the vitamin A deficient rat, and the failure of the animal to grow in this condition could be partly attributed to the absence of this substance.

The present work is a continuation of the investigation of the problem.

MATERIALS AND METHODS

Only albino rats of the Wistar strain were used during the course of this work. Experimental animals were the offspring of brother-sister matings, one male serving all brood females. Small litters were discarded; since, whenever possible, a single litter was divided to serve for both control and experimental animals. Any small or unhealthy looking rats were destroyed. Both male and female offspring were used, but they were divided equally so that the sex ratio in both control and experimental animals was the same.

The brood females were maintained in a large wire cage with the male, where they were given a stock rat diet ad libitum. Ordinary tap water was supplied twice daily. When the females became heavy, they were removed to smaller individual cages of wooden structure with one side covered with four mesh screen. These brood cages were so placed that they received direct sunlight during midday. Rats in such cages, receiving ample sunlight, seemed to maintain a much better physical condition than those kept in dark corners out of the sunlight. Wood shavings were kept on the floors of these cages and were replaced every three or four days.

Because pregnant rats deficient in vitamin A are prone to abort (Evans and Bishop, 1922; and Dann, 1934) and because the amount of vitamin A stored in the foetal rat is not influenced by the amount of carotin in the diet of the mother, pregnant females were given the stock diet until time of parturition. As Baumann, Rising, and Steenbock stated in 1934, the vitamin A reserve is built up in the young rats about three weeks after birth; and the vitamin A content of the milk during lactation is proportional to the amount of available vitamin A in the mother's diet throughout the lactation period. Therefore, after parturition the stock diet of the females was replaced by a standard vitamin A-free diet,[2] as prepared by Sherman and Munsel (1925). This food was also given ad libitum in small glass jars, having metal cones which prevented wasting of the food. These food jars were placed so that

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2. The vitamin A-free diet was prepared as follows:

Vitamin A-free casein	800.0 grams
Powdered dry yeast	400.0 grams
Osborne-Mendel salt mixture (Wesson modified 1934)	140.0 grams
Sodium Chloride	40.0 grams
Corn starch	2600.0 grams
Viosterol	3.6 grams

Vitamin A-free casein was prepared by placing dry commercial casein in a shallow metal pan, and heating in a constant temperature oven for seven days at one hundred and ten degrees Centigrade. It was stirred twice daily.

it was possible for the young rats to begin feeding as soon as they desired food other than the mother's milk. Water was given in shallow pans so that the young rats had ready access to it. These young were weaned as soon as they seemed to know how to eat and take care of themselves, which was usually about eighteen to twenty-five days after birth. It was found that the earlier the young rats were weaned, the shorter was the time before they became depleted of vitamin A.

The depletion and experimental pens measured ten inches by fourteen inches by eleven inches, and were so constructed that the whole cage was of screen, the bottom being of one-half inch mesh so that all feces could drop through to a receiving pan placed three inches below. In this way ingestion of urine or feces was impossible.

These cages were so placed that insects could not reach the rats by crawling up from the floor. The tops of the cages were made of tin so that it was impossible for insects to drop into the cages from the ceiling.

During the course of the experiment the colorimetric antimony trichloride ($SbCl_3$) test of Carr and Price (1926) for the presence of vitamin A was made on all materials suspected of containing vitamin A. Such a test was made on macerated whole cockroaches found in the animal room.

It was found that these roaches had definite quantities of the vitamin in their bodies. The test was performed as follows: A ten per cent solution of antimony trichloride ($SbCl_3$) was prepared in anhydrous chloroform ($CHCl_3$). To this mixture was added the dehydrated substance to be tested. As a standard, a ten per cent solution of copper sulphate ($CuSO_4$) was used. Since the color reaction is very unstable, the test was read immediately after mixing. The appearance of a color approaching that of the standard was taken as a positive test for vitamin A.

The hormone used, Antuitrin Growth, was supplied by the Parke Davis Company, in twenty cubic centimeter vials, biologically standardized to contain ten rat units per cubic centimeter.[3] During the early part of the experiment animals were injected both intraperitoneally and subcutaneously. Since no difference in the results was discernible, it was decided that the subcutaneous method of injection would be most advantageous, because it would eliminate the danger of rupturing the parchment thin intestines found in vitamin A depleted animals. All weighing was done by placing the rat in a small counter-weighted box. Daily

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3. One shipment of the hormone fell below the standard rat unit strength and was standardized to contain eight rat units per cubic centimeter instead of the usual ten. At this time, dosage was increased so that the same unit ratio was maintained.

weight records were kept in tabular form. For the purpose of comparison, photographs were taken at various times showing the animals in both typical and atypical conditions. At death an autopsy was performed on each animal, and the data recorded. The pituitary and adrenal glands, and sections of the uterus and ovaries in females were saved for histological study.

PROCEDURE

All rats were placed upon experiment at the time of weaning and since the vitamin A-free diet in the brood cage was the only food they had had beside the milk of the mother rat, no difficulty was experienced in getting them to eat properly.

The problem was divided into two series, Series I for the purpose of determining the action of Antuitrin Growth on rats which were depleted of vitamin A, and Series II was to determine the action of Antuitrin Growth in rats during the process of depletion.

The cages in which the rats were kept were numbered 1 to 4. Cages 2 and 4 were used for control animals, which were litter mates of the experimental animals. The experimental animals were placed in cages 1 and 3. The animals in cage 2 served as controls for the experimental animals in cage 1, while those in cage 4 served as controls for animals in cage 3.

In Series I, both control and experimental animals were given vitamin A-free diet and weighed daily. At the time when depletion was shown by extreme xerophthalmia, shaggy coat, and growth stasis, the animals in cages 1 and 3 were given injections of Antuitrin Growth. As

mentioned previously, during the early part of the experiment designated as Series I, the animals in cage 3 received intraperitoneal injections, while those in cage 1 received injections subcutaneously. Since no difference could be observed in the results obtained by these two methods, it was decided that the safest and easiest method would be to inject all animals subcutaneously, which was the method employed during the remainder of the experiment. The first litters of rats in Series I received six rat units of Antuitrin Growth. Since this appeared to have no immediate effect, the dosage was increased in successive litters until toward the end, ten rat units were being injected. The daily injections were continued until animals died or showed signs which indicated that they soon would die. At the time of death all rats were examined and the tissues were removed for histological study. Photographs were taken of both experimental and control animals at the time of extreme depletion. A total of thirty-eight animals was used in Series I.

The weights of all animals in cages 1 and 3 of Series I were averaged and plotted in graph form, and the average weight curve of all the control animals in cages 2 and 4 was superimposed upon this curve. (See Fig. 1.)

The rats of Series II were placed in the cages which were used for Series I, and the experimental animals occupied cages 1 and 3 while the controls were kept in cages 2 and 4. Treatment of the rats in this series was identical to that accorded those in Series I except that animals in cages 1 and 3 received subcutaneously, beginning at the time of weaning, ten rat units of Antuitrin Growth daily. These daily injections were continued until death of the animals. Pictures of both control and experimental animals were taken during the latter stages of vitamin A depletion. Tissues were removed from control and experimental animals and were preserved for histological study. A total of forty-seven rats was used in Series II. The weight record of all animals in cages 1 and 3 was averaged and plotted in graph form with the weight curve average of control animals in cages 2 and 4. (See Fig. 4.)

At intervals during the course of the experiment, rats in stock cages on normal diet were given subcutaneous injections of Antuitrin Growth. Weight records were kept of these animals along with weight records of litter mate controls; this was done as a check on the growth promoting activity of the extracts used.

RESULTS

In Series I, where the rats were injected only after they had become depleted of vitamin A, the results were quite uniform in that all but four of the 38 animals responded in a uniform manner throughout the experiment. The four that did not respond, died of anaphylactic shock. During the period of depletion the growth curves of control and experimental animals were almost identical (Fig. 1.). When the condition of the animals indicated that they were vitamin A-free and the weight curves reached a plateau, injections were started on all experimental animals. No change was noted in any of the injected animals when compared with those of control groups. With the exception of the four animals that died of anaphylactic shock there seemed to be no ill effects of the injected Antuitrin Growth, since no signs of toxicity were shown and no irritation was observed at the point of injection. The condition of the injected and control animals remained the same for the duration of the experiment. Both groups showed the progressively severe conditions which resulted from extreme avitaminosis A.

Death, which was directly attributed to the absence of vitamin A, occurred at approximately the same time for

control and injected animals in Series I. By comparing the growth curves of the control and injected animals (Fig. 1.) along with the photographic record of typical phases (Plate I), it was possible to deduce, that the albino rats depleted of vitamin A at the beginning of Antuitrin Growth injections, were not stimulated to growth by this treatment in doses up to ten rat units each day.

In Series II, where the experimental rats were injected with ten rat units of Antuitrin Growth daily throughout the period of vitamin A depletion, strikingly different results were obtained. Injected rats in this series responded at once to the growth preparation as evidenced by their greater growth rate when compared with that of the controls. This accelerated growth continued until the time of vitamin A depletion (See Fig. 4.). The plateau usually found in the growth rate of rats when they became vitamin A-free, was absent in the injected animals. They lost weight almost at once, and the onset of the conditions denoting extreme vitamin A deficiency was such that it caused the rats to die from four to eight days earlier than the control animals. Symptoms of nerve degeneration as described by Aberle (1934) were very prominent at a time early in the vitamin A depletions of the

injected animals in this group. In this series all injected animals showed a marked loss of hair in a region extending from the ears down across the top of the head and back along the nape of the neck. This bald pattern was very striking in that it persisted throughout this series in the injected animals (Plate II).

From studies of the growth curves of the rats in the control and experimental groups of Series II, along with the physical manifestations present in both groups, it was observed that the rats receiving Antuitrin Growth throughout the period of vitamin A-depletion showed an increased rate of growth, over the control group, only as long as vitamin A was present in the rat's body. After complete depletion, which was accelerated by injections of Antuitrin Growth, the growth promoting substance was without action in the animal body.

In Series I and Series II the following sequence of events occurred. Animals at first ate well, but after some time did not consume as much food as at the beginning of depletion. The first sign of a disturbance was the change of the feces from the typical oval rat pellet to a shapeless, pasty, foul-smelling, brown mass. Shortly after this, the hair lost its luster and grew

shaggy. Concurrently a reddening of the eye lids ensued, which in a few days developed into a definite scabby encrustation by which the vision of the rats was impaired. This cornified condition of the eyes is known as xerophthalmia. At the time of extreme xerophthalmia the rats failed to gain weight and a short period of time followed in which the animals neither gained nor lost weight. It was during this plateau period that the neurological conditions described by Aberle (1934) began to manifest themselves. After a latent period of four to seven days the animals began to lose weight and became very inactive, remaining curled up in their cages with their heads tucked under their bodies. Also at this time there was a continual seepage of urine which caused the genitalia to become encrusted with urine salts and to be highly inflamed. In these last stages the rats exhibited extreme photophobia and would try to avoid strong light, a condition which made photographing them very difficult.

In the animals of the injected group of Series II extensive hepatic hemorrhage was found at the time of death. A jaundiced condition accompanied this. In the control animals no hepatic hemorrhage was present but evidence of nephritic hemorrhage was observed and the

death of the control animal could be predicted several days in advance by the presence of bloody urine.

Histological studies of the pituitary gland gave a picture of increased numbers of basophilic cells as compared with those of normal animals. This condition was present in both control and injected animals throughout both of the series and agrees with the recent work of Sutton and Brief (1939), in which they explain the increase in basophilic cells as a result of damage to the gonads by the absence of vitamin A. Critical cell counts have not been made to determine whether or not any difference was noted in the conditions of eosinophiles present in animals receiving injections of Antuitrin Growth. Since the manufacturer stated that the extract contained, besides the growth promoting principle, small amounts of gonadotropic and thyrotropic hormones, no attempt was made to use the condition of the organs of reproduction as a criterion of vitamin A depletion. Temporary mounts of the retinas of rats of both the control and injected groups of both series showed marked rod destruction and complete absence of any visible visual purple as fixed by the method of Johnson (1939).

DISCUSSION

Because of the complex nature of the growth hormone, and its close association with other hormones of the anterior pituitary gland such as the sex stimulating factor and thyroid activating principle, side reactions, which might have been caused by residual quantities of these other substances, were not used as criteria of either the growth hormone of the anterior pituitary action, or vitamin A deficiency. The Parke Davis Company, from which the Antuitrin Growth was obtained, stated that small quantities of gonad stimulating substances and thyrotropic substances were present in the extracts used. In the presence of even small quantities of gonad stimulating substances some action upon the gonads could be expected and because of this action, it was felt that the vaginal smear method as described by Aberle (1933) and subsequently used by Wimmer and Ayers (1936) should not be used as an index of vitamin A depletion. Also Richter and Bruno (1939) have shown that the activity cycles persist in rats even though the vaginal smear is constantly cornified as a result of vitamin A depletion. The rate of growth was measured at all times by weight,

and since growth rate was used as an index of both the absence of vitamin A and the action of Antuitrin Growth, it was possible to determine whether any physiological interrelationship of the actions of these substances existed. It was not the purpose of this work to show how the vitamin A molecule permitted growth, nor how the growth hormone stimulated growth, but rather to ascertain if the failure of the animals to grow when depleted of vitamin A could be overcome by injections of the growth promoting substances of the anterior pituitary gland. Resumption of growth rate when the growth hormone of the anterior pituitary gland was injected into avitaminosis A rats showing growth stasis, would have indicated that some relationship existed in the growth regulating activities of the two substances. The results of early work of Wimmer and Ayers (1936) and Wimmer (unpublished) indicated that the condition described above might be the case and a theory was formulated that perhaps the vitamin A depleted pituitary gland produced no growth stimulating hormones, and that therefore the injection of the growth hormone might relieve some of the symptoms of avitaminosis A, and some growth might be induced. Because in the animals of both Series I and II no indication of any growth stim-

ulation was observed, it is believed that these two growth regulating factors are at least dissociated to the extent that the pituitary hormone which stimulates growth cannot act in the absence of the vitamin A molecule. It would seem that these two substances were both necessary for growth of the animal body, and that the function performed by each was separate and could not be compensated for by the substitution of the other.

The condition of the eyes in vitamin A deficiency, probably because of its striking nature, has been the object of much investigation. In spite of the fact that the presence of xerophthalmia was not concerned with growth and did not have a direct bearing on this problem, it was of interest to note that rats receiving Antuitrin Growth after depletion had a xerophthalmia which paralleled that of the vitamin A-free controls. Upon examination of the retinas of these animals a greater tissue destruction was found in the injected animals than in the controls. The greatest damage was found on the distal end of the rods; these structures were extremely wrinkled and in many cases ruptured. Antuitrin Growth seemed to accelerate this rod destruction, as the eyes of animals receiving injections were much more severly affected than were the eyes of the

animals which had received only vitamin A-free diet. This agrees with Hecht (1938) who found a definite rod and cone destruction in rats deficient in vitamin A.

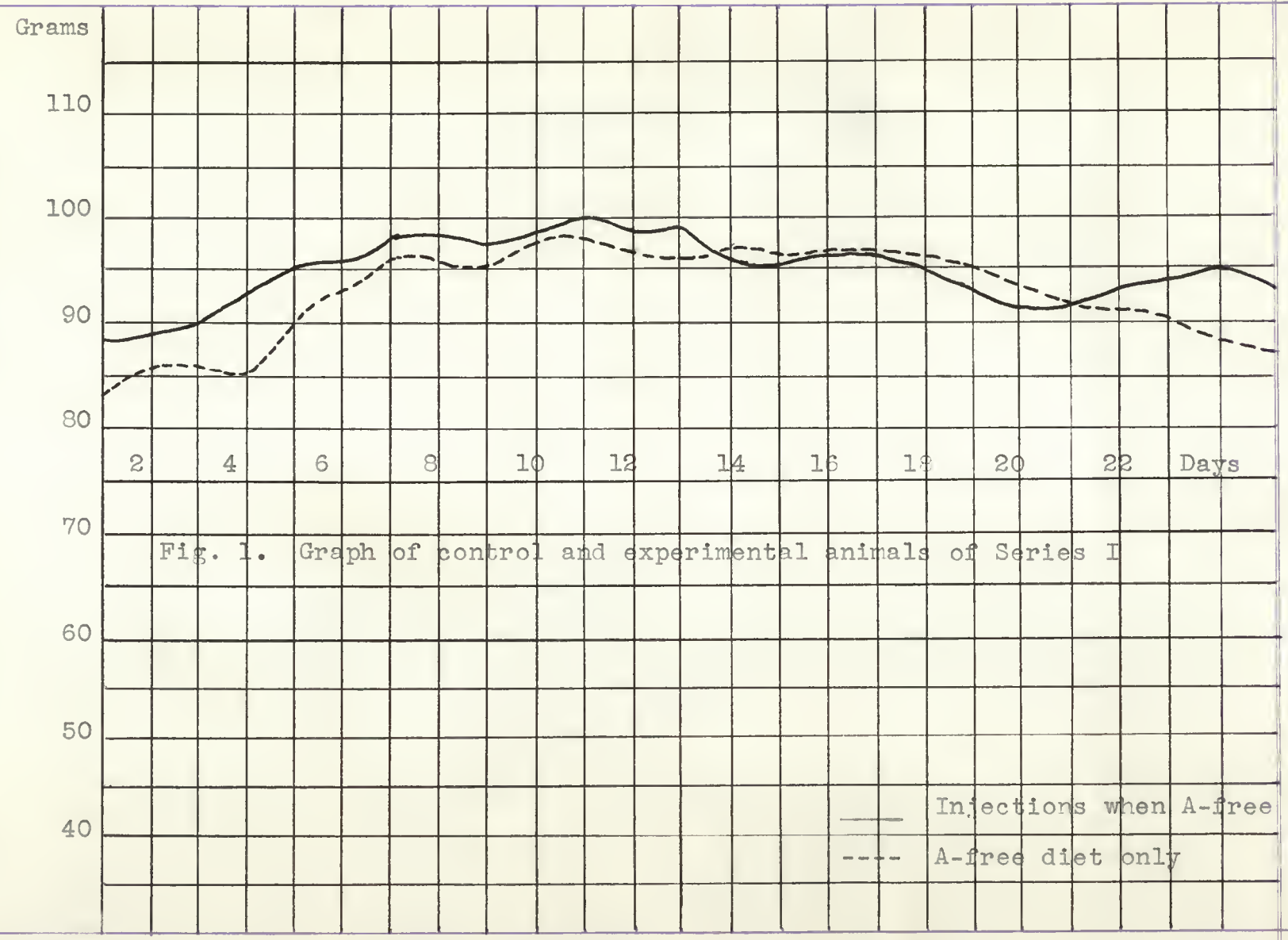

Fig. 1. Graph of control and experimental animals of Series I

EXPLANATION OF PLATE I

Fig. 2. Animal from the control group of Series I. This animal was maintained on vitamin A-free diet only and received no Antuitrin Growth at any time.

Fig. 3. Animal from the injected group of Series I. This animal received Antuitrin Growth daily from the time of vitamin A depletion until death.

PLATE I

Fig. 2.

Fig. 3.

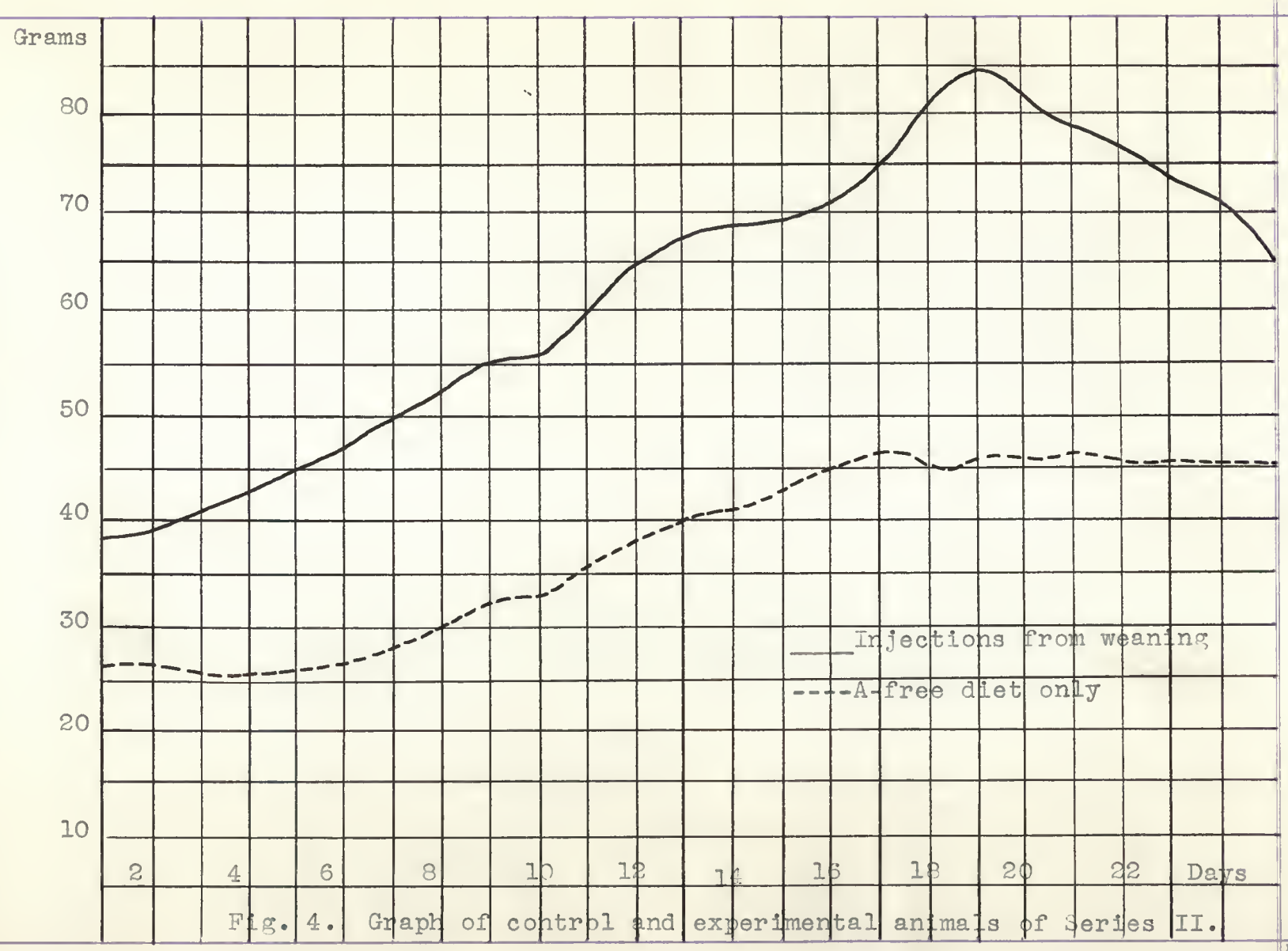

Fig. 4. Graph of control and experimental animals of Series II.

EXPLANATION OF PLATE II

Fig. 5. Control animal from Series II. This animal received only vitamin A-free diet with no injections of Antuitrin Growth.

Fig. 6. Animal from the injected group of Series II. This animal received daily injections of Antuitrin Growth throughout the period of vitamin A depletion. (note the bald condition)

PLATE II

Fig. 5.

Fig. 6.

SUMMARY

The experiment, Further studies of the action of Antuitrin Growth on albino rats deficient in vitamin A, was divided into two main series. In each an equal number of control animals was kept along with the experimental animals. Each series was divided into groups in which either single litters or combined litters of the same age were used. The animals in Series I were given injections of Antuitrin Growth only after they had become depleted of vitamin A, while the animals in Series II received injections of Antuitrin Growth during the progress of vitamin A depletion.

The injected animals in Series I, at no time during the experiment, showed growth differences when compared with their litter-mate controls. The animals in Series II, which received injections during vitamin A depletion, showed a marked increase in growth rate over their uninjected controls, up to the time they became completely depleted of vitamin A. After vitamin A depletion, all growth stopped and the animals died of extreme avitaminosis A, in many cases several days sooner than their litter-mate controls. The animals in Series II which received injections of Antuitrin Growth, developed a characteristic baldness which extended from the ear

region of the head back along the nape of the neck. This condition persisted throughout the experiment for the injected animals in Series II. In no case during the experiment was the condition of xerophthalmia improved by injections of the Antuitrin Growth.

Vitamin A and the growth hormone (or hormones) of the anterior pituitary gland are not related in so far as a replacement of vitamin A by an excess of the growth promoting substance of the anterior pituitary gland is concerned.

The presence of an excess of the growth promoting substance of the anterior pituitary gland in rats being depleted of vitamin A, lessens the time necessary for such a depletion.

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