

AN INVESTIGATION INTO THE POSSIBLE RELATIONSHIP
BETWEEN VITAMIN C AND THE ADRENAL CORTEX
OF THE GUINEA PIG

by

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B. S., Kansas State College
of Agriculture and Applied Science, 1948

A THESIS

submitted in partial fulfillment of the
requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1949

INTRODUCTION AND REVIEW OF LITERATURE

It has been suspected that there is some connection between the adrenal gland and vitamin C. The facts that point to a possible connection include the large stores of vitamin C found in the adrenal (Turner, 1948, p. 226) and the fact that vitamin C-deficiency and adrenalectomy cause symptoms which in some respects show surprising similarities. As an example, Turner (1948, p. 230, 232) reports that adrenalectomy causes a decrease in the volume of blood plasma and an interruption in reproductive functions, while for vitamin C-deficiency, Harman and Kordisch (1945) report a circulatory change that may be due to a decrease in blood volume, and Goettsch (1930) and Kramer, Harman and Brill (1933) report interference with reproduction and degenerative changes in ovaries and testes.

Additional indications of a relationship were found by Quick (1933), who reported marked hypertrophy of the adrenals in animals on vitamin C-deficient diets, and Deane and Morse (1948) who claimed, on the basis of histochemical evidence, that the cells of the cortex lose the power to secrete their steroids when vitamin C is not present. Dugal and Therien (1949) have recently reported that adrenal enlargement in rats exposed to the cold has been prevented by large doses of vitamin C.

Lockwood and Hartman (1933), investigating the effects of cortin on scurvy, found that cortin did not prevent scurvy but that it delayed its onset while Svirbely (1935) found on the

other hand that cortin had no effect in scurvy. In short, there seems to be ample evidence of some relationship, but the relationship has not been definitely established.

METHODS AND MATERIALS

Guinea pigs were selected as the experimental animals since they are among the few small experimental animals which respond to vitamin C-deficient diets. In this work, 29 guinea pigs obtained from the Department of Animal Husbandry were used. They were not specially selected and since no age records were kept, any reference to age that follows is a relative one based on the assumption that the heavier animals were the older ones, an assumption one has no reason to question.

The problem was approached from the angle that if the action of vitamin C-deficiency was to destroy or debilitate the adrenal, then perhaps measures which maintain adrenalectomized guinea pigs in normal condition would also maintain vitamin C-deficient guinea pigs.

Experimental Conditions

The experimental conditions chosen under which observations were made were as follows:

Normal Diet. "Rockland Guinea Pig Diet", (a pellet feed produced by Rockland Farms of New City, New York) with supplementary fresh green stuff was considered a normal diet, experience having demonstrated that this diet of pellets plus green stuff is one that will satisfactorily maintain normal animals.

With the thought that a strictly standard normal diet would be desirable, pellets alone were fed in many cases, but, as will be shown, the results obtained proved unsatisfactory.

Excess Vitamin C. The condition of excess vitamin C was established by oral administration of 5 mg per day of Cebione (crystalline vitamin C, Merck & Co.). This dosage was chosen because Harman and Miller (1939) found that .5 mg of Cebione per day per 100 gm of body weight would fill the vitamin C-requirements of guinea pigs on a vitamin C-deficient diet. Since the animals in this experiment weighed from 400 to 800 grams, it was considered that the 5 mg of Cebione administered per animal per day was more than enough to insure an excess.

Vitamin C-Deficiency. Sherman, LaMer and Campbell (1922) set forward the diet used to produce vitamin C-deficiency. It has been used and considered adequate by Harman and Miller (1939).

NaCl. One percent NaCl, administered as drinking water, was used as a form of adrenal replacement therapy. Various salt combinations have been used for adrenal replacement therapy (Rubin and Krick, 1934; Gaunt, Tobin and Gaunt, 1935), but

this one of one percent NaCl as drinking water was chosen as it seems to have proved as satisfactory as any for maintaining animals with nonfunctional adrenals.

Cortin. A second method of replacement therapy used was the subcutaneous injection of whole extract of adrenal cortex. Eschatin (whole extract of adrenal cortex, Parke, Davis & Co.) was employed in the dosage of $\frac{1}{2}$ cc once or twice a day. Since each cc contained 50 dog units of cortin (one dog unit being the amount required to maintain 1 Kg of adrenalectomized dog for one day), the dosage of from 25 to 50 dog units per day was considered more than ample to supply 400 to 800 grams of guinea pig. Unfortunately, no reference to the cortical extract requirements, expressed in dog units, of adrenalectomized guinea pigs was found in the literature so the adequacy of the dosage must remain an assumption.

Methods of Evaluating Results

Survival. In earlier work, survival time was one of the chief observations, with the animals being allowed to die of vitamin C-deficiency. In some few cases animals considered to be at the point of death were killed. Records of survival time were kept because it was thought that the beneficial effects of adrenal replacement therapy, if present, would show most clearly in increased survival of animals on a vitamin C-deficient diet.

In some later observations as noted below, the animals were not allowed to die but were taken only to the approximate beginning of weight loss and then killed.

Body Weight Curves. Body weight curves were kept with the thought that adrenal replacement therapy if beneficial would show its effects in increased ability to maintain weight. These curves were established by weighing the animals at intervals which varied from 2 to 7 days in various experiments.

Water Consumption. Water consumption was determined by weighing water cans at regular intervals, computing the average consumption per day over that period and recording the result on a graph. It was thought that these records might prove of value since Mark (1943) has already established a relationship between adrenal activity and water consumption.

Blood Sodium Level. If vitamin C-deficiency affects the adrenals it would be quite apt to affect the blood sodium level, for one of the chief functions of the adrenals is the control of the sodium level in the blood. With this in mind, 2 to 5 ml blood samples drawn immediately after death from the heart, and occasionally the post cava, were turned over to the Biochemistry Department for analysis of total blood sodium.

Examination of Testes and Ovaries. As was mentioned in the Introduction, the gonads are affected both by vitamin C-deficiency and adrenalectomy. For that reason, samples of testes from experimental animals were fixed in Flemming's Strong Solution and ovaries were fixed in Bouin's Solution.

Specimens were then imbedded in paraffin and one slide of serial sections was prepared from each piece of tissue. These slides were mordanted in iron alum for 24 hours and then stained in Haedenheim's Iron Haemotoxylin for 24 hours before being destained and covered.

General Gross Observations. As a source of information, general observations on such things as appearance, movements, eating habits and autopsy findings were recorded.

Outline of Experiments

In the following recording of results, the work is classified in three experiments.

Experiment I. All animals in this experiment were kept on a scoubutigenic diet until they died of scurvy. While on the scorbutigenic diet they were treated with sodium chloride and cortin as noted. Water consumption records were kept on these animals.

Experiment II. After experience was gained in working with the animals of Experiment I, Experiment II was run. The only differences between the two experiments were the administration of $\frac{1}{2}$ cc of cortin twice a day, instead of once a day, to some of the animals of Experiment II, the fact that no water consumption records were kept during Experiment II, and the modification of a few points of technic as dictated by experience with Experiment I.

Experiment III. In this last experiment were animals treated with all possible regimens as outlined above. These animals were not allowed to die of scurvy but were killed at the point it was felt that weight loss was about to set in. This was done in an attempt to separate the effects of scurvy from the effects of starvation. Water consumption records were kept.

Only those animals that actually lived long enough to become vitamin C-deficient are recorded in this paper. There were some animals that refused to eat the vitamin C-deficient diet, and as a consequence died of starvation. There are also a few animals omitted that died of abortions, infections and unknown causes while still in the first few days of the experiment.

RESULTS OF OBSERVATIONS

Survival

The plotted points in Fig. 1 show the weight of each significant animal of Experiments I and II at the start of the experiment and the number of days it lived before dying of scurvy. The key indicates the treatment given each animal.

This figure shows that there is a relationship between the weight at the start of the experiment and the survival time, with the heavier, older animals withstanding the effects

of vitamin C-deficiency for a longer time. There is also an interesting grouping of animals receiving cortin at the lower right of the graph. The animals receiving $\frac{1}{2}$ cc of cortin twice a day show the longest survival per gram of weight of any of those recorded. It should be noted that animals 5-2, 6-2 and 7-2 are littermates.

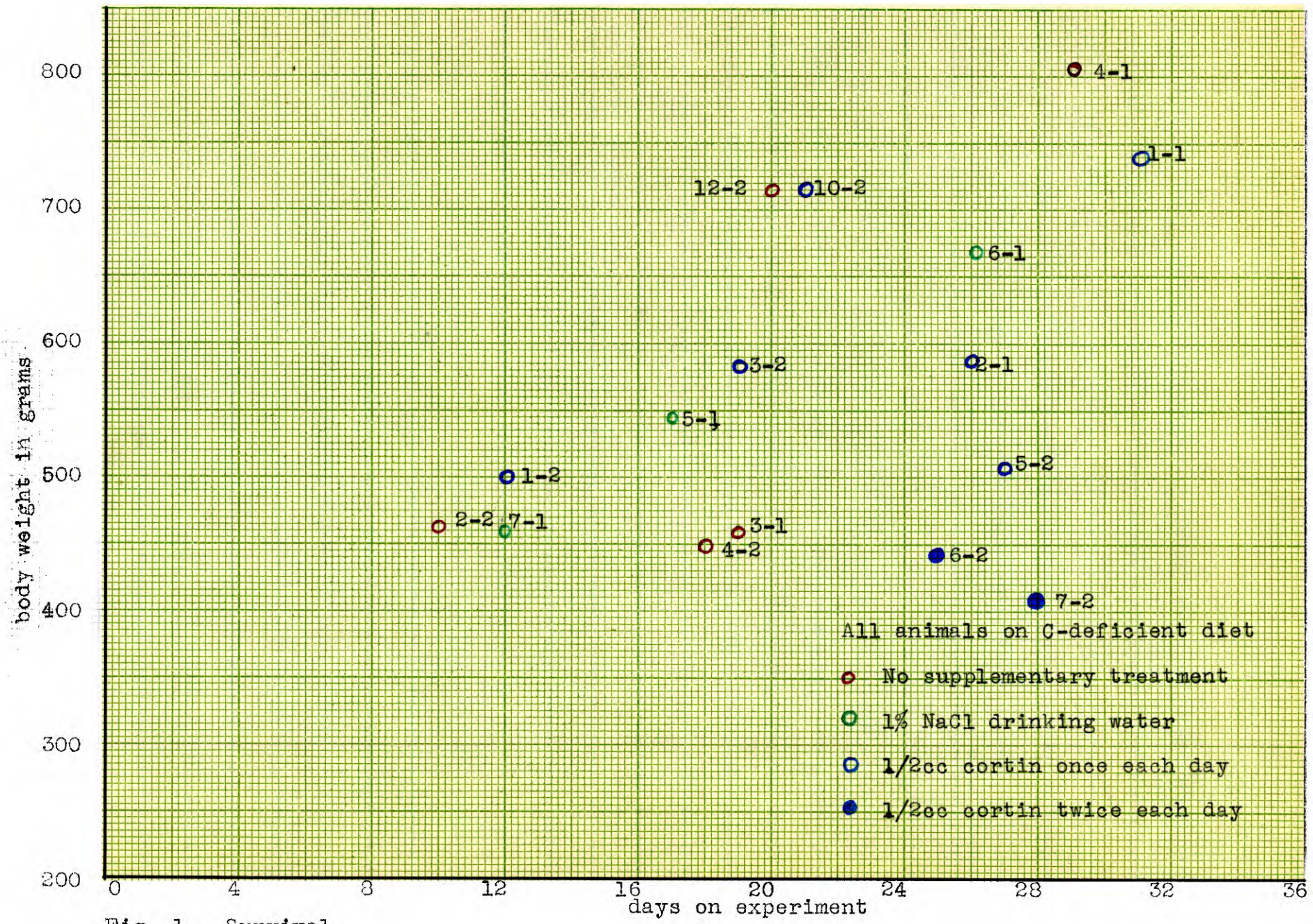


Fig. 1. Survival.

Body Weight Curves

The body weight curves of Fig. 2 show that animals on a vitamin C-deficient diet are maintained from two to four weeks at a fairly constant weight. After that period of constant weight there is a rather sharp break and the animals then lose weight at a constant high rate until death. These curves show no obvious relation between the shape of the body weight curve and the treatment given the animal.

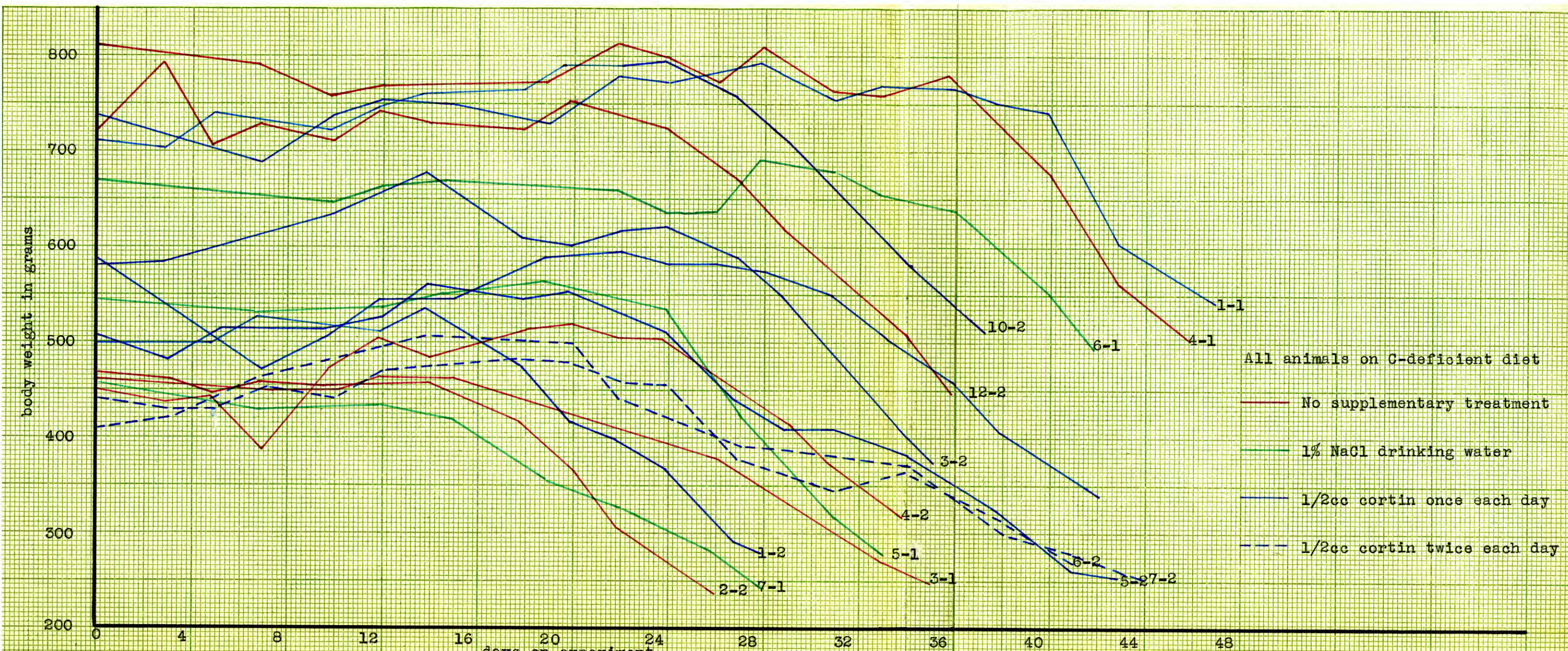


Fig. 2. Body weight curves, experiments I and II.

Figure 3, showing the relation between the weight of each animal of Experiments I and II at the start of the experiment and the approximate day on experiment when it began its sharp weight loss, was constructed to aid in the interpretation of the body weight curves. It shows that the heavier, older animals are maintained longer at a constant weight and it contains a faint suggestion that cortin may delay the processes of scurvy to a slight extent.

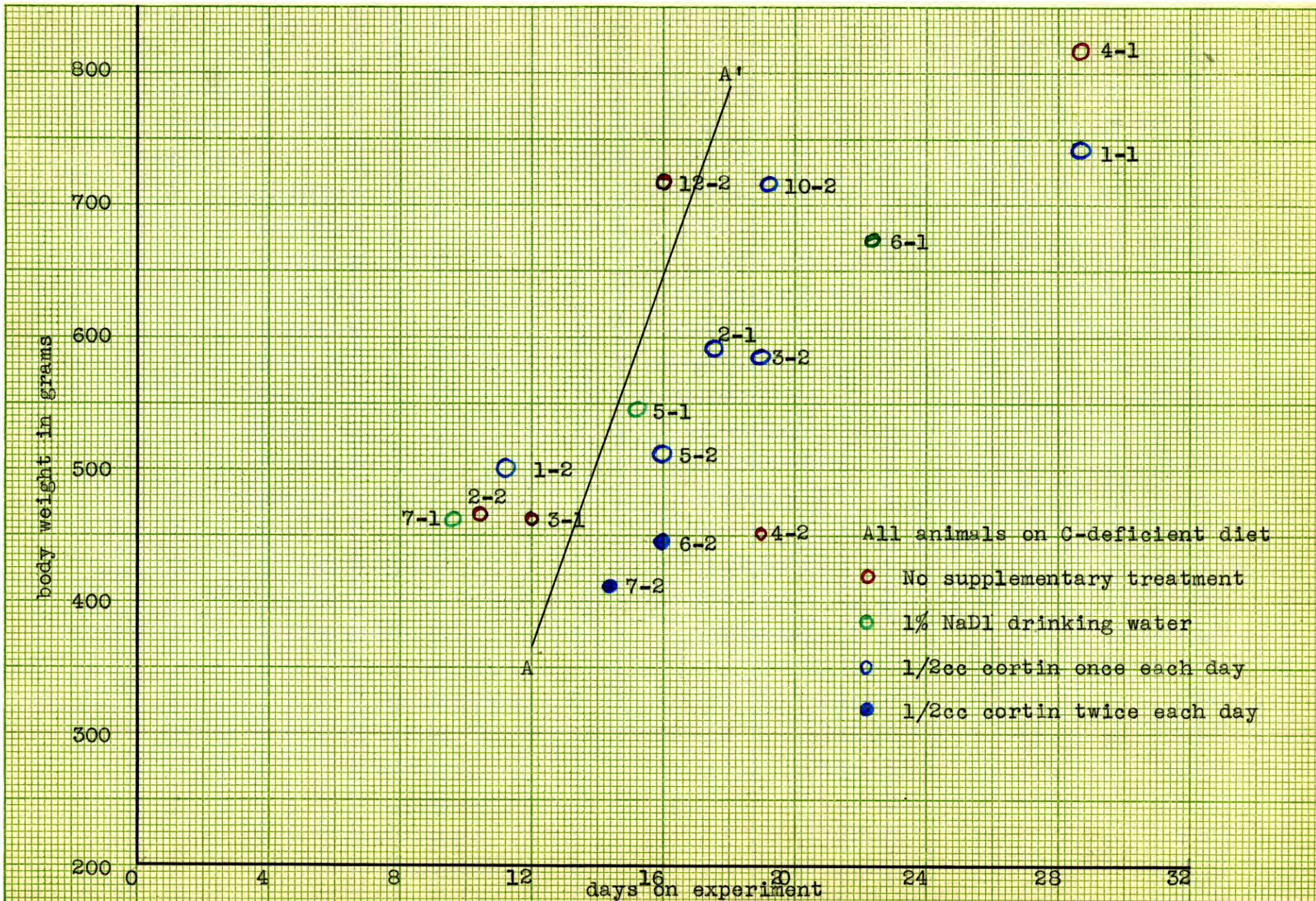


Fig. 3. Days until loss of weight set in as a result of C-deficiency.

A comparison was made between the terminal portions of the body weight curves of Fig. 2 and the starvation body weight curves of Fig. 5. The marked similarity brought up a new problem. Are the changes being observed due to vitamin C-deficiency or are they secondary changes produced by starvation which in itself seems to be a result of vitamin C-deficiency? Experiment III was run to investigate the problem, and in Fig. 4 the body weight curves of Experiment III are given.

This figure shows that all animals in Experiment III were killed before any large starvation effects had set in. Animals 27-3 and 29-3 started losing weight about the twelfth day as could have been predicted by an examination of Fig. 3, but the weight loss was not excessive at the time of death.

Animal 28-3 was given excess vitamin C to offset the effects of the scorbutigenic diet and the rising body weight curve for this animal stands in marked contrast to the falling curves for all other animals on this diet. In fact, it shows a greater gain in weight than might be predicted through an examination of normal body weight curves in Fig. 5.

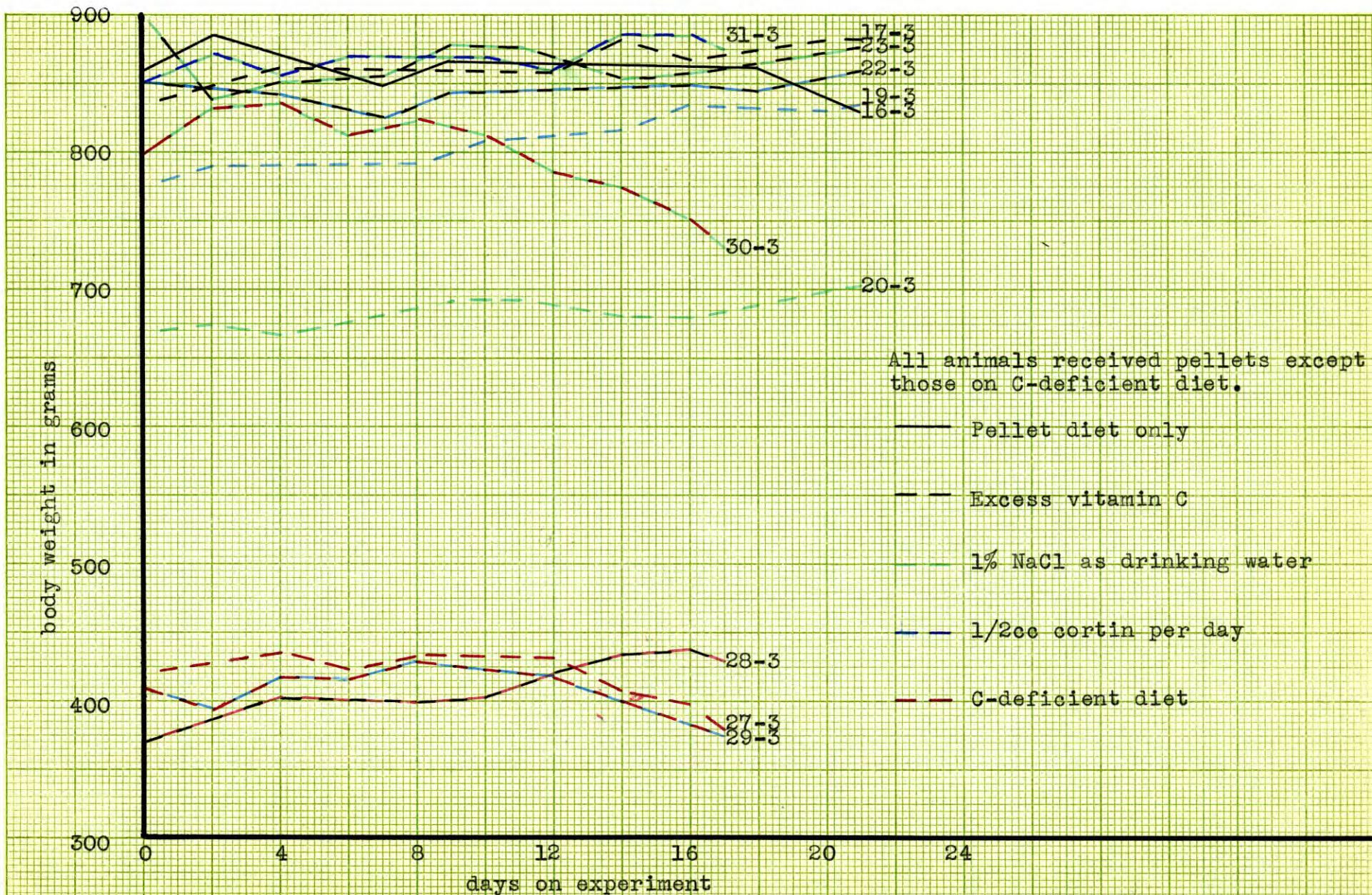


Fig. 4. Body weight curves, experiment III.

Figure 5 shows body weight curves of normal guinea pigs of various weights as worked out by Bessesen and Carlson (1923) and also a few curves from our experience of animals given water but no food. These curves are presented to give a standard with which to compare previous body weight curves.

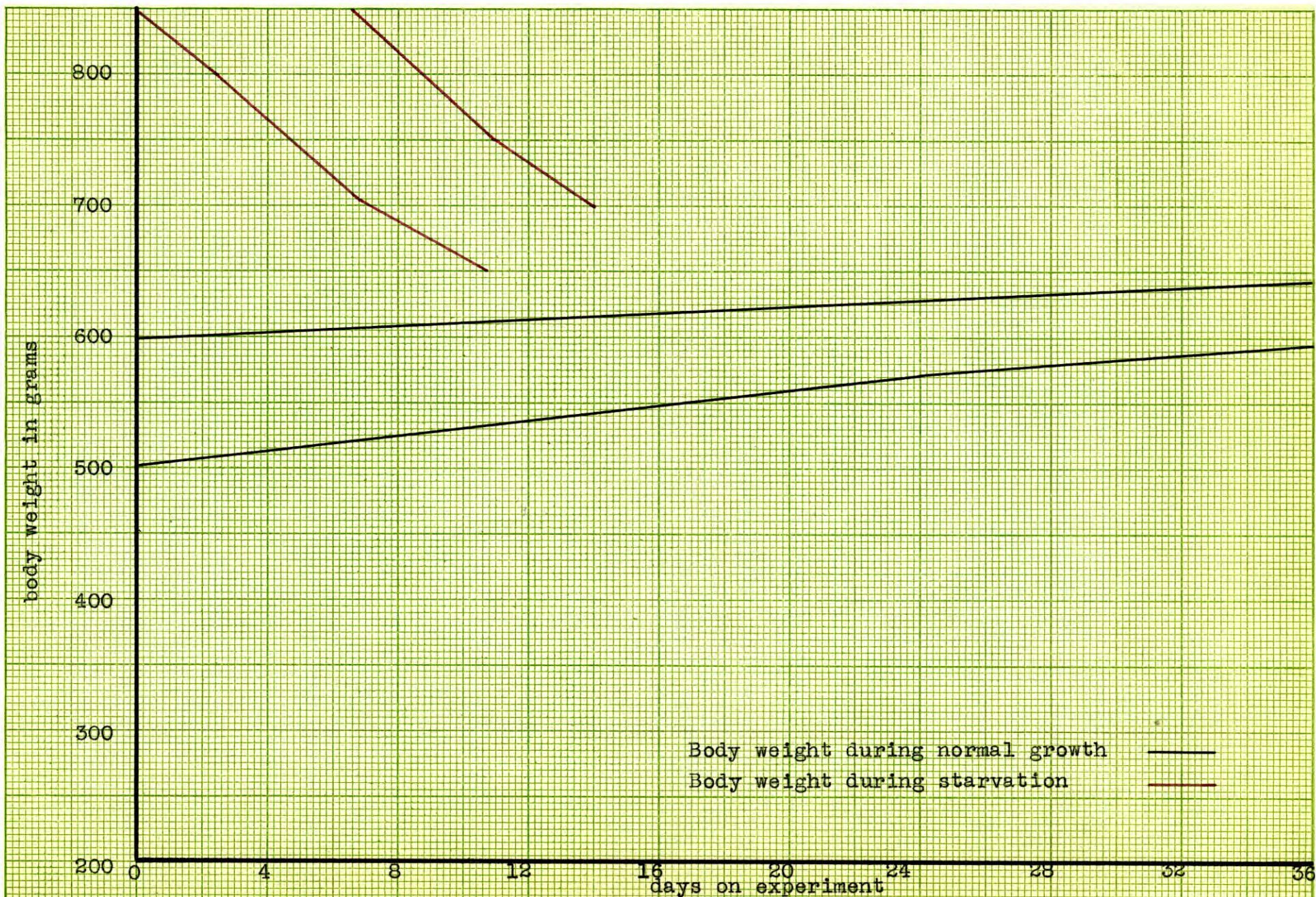


Fig. 5. Body weight curves, normal growth and starvation.

Water Consumption

Graphs were kept of water consumption but they are not included in this paper since they are highly irregular and complex, and reveal little information. However, one or two general statements can be made as a result of a study of the original data.

There was no apparent correlation revealed between treatment and water consumption save for the fact that animals on vitamin C-deficient diet, no matter what additional measures were used, seemed gradually and rather uniformly to decrease their water consumption from about 80 ml per day to about 40 ml per day, with the decrease in water consumption starting at approximately the time the weight loss set in. This, however, is only a general observation and correlations with treatment may be present, but if so they are hidden by individual, day to day changes in consumption.

Blood Sodium Levels

Figure 6 shows the blood sodium levels found for the animals of Experiment III. The treatment of each individual was that indicated by the key.

Samples included in Group A were collected May 16 and sent to the Biochemistry Laboratory for analysis. Samples in Group B were collected June 16 from different animals and sent to the Laboratory where they were analyzed by a different technician. It will be noticed that there is no overlapping of values between the two groups.

There is no apparent correlation between the blood sodium levels and experimental conditions save for the grouping of vitamin C-deficient animals at the top.

Though it is not recorded in Fig. 6 because it was not a part of a regular experiment, a blood sample was collected from a healthy, pregnant guinea pig, the discard from a previous piece of work in which all but a very small tab of its adrenal tissue had been removed some time previously. This blood sample showed the exceptionally low value of 45 mg Na/100 ml blood.

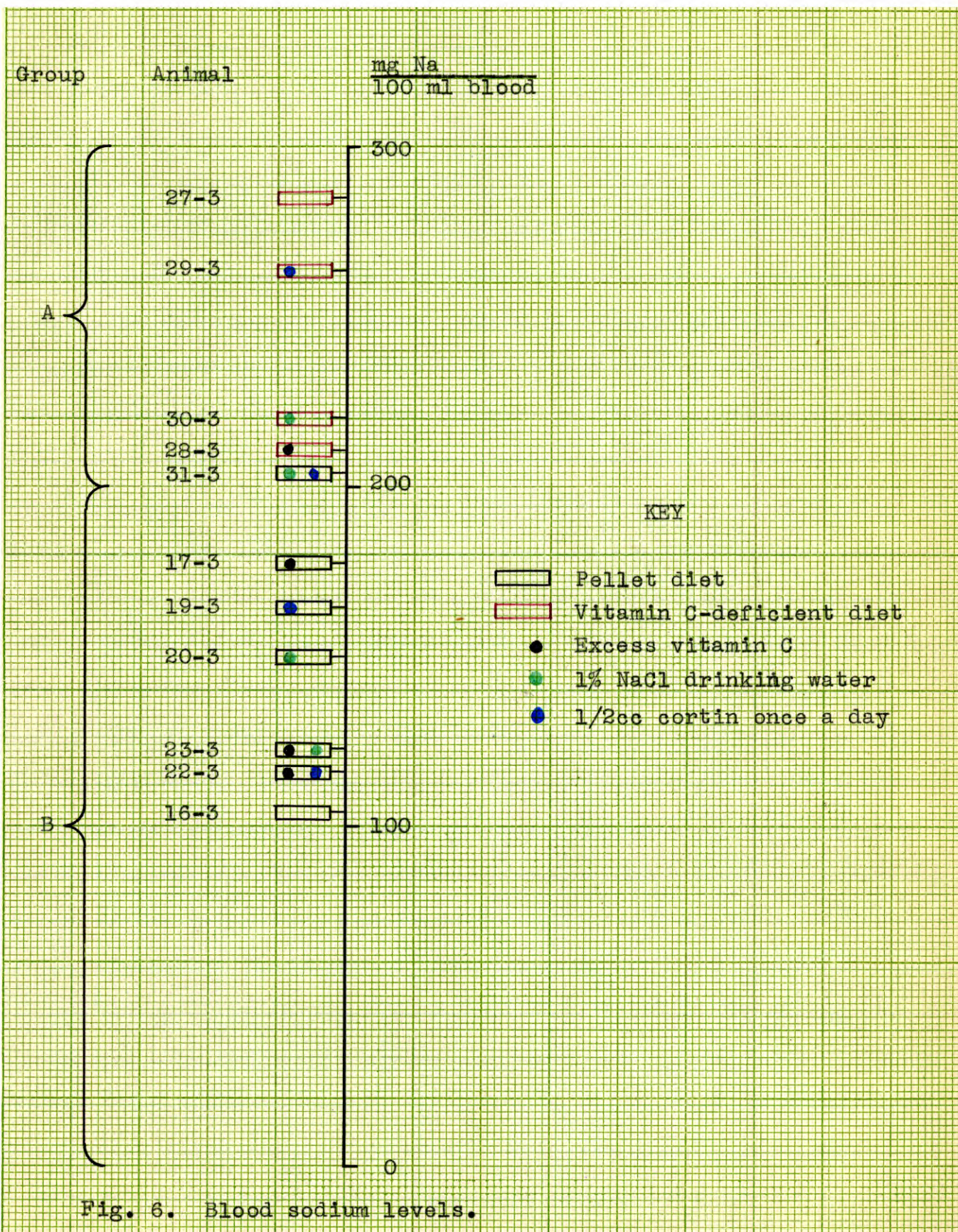


Fig. 6. Blood sodium levels.

Observations of Gonads

Table 1. Appearance of testes.

Animal:	Appearance of testes	:	History
9-3	Lumen clear, chromatin in open arrangement. Abundance of nearly mature spermatids with tails extending into lumen. In general, similar in appearance to what is usually considered normal tissue.		Discarded from previous work in which adrenalectomy was attempted but judged unsuccessful for animal gained weight afterwards and adrenal tags were found at autopsy. Given diet of pellets and green stuff.
19-3	Many lumena not open. Some clumping of chromatin. Relatively fewer mature sperm present than in slide from animal 9-3.		Given diet of pellets only and injection of $\frac{1}{2}$ cc cortin per day for 21 days.
22-3	Openness of lumena intermediate between that of 9-3 and 19-3. Some clumping of chromatin. About as many sperm as 19-3.		Given diet of pellets only. 200 units per day of vitamin C and $\frac{1}{2}$ cc per day of cortin administered for 21 days.
23-3	Very few lumena open. More clumping of chromatin than in any of the other slides. Some mature sperm present but fewer than in any other slide.		Given diet of pellets. 200 units of vitamin C per day and 1 percent sodium chloride drinking water administered for 21 days.

In Table 1, the only animal with completely normal looking tissue is the animal which received green stuff to supplement its diet, and the animal with the worst looking tissue is the one which received pellets and NaCl. Pellets and vitamin C gave a fairly normal looking tissue, and pellets and cortin somewhat less normal.

Table 2. Appearance of ovaries.

Animal:	Appearance of ovary*	Days on experiment:	Experimental conditions
15-3	1	-	Survived partial adrenalectomy. Fed pellets and green stuff.
16-3	4	21	Fed diet of pellets only.
117-3	1	21	Fed diet of pellets, 5 mg vitamin C per day.
20-3	2	21	Fed diet of pellets, 1 percent NaCl in drinking water.
27-3	2	17	Fed vitamin C-deficient diet.
28-3	3	17	Fed vitamin C-deficient diet, 5 mg of vitamin C per day.
29-3	3	17	Fed vitamin C-deficient diet, $\frac{1}{2}$ cc cortin per day.
30-3	4	17	Vitamin C-deficient diet, 1 percent NaCl in drinking water.
31-3	1-4	17	Pellet diet 1 percent NaCl in drinking water, $\frac{1}{2}$ cc cortin per day.

* Explanation of numerical scale of appearance.

- 1 - This indicated an ovary considered normal in appearance. The cells of the follicular walls are large and clear with distinct nuclei, and mitotic figures are present. The eggs are clear with well defined nuclei and chromatin material.
- 2 and 3 - These represent shades of difference between the extremes outlined for 1 and 4.
- 4 - This indicates an ovary considered abnormal in appearance. The cells of the follicular wall are small, shrunken, and show signs of degeneration. Mitotic figures are not present and the nuclei are pycnotic. Cellular debris is scattered throughout the follicular cavity.

The only completely normal ovarian tissue came from animals given pellets with a supplement of green stuff or vitamin C. Animal 28-3 received a vitamin C-deficient diet with a supplement of crystalline vitamin C, yet failed to develop normal tissue. Animal 31-3 had both normal and abnormal tissue in its ovary. NaCl and unsupplemented pellets seem to be associated with abnormal ovaries. Supplements of NaCl and cortin did not prevent changes in the ovaries of animals on vitamin C-deficient diets.

General Gross Observations

In guinea pigs which were carried on the vitamin C-deficient diet to the point of death, there were marked external changes. Approximately at the point of downward deflection of the growth curve the coats of the animals began to look dull and rough, they all but stopped eating, swelling began to be evident in the perineal region and loose stools were found in the cage. As the scurvy became more severe, these changes became more and more marked. Blood appeared in the stools, the animals became quiet, movement was made awkwardly and in the terminal stages they were able to regain their feet only with great difficulty. Their eyes became dull, the eyelids more or less completely shut, a white exudate frequently appeared around the eyes and the animals finally died quietly. Administration of sodium chloride or cortin failed to prevent

the appearance or change the sequence of these events.

Animals killed before the weight loss set in remained normal in all external appearances regardless of the treatment given.

At autopsy, animals which had died of vitamin C-deficiency showed the same absence of fat in the abdominal cavity that has been exhibited by animals which died of starvation. In addition, those that died of vitamin C-deficiency had loose teeth, weak tissue and bone, and in the large bowel, bloody material which seemed to have its origin as high as the junction between the large bowel and the caecum.

DISCUSSION

Survival and Body Weight Curves

As was mentioned in the Introduction of this paper, there is a disagreement in the literature as to the effects of cortin on scurvy. An inspection of Figs. 1 and 3 might serve to indicate that cortin did actually slow up the processes of scurvy to some extent. If this was truly the case, the results would agree with the results of Lockwood and Hartman (1933). If it is felt that there is no difference, the agreement would lie with the results of Svirbely (1935). Actually, a recognition of the small number of individuals represented by the graphs and an inspection of Fig. 2, which shows the extreme

individual variations in body weight curves, will establish the fact that the data obtained should not be considered conclusive one way or the other. It is of particular interest to notice that 5-2, 6-2 and 7-2 are littermates. This brings in the possibility of an hereditary resistance to vitamin C-deficiency instead of a cortin-induced resistance.

Water Consumption

As a point of technic, it was learned that water cans must be emptied, cleaned thoroughly and refilled every two to three days if consumption curves with any degree of smoothness are to be obtained.

Mark (1943) found that the water appetite of rats fell off somewhat after adrenalectomy. The loss in water appetite noted in the observations on water consumption may or may not be further evidence of a relationship between vitamin C and the adrenals.

Blood Sodium Level

This is an aspect of the problem that might have proved of value had technics been worked out satisfactorily, for the relation between blood sodium level and adrenal activity is rather well established (Swingle and Remington, 1944, Deane, Shaw and Greep, 1948).

The values obtained in this experiment are plotted in Fig. 6, but the fact that there is little correlation with experimental conditions raises a question as to their validity. The blood samples of Groups A and B were collected a month apart by the author, an inexperienced technician at best, and turned over to the Biochemistry Department for analysis by a different laboratory assistant each time. The above set of circumstances, coupled with the fact that there is no overlapping of values obtained from the two determinations, make the data of doubtful value.

However, while there is considerable doubt as to the validity of these figures, there is enough information in them to raise the question of the exact behavior of blood sodium levels in vitamin C-deficiency, pregnancy and partial adrenalectomy of the guinea pig, for all animals on vitamin C-deficient diets had high blood sodium levels, the animal which received vitamin C to supplement its deficient diet had the lowest level recorded in Group B and the lowest determination of all was that of the partially adrenalectomized, pregnant female.

Testes

There are not enough cases in Table 1 to permit any generalizations or conclusions, but the results obtained suggest that a further investigation into the effects on the testes

of pellet diet and the various treatments used might prove of value.

A number of other specimens of testis tissue were collected, but all those not included in Table 1 were taken from animals which had died, and since there were evident differences between these specimens and those taken from freshly killed animals, it was concluded that the differences were due to necrotic changes and that they were of such magnitude as to invalidate any possible observations that might be made.

There is, according to Parkes (1945), a functional relationship between the adrenal cortex and the gonads which is not yet established. Had more evidence been obtained, a parallel might have been drawn between the effects of vitamin C on the testis and the effects produced by adrenalectomy as set forth by Herrick and Torstveit (1938).

Ovaries

As in the case of Table 1 on the testis, this table deals with a small number of animals so conclusions must necessarily be drawn with caution. However, the fact that the only completely normal tissue obtained, either testicular or ovarian, was from animals which had received pellets plus fresh green stuff or pellets plus vitamin C, while the ovary from the animal receiving only pellets presented an abnormal picture very

much like that produced by prolonged vitamin C-deficiency, would make it seem that the pellet diet is adequate only when supplemented by green stuff and perhaps by vitamin C.

On the other hand, the fact that abnormal ovaries were taken from the animal given a vitamin C-deficient diet supplemented by crystalline vitamin C indicates the distinct possibility of a lack of something more than vitamin C in this diet. Abnormal ovaries from this particular animal also suggest that weight alone may be no criterion of sufficiency of diet, for this animal had a better than normal growth curve.

It is evident that neither sodium chloride, cortin, nor an excess of vitamin C are enough of a replacement for the deficiencies of the "vitamin C-deficient diet" to permit the maintenance of normal ovarian tissue.

In addition, there is an indication, though not so obvious, that cortin does not completely make up the deficiencies of the pellet diet and that sodium chloride may actually exert a negative effect on gonadic tissue. The lack of clear-cut results was especially evident in animal 31-3 which received pellets, sodium chloride and cortin. It showed a growth curve of well maintained weight, yet its ovaries presented a unique combination of normality and abnormality side by side.

These observations of ovarian changes in the vitamin C-deficient animals of this study seemed to confirm the work of Kramer, Harman and Brill (1933), and they indicate that the

relation between vitamin C and the gonads is probably not caused entirely by a scurvy-induced shortage of cortin.

The abnormalities observed in animals on supposedly normal or fully supplemented diets also suggest that a re-evaluation of the diets and the crystalline vitamin C used should be undertaken, before further work is done on vitamin C-deficiency, particularly in view of recent work with the "grass factor" (Cannon and Emerson, 1939).

General Gross Observations

Turner (1948, p. 227-233) gives, as effects of adrenalectomy, a number of symptoms which include loss of appetite, diarrhea, bloody stools, muscular weakness, lack of interest in surroundings and failure to maintain normal weights and growth rates. In this study it was observed that vitamin C-deficient guinea pigs refuse to eat, suffer diarrhea and bloody stools, become quiet, move with difficulty and undergo a marked reduction in weight. Whether or not these similarities are significant, they are at least interesting.

Future Work

As a portion of this problem, many slides were made of adrenal tissue, but lack of time and the considerable variation found even in animals receiving the same treatment forced

the abandonment of this line of approach. It is felt, however, that this aspect of the problem could also be an interesting and fruitful one in future work, particularly if some of the newer and truly remarkable histochemical techniques were utilized (Deane, Shaw and Greep, 1948).

A number of adrenalectomies also were attempted in working out this problem, but no fully satisfactory results were obtained. However, it is believed that by using the technic of adrenalectomy worked out by Schachter and Bebee (1939), and by administering cortin from the time of operation until healing has taken place, the result could be a successful and valuable procedure.

In short, it is felt that this line of investigation holds further promise, in spite of the fact that the results obtained here, while interesting, are not sufficiently definite to warrant the drawing of many conclusions.

CONCLUSIONS

1. A one percent sodium chloride solution administered as drinking water does not seem to alleviate the effects of scurvy. There is even some indication that it has a detrimental effect upon the gonads.

2. Injection of 25 to 50 dog units of cortin per day does not prevent scurvy, although there is a possibility that the larger dose may delay the effects of scurvy somewhat.

3. These facts may be taken as partial proof that scurvy does not act solely through debilitating the adrenals.

4. It is felt that a re-examination of diets and the crystalline vitamin C used in the experiment is in order in view of some of the results obtained on supposedly complete diets.

ACKNOWLEDGMENTS

The author greatly appreciates the efforts of Dr. Mary T. Harman, Dr. E. H. Herrick and others who have, by their work, counsel, and encouragement, made this paper possible.

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