AN ABSTRACT OF THE THESIS OF

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Title: Evidence for the Nonessentiality of Ascorbic Acid in the Diet of the Rainbow Trout, Salmo gairdneri.

Abstract approved:

Professor Charles E. Warren

Numerous tests were applied to rainbow trout of eleven months of age to determine whether ascorbic acid is an essential element of the diet of these fish. Although previous work had been done on this question, conflicts in the results, the large reliance upon abnormal symptoms such as lordosis and scoliosis for the diagnosis of scurvy, failure to determine the nutritional status of the experimental animal, and the incompleteness of the tests made for scurvy, seemed to warrant this further effort. Tests showed that the experimental fish were depleted of ascorbic acid significantly below a level of the control trout; however, no effects upon growth, formation and replacement of collagen, haematological condition, spleen development, fat metabolism, or mortality rate, could be detected.

Specifically, lordosis and scoliosis failed to develop not only in fish of an initial age of eleven months but also in fish of an initial age of two months, with the exception of one case of scoliosis out of a total population of three hundred. It is hypothesized that the results of others that show the dietetic requirement of ascorbic acid by the salmonoids may be caused by an interrelationship of ascorbic acid with another vitamin. Further work to verify the findings of this experiment and the hypotheses rising therefrom are planned.

Evidence for the Nonessentiality of Ascorbic Acid in
the Diet of the Rainbow Trout, _Salmo gairdneri._

by

Edward Rudolph Joseph Primbs

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Dean of Graduate School

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Acknowledgments

In a very factual sense, the author of an experiment is but a coordinator of the contributions of many. Thus, this experiment would not have been possible without the fellowship granted by the Bureau of Commercial Fisheries, nor without the laboratory facilities, equipment, fish, and feed generously provided by the Department of Food Science and Technology of Oregon State University. More subtle, perhaps, but of no less value, has been the stimulating environment and the readiness to inquire that prevails under Dr. Harold W. Schultz's leadership.

More specifically, Professor Russell O. Sinnhuber has been of inestimable value in all of the following work, and its success is due to him more than to any other. Credit must also be given to Professor Joseph H. Wales for his suggestions, criticisms, and advice, and to Mrs. June L. Hunter for her assistance. Dr. Jong Lee translated articles written in Japanese, a contribution of no small value. George B. Putman has been a helpful colleague, and to Theodore Wales and Richard Foster credit for the preparation of the diets and daily feeding of the fish must go.

Finally, thanks to my wife, Julia, are expressed for her patience, encouragements, and suggestions.

Evidence for the Nonessentiality of Ascorbic Acid in the Diet of the Rainbow Trout, *Salmo gairdneri*.

Introduction

In the decade 1947-1958 some effort was made to determine whether ascorbic acid was a dietetic requirement of the salmonoids. The work in 1947 of McLaren (McLaren, 1947), which actually had been preceded by an experiment on ascorbic acid requirements of trout of 1924 even before ascorbic acid had been isolated (Davis and James, 1924) and by an experiment of 1937 (Hewitt, 1937) during a period of active investigation of vitamin C, evidenced not only that ascorbic acid is a dietetic requirement but also that an excess of the vitamin had depressing effects on growth. McLaren's work undoubtedly had been suggested by Elvehjem's suspicions that the trout is dependent on a dietary intake of ascorbic acid. (Field, 1943) However, later experiments of Wolf (Wolf, 1951), Halver (Halver, 1957), and Coates (Coates and Halver, 1958) clearly contradicted that of McLaren and of Hewitt, but the results of these efforts are questionable, because of (1) the failure to appreciate algae as a source of ascorbic acid, (2) the termination of the

experimental period short of McLaren's time, (3) the interference with the established criteria by disease, and (4) the failure to test diets for the presence of ascorbic acid.

In 1965 and 1967 a Japanese team reported the results of experiments that suggested that rainbow trout require L-ascorbic acid in the diet. (Kitamura, 1965, 1967) The symptoms accepted as the criteria for the development of scurvy were accompanying scoliosis, lordosis, abnormal mortalities, a decrease in growth rate, hemorrhages, and an incomplete development of the operculum. Following these findings, Halver recently with a diet deficient in ascorbic acid also developed curvatures of the spinal column of silver salmon, as well as change in skin pigmentation, susceptibility to subcutaneous and intramuscular hemorrhage, loss of coordination, a deficient cartilage development in gill and eye structures, and hyperplasia of adrenal cortical cells. (Halver, 1967) (Ashley, 1967) (Halver, 1968)

The Cortland laboratory also reversed their previous conclusions on the need of ascorbic acid by trout after the Japanese publications: Poston found lordosis and scoliosis, internal hemorrhaging, and a

higher condition factor among brook trout fed a diet
lacking ascorbic acid. (Poston, 1966)

These experiments previously performed, however,
have been incomplete tests for the scurvy syndrome.
Moreover, with the exception of Hewitt, none have
mentioned any effort to test whether their subject
animals actually were in a state of ascorbic acid
deficiency. Hence this experiment was undertaken to
attempt to reach a conclusion that could be applicable
to hatchery diets and that could provide necessary
data as a basis for future research into the needs for
and function of ascorbic acid in fish in general and
other animals.

Materials and Methods

The water used was that of a well (40 feet deep) at the Food Toxicology and Nutrition Laboratory of Oregon State University. After an examination of the water for the type of endemic organisms, an assay of this biomass for ascorbic acid concentration was made by the method of Roe, Kuether, Oesterling, and Mills (Roe, 1954), as reported and modified by the Association of Vitamin Chemists. (Freed, 1966) Light in the indoor fish tanks was reduced below the level at which endemic organisms were collected, and the tanks were scrubbed whenever any slight growth was apparent and cleaned at least once a week.

Two hundred rainbow trout (*Salmo gairdneri*), approximately 11 months of age and 43 grams in weight, of a Mt. Shasta strain were randomly taken from the population of the Food Toxicology and Nutrition Laboratory and randomly divided into four lots of 50 each, and four hundred trout, approximately two months of age and 2.30 grams in weight, from the population of the following year were randomly divided into four lots of 100 each. From time of segregation into lots, two lots from each age group were fed ad libitum the Oregon Test Diet No 1 (Table No 1) which had the

additament of 1.2 mg. of ascorbic acid per gm of diet,

and two lots were fed the same diet without the

ascorbic acid.

The casein

used was Vitamin-

free Casein, a

product of NBC,

which had

previously been

tested in

ascorbic acid

deficient diets.

(NBC, 1967) The diet in toto and specific suspected

components were assayed for ascorbic acid concentration

by the method of Roe

(Roe, 1954), as

modified by the

Association of

Vitamin Chemists.

(Freed, 1966) The

concentration of

ascorbic acid in the

blood plasma was

measured by the

method of Roe for

Table I
Oregon Test Diet No 1

Nutrient	% of Diet
Casein	49.4
Gelatin	8.7
Dextrin	15.6
Salmon Oil	10.0
Salt Mix No 2 USP XII	4.0
Calcium Carbonate	0.9
Carboxymethylcellulose	1.3
Cellulose (NBC)	6.4
Choline Chloride (70%)	1.0
Vitamin A	0.1
Vitamin E, Conc (110IU/g)	0.6
Vitamin Mix	2.0

Table 2
Vitamin Mix (mg/kg diet)

Alphacel	14,716
Ascorbic Acid	1,200
Butylated Hydroxyanisole	15
Butylated Hydroxytoluene	15
B_{12} Supp.	53
Biotin	2
Ca Pantothenate	288
Folic Acid	19
Inositol	2,500
Menadione	16
Niacin	512
P-Aminobenzoic Acid	400
Pyridoxine	48
Riboflavin	144
Thiamine	64
Vitamin D	8

indicating immediate intake of the vitamin. Tests for the state of ascorbic acid deficiency were made and the degree of deficiency measured by determination of ascorbic acid concentrations in the liver, brain, and spleen by the Roe method.

From the 24th to the 35th week after initiation of the feeding trial, samples of fish from the 43-gram age group were examined for scurvy symptoms as defined by the following criteria:

1. *Decrease in growth*

Total fish were weighed biweekly, and calculations of condition and conversion factors were made.

2. *Decrease in formation and replacement of collagen*

(i) Bone abnormalities.

Plural ribs were extracted and examined for (a) "ground glass appearance", (b) the "white line of Frankel", (c) the "corner sign", and (d) fractures.

(ii) Failure of wound healing process.

Vertical wounds, immediately above the lateral line and directly vertical to anterior margin of dorsal fin, were made with scalpel on samples of each lot under anesthesia (MS-222). Wound length was ten mm, and depth was six mm. After 15 days wounds were sutured, and subsequently excised on the 23rd day after wounding, preserved in Bouin's solution, sectioned with a

microtóme, stained with Masson trichrome connective tissue stain, and compared at 20X, 128X, and 800X.

(iii) Weakness in collagenous structures.

Gas bladders of samples of all lots were removed and tested for relative strength by application of air pressure, which was measured by a manometer.

(iv) Hemorrhagic tissue.

Gross examination of liver, intestine, and epidermis was made.

(v) Development of lordosis and scoliosis.

Gross examination of all fish, including fish of the 2.30-gram age group, was made. Dorsal and lateral radiograms were taken of all fish apparently deformed.

3. Development of anemia.

(i) Erythrocytes were counted by use of a Fisher autocytometer and the B. D. Unopette diluting technique.

(ii) Hematocrit readings were determined by a clinical centrifuge.

(iii) Erythrocytes were measured with a micrometer (800X) after staining with Wright's.

(iv) Hemoglobin gm. % was determined by use of Hycel Cyanmethemoglobin reagent and standard solutions with a Beckman D. U. spectrophotometer at 540 m. u. wavelength.

4. Enlargement and congestion of spleen.

A spleen index was computed by determination of the

ratio of the weight of a spleen to the weight of the subject animal.

5. *Fat deposits in liver.*

Extracted livers were fixed in Bouin's solution and sectioned with a microtome. Samples were stained with Sudan IV and compared at 800X.

6. *Mortalities.*

All mortalities were examined for abnormalities and recorded.

Results

The sources of ascorbic acid for the test lots were investigated. Ninety to ninety-five percent of the biomass in the water consisted of diatoms (<u>Naviculu minima</u> Grünow and <u>Achnonthes minutissima</u> Kutzing) (McIntire, 1967), which contained approximately 34 mg. of ascorbic acid per 100 g of algae (dry basis). This determination compares favorably with that of Pratt, who found 39 mg per 100 g in <u>Chlorella</u> (dry). (Pratt, 1967) However, the growth present in any one tank could be estimated at no more than a few milligrams, and this was promptly eliminated when the growth became apparent.

The concentration of ascorbic acid in the Oregon Diet without added vitamin C was also suspected to be insignificant: the diet was found to have on the average seven ug per g of diet with a range of five to eight ug.

Table 3
Ascorbic Acid in Diet Components

Component	ug/g Diet
Alpha Cellulose	0.5
Casein	1.0
Dextrin	0.3
Salmon Oil	0.4
Vitamin Mix	1.3

Five of the major components of the diet contributed at least half of this amount. (Table 3)

From the time of initiation to the time of

termination of the experiment 66,740 grams of the Oregon Diet was fed to the test group. Thus, the diet provided but 467 mg to 100 fish over a period of approximately six months, or approximately 26 ug per fish per day.

Measurements of concentrations of ascorbic acid in the blood plasma of the test and control groups indicated a significant difference in intakes of the vitamin. (P < .005)

Table 4

Ascorbic Acid Concentration in Blood Plasma

	Test	Control
Mean (mg/100 cc)	1.02	4.57
Standard Deviation	.69	1.42
Number of Samples	19	19

(Table 4) Two zero concentrations were found among the test lots. Figure 1 shows the distribution graphically.

A distinctly defined state of ascorbic acid deficiency in the test groups was found by the measurements of ascorbic acid concentrations in
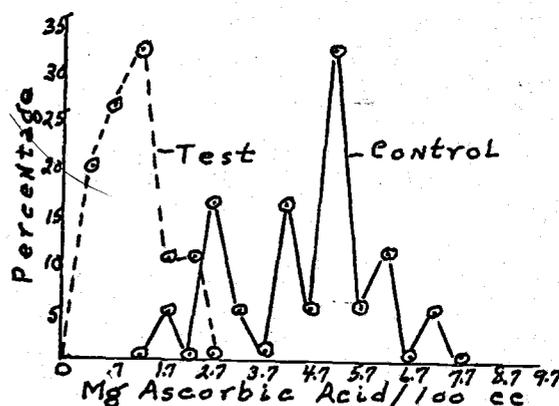
Fig. 1. Frequency of Plasma Ascorbic Acid Levels in Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Groups.

the liver, brain, and spleen. The differences between
test groups and control groups were significant in each
case.

Table 5
Ascorbic Acid Concentrations in Organs

	Test	Control
Liver		
Mean (mg/100 g liver)	1.43	18.00
Standard Deviation	.85	6.85
Number of Samples	14	12
P < .005		
Brain		
Mean (mg/100 g brain)	9.00	36.75
Standard Deviation	1.60	12.20
Number of Samples	8	8
P < .005		
Spleen		
Mean (mg/100 g spleen)	6.75	45.75
Standard Deviation	1.50	8.26
Number of Samples	4	4
P < .005		

Of particular
interest were the
differences between
standard deviations.
Whereas the
distributions of values
within the test groups
lie within a narrow
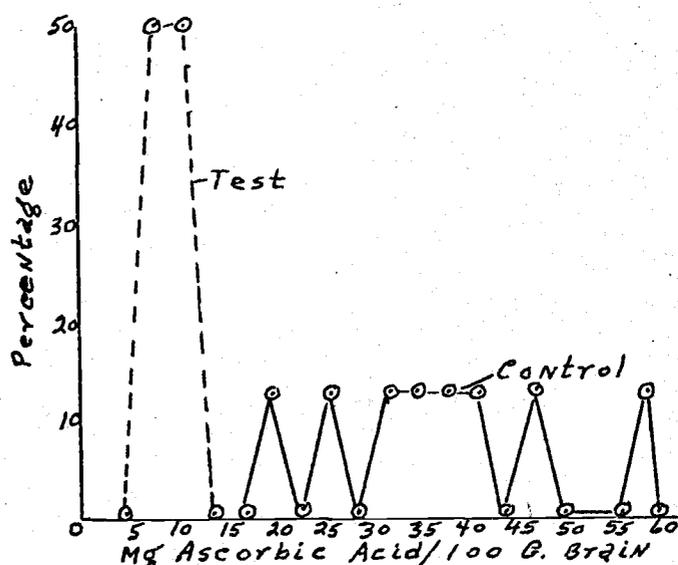range, the distributions
of values within the
control groups encompass
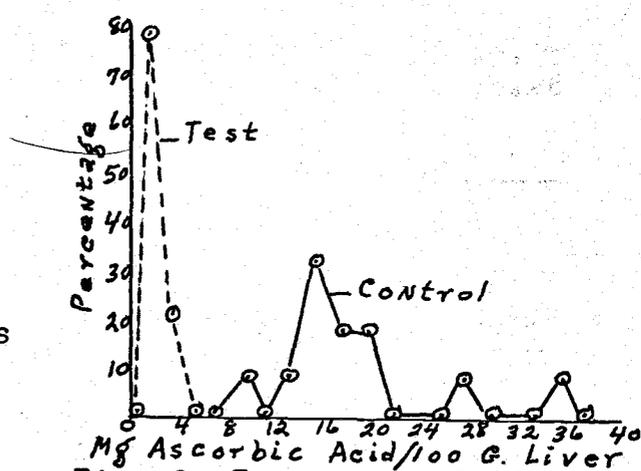
Fig. 2. Frequency of Liver
Ascorbic Acid Levels in
Ascorbic Acid Deprived (Test)
and Ascorbic Acid Fed (Control)
Groups.

considerable scope.

This is seen in

figures 2, 3, and 4.

   Of equal

interest was the

large gradual

increase in the

ascorbic acid

concentration in the

liver of the control

groups over a period

Fig. 3. Frequency of Brain Ascorbic Acid Levels in Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Groups.

of 50 days, while the ascorbic acid concentration in

the liver of the test groups remained fairly constant

over the entire period with the exception of a slight

decline within the last ten days. (See figure 5.)

   Both, the large

differences in the

standard deviations of

the data of the three

organs examined and

the difference in

change of ascorbic

acid concentrations in
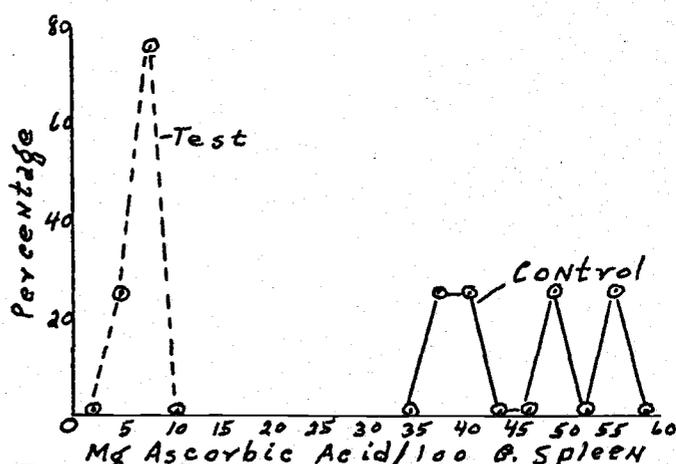
Fig. 4. Frequency of Spleen Ascorbic Acid Levels in Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Groups.

the liver with time,

suggest that the major

portion of
the ascorbic
acid in the
organs is
without
function
and is
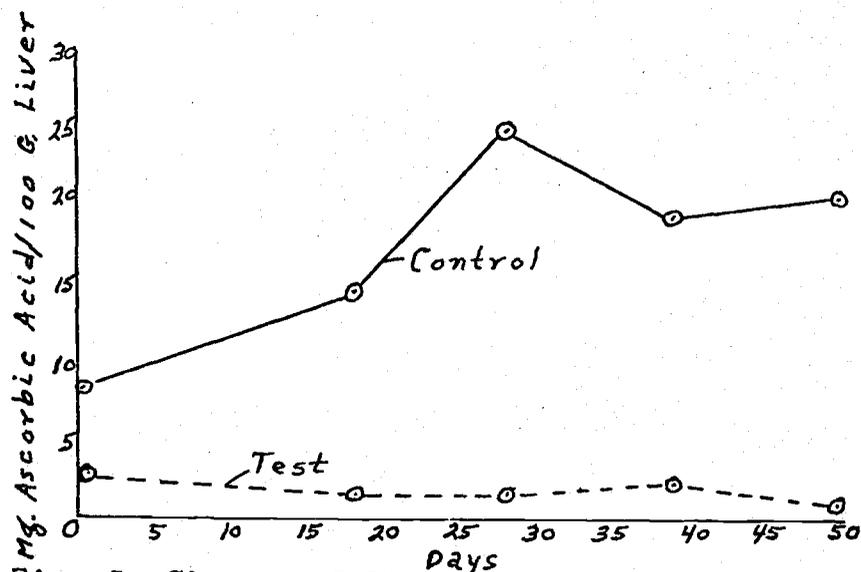deposited
in these
tissues
only for
storage.

Fig. 5. Change of Liver Ascorbic Acid with
Time in Ascorbic Acid Deprived (Test) and
Ascorbic Acid Fed (Control) Groups. (0
day is 1st day of 26th week of experimental
period.)

Upon examination of growth data and calculation of

the index food conversion factor no significant

Table 6

Comparison of Growth between Ascorbic Acid Deprived
and Ascorbic Acid Fed Groups

	Test	Control
Total Fish	100	99
Total Weight Increment (g)	23,216	22,895
Final Average Wt. (g) (58 fish)	300	308
Food Conversion Factor	1.01	0.98

differences were found between the control and test

groups. (Table 6)

Moreover, no significant difference was found

between the condition factors calculated for the test

and control groups, although the condition factors of

both groups were larger than either of the two
reported by Poston as indicating ascorbic acid
deficiency. (Poston, 1966) The mean condition factor
for 50 test fish is 1.62 and the mean condition factor
for 50 control fish is 1.65. (P > .10)

No distinction up to 40X could be made between the
bone structures and composition of the pleural ribs of
the test and control groups, and none of the
traditional scurvy symptoms of the costochondral
junctions could be discerned.

Statistical measurement showed a high probability
(P > .25) of no difference between the mean air
pressures required
to break the gas
bladders of the
test and control
groups. This
result suggests
that there was

Table 7

Comparison of Gas Bladder
Breaking Points of
Ascorbic Acid Deprived and
Ascorbic Acid Fed Trout

	Test	Control
Mean (mm Hg)	217.9	215.6
Standard Deviation	105	116
Number of Samples	34	35
P > .25		

no interruption in the formation and replacement of
collagen in the ascorbic acid deprived fish. (Table 7)

External and internal gross examination of both
test and control groups revealed no difference between
the two groups, and no hemorrhages were observed.

An incomplete development of the operculum, as noted by Kitamura as a symptom of ascorbic acid deficiency, was observed in both the test and control groups.

Fig. 6. Gas Bladder Breaking Points of Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Trout.

Hematological studies revealed no conclusive evidence of any of the types of anaemia found in scorbutic animals. Thus, no significant difference $(P > .10)$ was found between the mean erythrocyte counts of the ascorbic
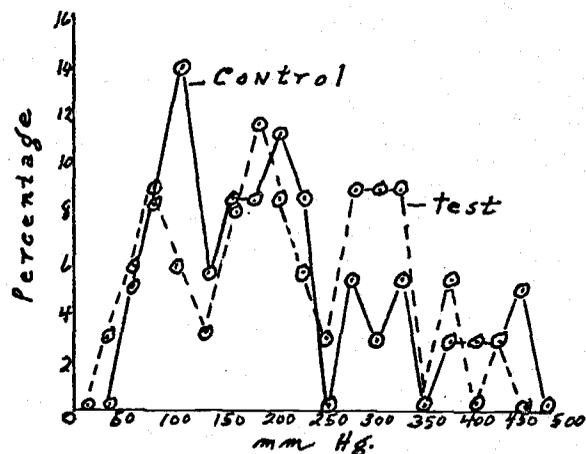
Table 8

Comparison of Erythrocyte Counts of Ascorbic Acid Deprived and Ascorbic Acid Fed Trout

	Test	Control
Mean (per cu mm)	1,044,000	1,083,000
Range (X 100,000)	5.25-14.25	5.25-14.25
Standard Deviation	2.04	1.67
Number of Samples	39	39
$P > .10$	—	—

acid deprived and ascorbic acid fed groups. (Table 8)

The dissolved oxygen of the various tanks, which can affect the number of erythrocytes (Smith, Lewis, & Kaplan, 1952) did not vary more than 0.4 mg/l among the tanks.

Supporting the results of the erythrocyte counts, the mean hematocrit of the test group is not significantly different from the mean hematocrit of the control group. (Table 9)
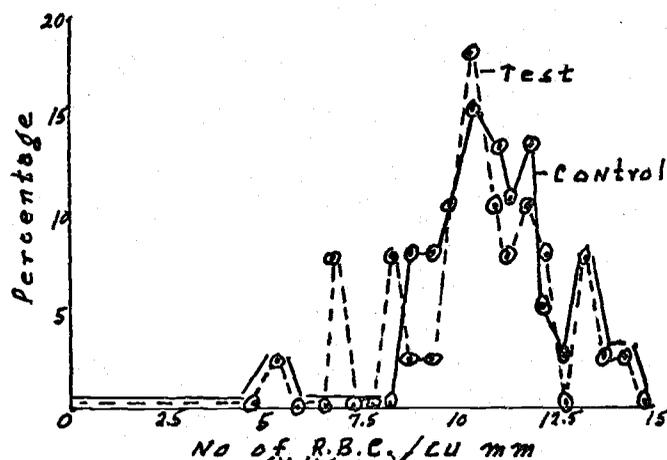
The type of anaemia generally found in the scurvy syndrome is normocytic, but other forms do occur. Thus, a decrease in hemoglobin relative to R.B.C. counts is frequently attributed to ascorbic acid deficiency. (Chakrabartz, 1963)

Fig. 7. Erythrocyte Counts of Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Trout.

Table 9

Comparison of Hematocrits of Ascorbic Acid Deprived and Ascorbic Acid Fed Trout.

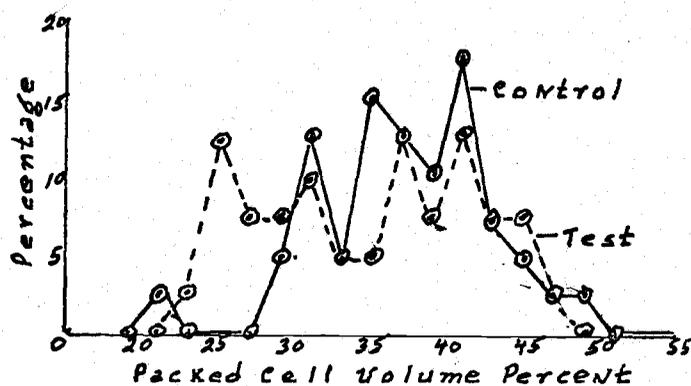|	Test	Control
Mean	34.85	37.26
Range	23-47	21-49
Standard Deviation	6.98	5.65
Number of Samples	40	39
$P = .05$	-	-

Fig. 8. Hematocrits of Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Trout.

In this experiment no significant difference was measured between the

Table 10

Comparison of Gram Percent Hemoglobin of Ascorbic Acid Deprived and Ascorbic Acid Fed Trout

	Test	Control
Mean	7.88	8.16
Range	4.8-13.3	5.8-11.3
Standard Deviation	1.81	1.34
Number of Samples	39	38
$P > .10$	-	-

means of the gram percent hemoglobin of the test and control trout. (Table 10)

Macrocytic anaemia also has been produced by a diet lacking ascorbic acid. (Chakrabartz, 1963) This type of anaemia involves an abnormal enlargement of the red blood corpuscles. However, again the mean of

Fig. 9. Gram Percent Hemoglobin of Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Trout.

the calculated volumes of red blood cells of the test groups did not significantly differ from the mean of the volumes of red blood cells of the control. (Table 11)

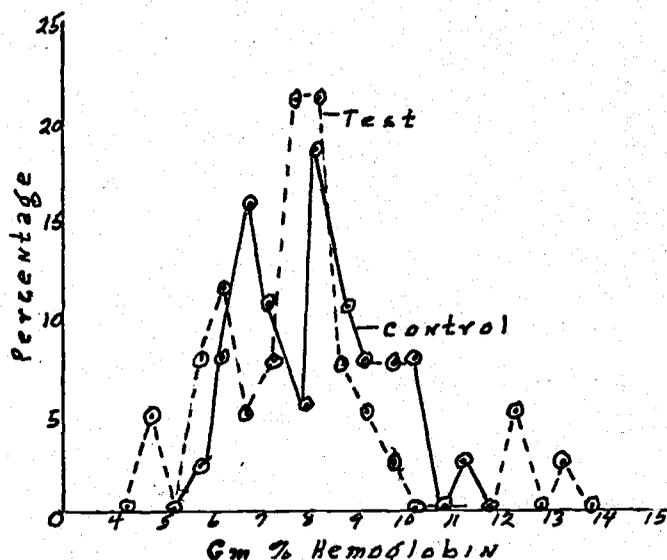The mean diameter of 286 red blood cells (microscopic measurements) of the test groups is 12.39 μ,

the mean
diameter of
320 red
blood cells
of the
control
groups being
12.73 u.

No enlargement
of the spleen was
shown by
calculation of the
spleen index: the
mean of the test
groups did not
differ
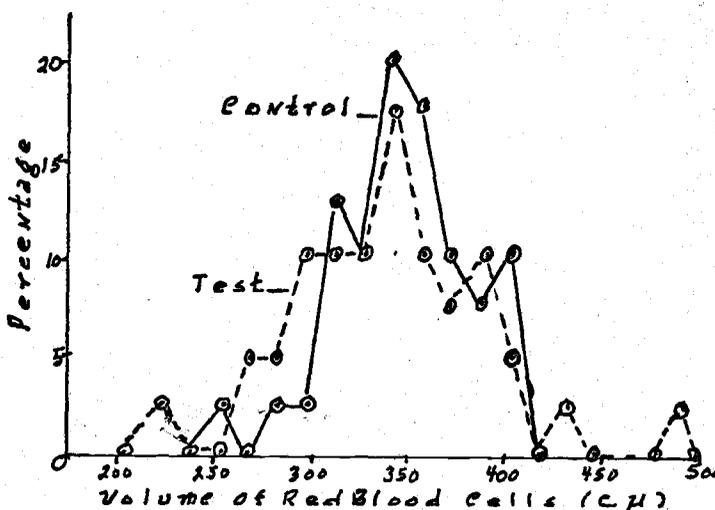significantly
from the mean of
the control
groups. (Table 12)

Microscopic
comparison of
sections of the

Table 11

Comparison of R.B.C. Volumes of Ascorbic Acid Deprived and Ascorbic Acid Fed Trout.

	Test	Control
Mean (cu)	341	348
Range	223-493	253-403
Standard Deviation	50	34
Number of Samples	39	39
P = .25	–	–

Fig. 10. R.B.C. Volumes of Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Trout.

Table 12

Spleen Index of Ascorbic Acid Deprived and Ascorbic Acid Fed Trout

	Test	Control
Mean	7.42	7.41
Standard Deviation	3.1	3.9
Number of Samples	43	39
P > .25	–	–

liver (sections from four test fish and from four

control fish)

revealed no

abnormal fat

deposits in the

liver.

   Even though

all lots of the

43-gram fish

developed fin rot
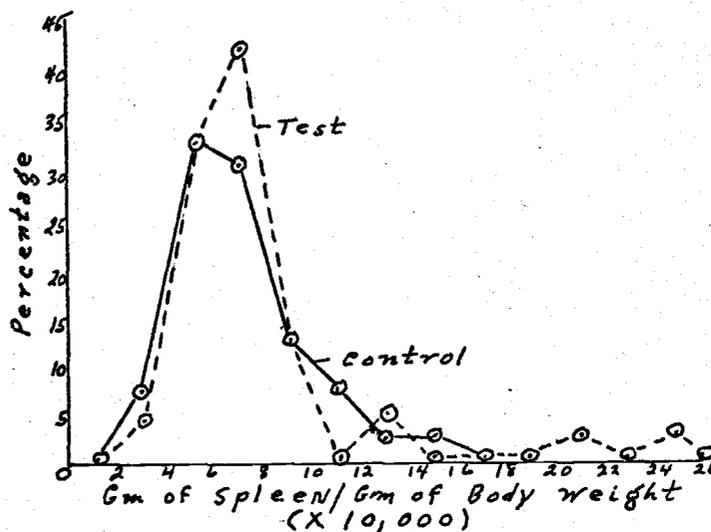
during the course

of the experiment,

Fig. 11. Spleen Index of Ascorbic Acid Deprived (Test) and Ascorbic Acid Fed (Control) Trout.

the mortality was low in all groups. Of the total

yearlings, only one death of an ascorbic acid deprived

fish out of a total population of 100 occurred to give

a percent mortality of one percent, and among the

controls only two deaths of ascorbic acid fed fish out

of a population of 99 to give a percent mortality of

two percent.

   A comparison of sections of wounds of ascorbic

acid deprived and ascorbic acid fed groups at 20X, 128X,

and 800X evidenced that healing and thus the formation

of collagen was normal in the test group: collagen was

widely dispersed, collagenous fibres were formed, and

fibroblast cells were abundant. (cf: figures 12 through

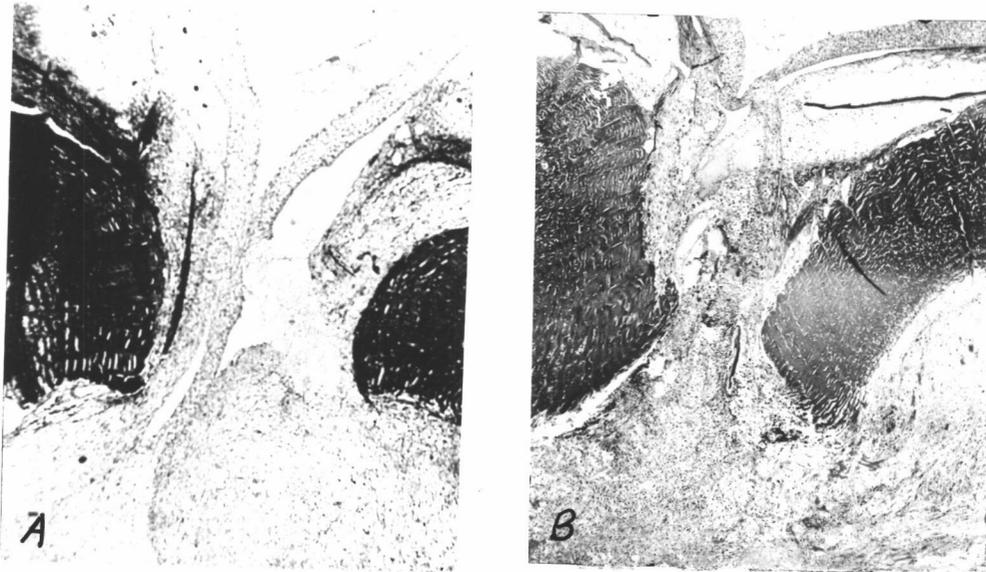*14) There was some variation in the degree of healing,*

Fig. 12. Comparison of cross sections of wounds of (a) ascorbic acid fed and (B) ascorbic acid deprived trout. Complete regeneration of epithelium and closure of wound by granulation tissue present in both cases. H.& E.. X 20.
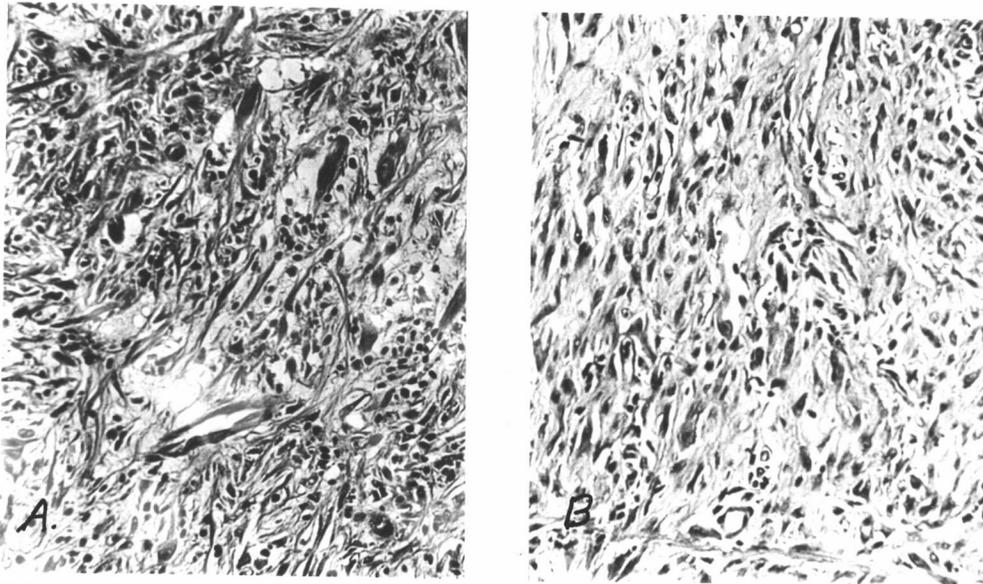

Fig. 13. Comparison of granulation tissue of wounds of (A) ascorbic acid fed and (B) ascorbic acid deprived trout. Proliferating fibroblasts, inflammatory cells, and collagenous fibres abundantly present in both cases. Masson Trichrome. X 128.
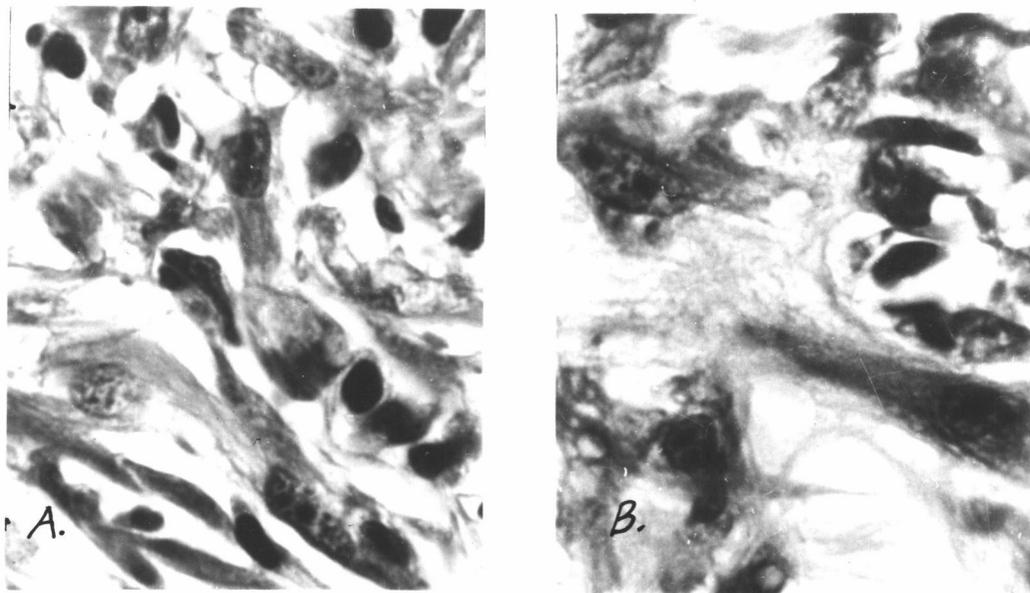
Fig. 14. Comparison of granulation tissue of the
wounds of (A) ascorbic acid fed and (B) ascorbic acid
deprived trout. Numerous fibroblasts, some undergoing
mitosis, embedded in collagenous fibers in both cases.
Masson Trichrome. X 800.

but the wounds of some of the test fish tended to show a more advanced stage of healing than the wounds of the control fish. Probably the variation originated in divergencies in suturing, in sectioning, or in mechanical stresses in vivo.

Among the 100 ascorbic-acid-deprived trout of the initial-43-gram group, neither scoliosis nor lordosis was observed, and within the 200 ascorbic-acid-deprived trout of the initial-2.30-gram group, only one case of scoliosis occurred after the expiration of 26 weeks of the experimental period. (See appendices one and two.)

Discussion

The results of this experiment evidenced that ascorbic acid is not essential in the diet of the rainbow trout. It is difficult to evaluate these findings in relationship to those of Kitamura's (1965, 1967) and Halver's (1967), since neither of these authors has indicated that they had tested their diets for the presence of ascorbic acid and had determined the relative degree of deficiency of their test animals.

The elimination of ascorbic acid completely from the diet is not accomplished simply by excluding the nutrient from the constituents used to prepare a purified ration. As far as it is known, ascorbic acid is present in all animal and plant cells, (King and Becker, 1959) and may be carried in oils as a suspension. (Hewitt, 1937) Thus, it is at least doubtful that the diets of McLaren, Kitamura, and Halver were actually free of measurable amounts of ascorbic acid.

Although recommended allowances for man have a range from 30 mg per day (Medical Research Council, 1953) to 1.8 g (Stone, 1966), the minimum amount required to avoid definitely associated reactions is 10 mg per day. (Medical Research Council, 1953) The minimum amounts required to avoid clinical scurvy

symptoms in the guinea pig and the rhesus monkey have also been established: 5 mg/kg body weight/day and 2 mg/day, respectively. (National Academy of Science, 1962) McLaren (1947) found that 0.25 to 0.50 mg per g of ration produced the least undesirable results in the case of the rainbow trout. Relative to these levels, the amount of ascorbic acid in the diet, 26 ug/day or 7 ug/ g of diet, used in this experiment does not seem to be significant.

Indeed, in this work a state of deficiency was definitely defined by analysis of various organs for ascorbic acid concentrations. It has long been thought that the high concentration of ascorbic acid in organs of animals - the liver, the brain, and the adrenal cortex, e.g. - suggests special functions of ascorbic acid in these organs. (Long, 1946) (Chalopin, Mouton, and Ratsimamanga, 1966) However, the wide variance in ascorbic acid concentrations among organs of those fish fed ascorbic acid and the narrow range in concentrations of those fish deprived of added ascorbic acid as well as a large gradual increase in ascorbic acid concentrations in the liver of the control fish indicate that the large concentrations of ascorbic acid normally associated with the organs are largely functionless and

only represent storage of ascorbic acid.

On the other hand, the stability of liver ascorbic acid and the narrow variance of values of organ ascorbic acid in general in the fish deprived of dietetic ascorbic acid suggest a minimum level for survival as well as an endogenous source, or an untested exogenous source - for example, bacterial synthesis. In view of recent evidence, however, bacterial synthesis is unlikely. (Levenson, 1962)

In spite of the apparent state of deficiency developed in the test fish, moreover, no evidence of an impairment in the general health or an incapacity for normal functioning of the organism was obtained upon administration of a number of tests for symptoms of scurvy. Here again it is difficult to evaluate these results in relationship to those of previous workers, since the previous work was concluded on the basis of a very limited set of symptoms for scurvy, few, if any, of which are specific for an ascorbic acid deficiency. McLaren, for example, reached her conclusions just by application of the criteria of growth, mortality, hemoglobin, liver fat, and hemorrhagic tissue. Beyond these, Kitamura has only observed lordosis and scoliosis and an incomplete development of the operculum.

Although lordosis and scoliosis have not
traditionally been included in the scurvy syndrome
(Follis, 1948) (Bickness and Prescott, 1953) (Vilter,
1960) (Goldsmith, 1964), these diseases have been
associated with inadequate nutrition, as well as with
inadequate metabolism, for over one hundred years.
(Risser, 1964) Moreover, the effects of ascorbic acid on
bone development as well as on metabolism seem to
suggest a role of a deficiency of this vitamin in the
development of the deformity (Thornton, 1968), but the
role may not be a direct one.

A. F. Gardner (Gardner, 1966) produced lordosis in
rats by massive (25 mg.) dosages of vitamin D_3, and it
has been observed that ascorbic acid may detoxify toxic
dosages of other vitamins. (Rosenberg, 1945) Numerous
other observations have confirmed a substance (Vitamin
A or Vitamin D?) in the liver or liver oils (cod liver
oil, e.g.) of animals which has an inhibitory effect
upon the activity of ascorbic acid. (Collett and Eriksen,
1938) (Vedder and Rosenberg, 1938) (Rodahl, 1949)
Poston has not found any symptoms of hypervitaminosis
yet in brook trout after feeding massive doses of D_3 to
these fish for thirty weeks (Poston, 1968), but
undoubtedly his diet includes ascorbic acid. It may well
be that not only the lordosis and scoliosis but also the

scurvy symptoms experienced in previous work have had as their common cause hypervitaminosis A or D.

Neither lordosis nor scoliosis was reported by McLaren, who had included cod liver oil in her diet. Since Kitamura began with fish of 0.60 g weight and Poston with 1.45 g, whereas McLaren began with fish of 3.5 g and in this experiment we started at 43.00 g and 2.30 g, the deformity of spinal flexure may be limited to trout of an age less than two months. In man and some other animals, at least, the problem has been one of preskeletal maturity (MacEwen and Shands, 1967), with evidence in tadpoles of scoliosis having a genetic origin. (Underhill, 1966)

Otto Bessy in the early 'thirties had found fatty degeneration of the liver in scorbutic guinea pigs. (Bessy, Menten and King, 1933) Hewitt also reported the symptom. (Hewitt, 1937) However, Baldwin showed in 1944 no significant difference in tissue lipids between scorbutic and normal animals. (Baldwin, Longenecker and King, 1944) McLaren employed this criteria of scurvy, but the negative results were not consistent with her other data. Comparison of liver sections in this experiment also revealed the livers of ascorbic acid deprived subjects to be normal, both controls and test groups having only very slight deposits of fat.

The red blood cell count, the hemoglobin gm %, and
the mean cell volume, which were determined in this
experiment, compared favorably with the blood values of
the normal trout as determined by Field (Field, Elvehjem,
and Juday, 1943). The hematocrits, however, differed by
eight percent. The red blood cell counts made by Halver
exceeded Field's even in the controls by over 200,000.
A low dissolved oxygen content would account for the
abnormally large R.B.C. count and differences between
the experimental and control groups, polycythemia; on the
other hand, an increase in the number of red blood cells
has been observed in vitamin-C-deficient guinea pigs, and
explained as a response to loss of blood (Constable,
1960). But Constable's experiment also showed that
scorbutic guinea pigs did not develop anaemia. In accord
with this finding, Kitamura's R.B.C. count of the rainbow
trout, supposedly scorbutic, was quite similar to the
control.

An experiment, later than Constable's (Chakrabartz
and Banerjee, 1963), however, produced anaemia in 13 of
a total number of 14 scorbutic guinea pigs. Although the
anaemia of scurvy is not completely understood (Kahn
and Bradsky, 1966), it is fairly well established
experimentally that ascorbic acid deficiency
significantly affects (1) iron metabolism (Mazur, Green

and Carleton, 1960) (Mazur, 1961) (Hallberg, Salvell and Brise, 1966) and haem biosynthesis (Lochhead, 1959) and (2) folic acid metabolism and thus D.N.A. biosynthesis by the red blood cells. (Vilter et al, 1963) Thus, the scurvy syndrome still commonly includes anaemia under several of its various forms (Woodruff, 1964) (G. C. Chatterjee, 1967): (1) normochromic and normocytic (a decrease in normal R.B.C. count and gram percent hemoglobin) (Vilter, 1967), (2) microcytic, hypochromic (a decrease in hemoglobin and cell size) (Vilter, 1967), or (3) macrocytic (a failure in growth of the nucleus) (Vilter, 1963). A brief mention of symptoms of microcytic anaemia being eliminated among chinook salmon by adding ascorbic acid to the Abernathy diet was reported recently. (Burrows, 1968); however, the data were not given.

Not only a decrease in the rate of growth but also a rapid loss of weight has been a traditional symptom of scurvy. (Knox and Goswami, 1961) This effect upon growth has been attributed not only to a decrease in appetite but also to a direct effect upon metabolism. (Ram, 1966) (Evans and Hughes, 1963) And yet except for McLaren, who showed a decrease in the rate of growth under ascorbic acid deficiency, no one has yet reported any data indicating a significant difference. Kitamura (1965) reported retarded growth, but gave no data, and later

(1967) showed graphically what appears to be an insignificant difference between the vitamin-C-deficient fish and his control. (No test of significance was mentioned.) Halver (1968) mentioned unfavorable growth and food conversion in fish on the vitamin-C-deficient diet, but also gave no supporting data. Poston (1967), though finding no difference in average body weights, did find a significant difference in the condition factors.

Scurvy, untreated, terminates in death. (Wohl and Goodhart, 1964) Both McLaren and Kitamura (1965) found differences in rate of mortality, supporting their diagnoses of scurvy in rainbow trout. Hemorrhagic tissue, another common indication of ascorbic acid deficiency, was found again by McLaren and Kitamura (1967).

Although the gill filament cartilage rods were not microscopically examined by us, and thus the deformation in this structure as noted by Halver (1968) was not contradicted, other tests of collagen formation and replacement were made with positive indications of normalcy. The failure of the operculum to fully develop, as observed by Kitamura, was a defect found by us in both the experimental and control groups, and thus a cause other than ascorbic acid deficiency was assumed.

Although the results of this experiment indicate

*that there exists no relationship between the large
concentration of ascorbic acid in organs and a function
of ascorbic acid in those organs as often suggested, yet
this interpretation does not contradict to any degree
the significant body of evidence that implicates
ascorbic acid in the activity of the adreno-cortical
gland. (Chalopin, 1966) On the other hand, there is
evidence that strongly suggests that the changes induced
in the adrenal gland by vitamin C deficiency are caused
by stress attributable to the scorbutic state, which
acts upon the adrenal gland by excessive stimulation by
ACTH and not directly by the depletion of ascorbic acid
in the adrenal gland (Howard and Cater, 1959), the
hypertrophy being more of a sign of exhaustion than of
activity. (Dugal, 1961)*

*The changes in the adrenal gland observed during
avitaminosis C are hyperplasia, hypertrophy, and
decreased and irregular lipid distribution. (Howard,
1959) Halver (1968) observed hyperplasia in the adrenal
cortex of coho salmon, supposedly deficient in ascorbic
acid. The adrenal cortex in salmonoids is located along
the posterior cardinal veins only as a layer of one or
two cells in the region of the head kidney. (Rasquin
and Rosenbloom, 1954) To reduce the probability of a
misinterpretation, confirmatory evidence, such as*

measurement of blood ACTH (Clayton, Hammond and Armitage, 1957) or observation of increased mitoses, should have been taken, especially since only several samples were compared.

Summary

The evidence of this experiment supports the conclusion that ascorbic acid is not essential in the diet of the rainbow trout under normal conditions. It is suspected that previous results that tended to show that salmonoids require ascorbic acid in the diet are attributable to interrelationships of ascorbic acid with other vitamins and to hypervitaminoisis A or D, and thus under the abnormal conditions of the inactivation of endogenous ascorbic acid by excessive dosages of other vitamins scurvy symptoms can be produced. It is also suspected that lordosis and scoliosis are not caused by an ascorbic acid deficiency, but rather by another nutritional abnormality related to ascorbic acid metabolism.

Further confirmation of the conclusions of this experiment, as well as tests to verify the explanation given here of the causes of some scurvy symptoms in salmonoids reported by others, is planned.

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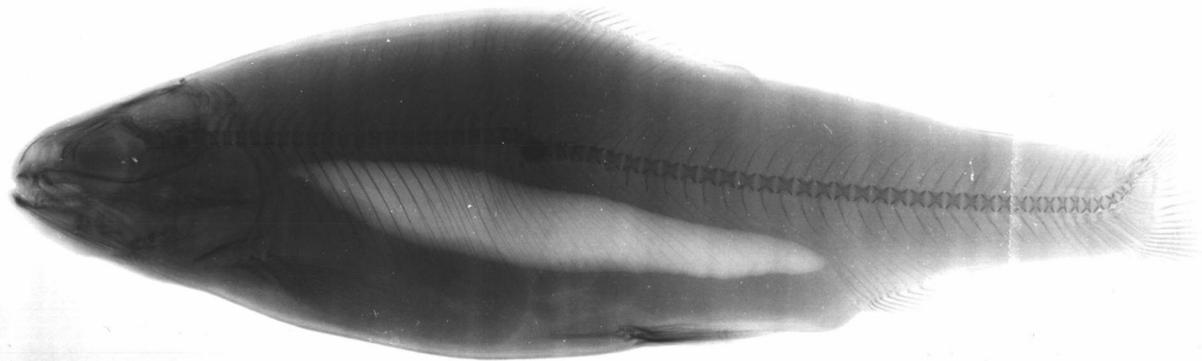
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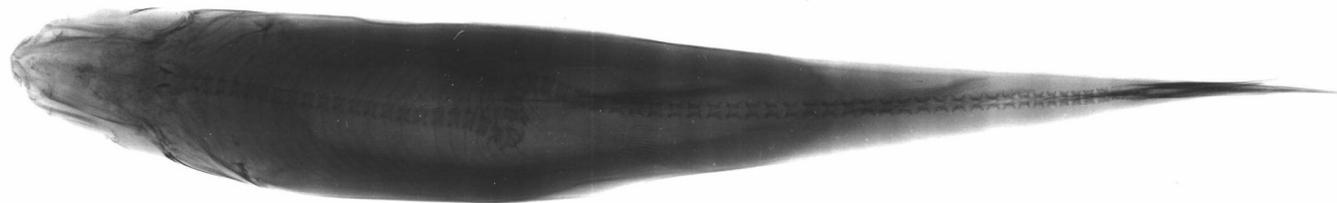
Appendix I. Dorsal Radiogram of an Ascorbic-Acid-Deprived Trout after 26 Weeks from Time of Initiation of Experiment.

5

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Appendix 2. Lateral Radiogram of an Ascorbic-Acid-Deficient Trout after 26 weeks from Time of Initiation of Experiment.

7