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.

by

Edward Rudolph Joseph Primbs

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Redacted for privacy

Professor of Fisheries in charge of major Redacted for privacy

Head of Department of Fisheries & Wildlife Redacted for privacy

Dean of Graduate School

Date thesis is presented... Oct 15 - 1968...
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.

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

<table>
<thead>
<tr>
<th>Vitamin Mix (mg/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphacel</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
</tr>
<tr>
<td>Butylated Hydroxyanisole</td>
</tr>
<tr>
<td>Butylated Hydroxytoluene</td>
</tr>
<tr>
<td>B₁₂ Supp.</td>
</tr>
<tr>
<td>Biotin</td>
</tr>
<tr>
<td>Ca Pantothenate</td>
</tr>
<tr>
<td>Folic Acid</td>
</tr>
<tr>
<td>Inositol</td>
</tr>
<tr>
<td>Menadione</td>
</tr>
<tr>
<td>Niacin</td>
</tr>
<tr>
<td>P-Aminobenzoic Acid</td>
</tr>
<tr>
<td>Pyridoxine</td>
</tr>
<tr>
<td>Riboflavin</td>
</tr>
<tr>
<td>Thiamine</td>
</tr>
<tr>
<td>Vitamin D</td>
</tr>
</tbody>
</table>
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
microtome, 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.
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 (*Navicula minima* Grünow and *Achnonthes minutissima* 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 *Chlorella* (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. 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

**Table 3**

<table>
<thead>
<tr>
<th>Component</th>
<th>ug/g Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha Cellulose</td>
<td>0.5</td>
</tr>
<tr>
<td>Casein</td>
<td>1.0</td>
</tr>
<tr>
<td>Dextrin</td>
<td>0.3</td>
</tr>
<tr>
<td>Salmon Oil</td>
<td>0.4</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1.3</td>
</tr>
</tbody>
</table>
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) 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

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Ascorbic Acid Concentration in Blood Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test</td>
</tr>
<tr>
<td>Mean (mg/100 cc)</td>
<td>1.02</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>.69</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>19</td>
</tr>
</tbody>
</table>

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

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

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
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 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 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. Fig. 5. Change of Liver Ascorbic Acid with Time in Ascorbic Acid Deprived (Test) and only for 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 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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Fish</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Total Weight Increment (g)</td>
<td>23,216</td>
<td>22,895</td>
</tr>
<tr>
<td>Final Average Wt. (g) (58 fish)</td>
<td>300</td>
<td>308</td>
</tr>
<tr>
<td>Food Conversion Factor</td>
<td>1.01</td>
<td>0.98</td>
</tr>
</tbody>
</table>
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 no interruption in the formation and replacement of collagen in the ascorbic acid deprived fish. (Table 7)

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (mm Hg)</td>
<td>217.9</td>
<td>215.6</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>105</td>
<td>116</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>$P &gt; .25$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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. 

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 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.

Table 8

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (per cu mm)</td>
<td>1,044,000</td>
<td>1,083,000</td>
</tr>
<tr>
<td>Range (× 100,000)</td>
<td>5.25-14.25</td>
<td>5.25-14.25</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.04</td>
<td>1.67</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>( P &gt; .10 )</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
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)
In this experiment no significant difference was measured between the 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 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)

Table 11

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (cu)</td>
<td>341</td>
<td>348</td>
</tr>
<tr>
<td>Range</td>
<td>223-493</td>
<td>253-403</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>39</td>
<td>39</td>
</tr>
</tbody>
</table>

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

Table 12

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.42</td>
<td>7.41</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>3.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>P &gt; .25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Spleen Index of Ascorbic Acid Deprived and Ascorbic Acid Fed Trout

Microscopic comparison of sections of the 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, 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 hypervitaminosis 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.
BIBLIOGRAPHY


44. Poston, H. A. 1968. Massive doses of vitamin D₂ in
brook trout diets. In: Progress in sport fishery
Fish and Wildlife Service. Bureau of Sport Fisheries
and Wildlife. Resource Publication no. 64)
45. Pratt, R. and E. Johnson. 1967. Vitamin C and
choline content of Chlorella vulgaris and C.
pyrenoidosa. Journal of Pharmaceutical Sciences
56:536-37.
utilization in vitamin C deficiency. Indian Journal
of Medical Research 54:964-970.
47. Rasquin, P. and L. Rosenbloom. 1954. Endocrine
imbalance and tissue hyperplasia in teleosts
maintained in darkness. Bulletin of the American
Museum of Natural History 104:363-430.
Nature 164:531.
50. Roe, J. H. 1954. Chemical determinations of ascorbic,
dehydroascorbic, and diketogulonic acids. In:
Methods of biochemical analysis, ed. by D. Glick.
51. Rosenberg, H. R. 1945. Chemistry and physiology of
A comparative morphologic and physiologic study of
fish blood. The Progressive Fish-Culturist 14:169-
172.
53. Stone, I. 1966. Hypoascorbemia, the genetic disease
causing the human requirement for exogenous ascorbic
acid. Perspectives in Biology and Medicine 10:133-
134.
effected by ascorbic acid. Proceedings of the
Society for Experimental Biology and Medicine 127:
1096-1099.
caudal scoliosis in tadpoles of Rana pipiens.
Schreber, Copeia, no. 3, p. 582-583.
the toxicity of vitamin A. The Journal of Nutrition
16:57-68.
57. Vilter, R. W. 1960. Vitamin C (ascorbic acid). In:
Modern nutrition in health and disease, ed. by M.
G. Wohl and R. S. Goodhart. Philadelphia, Lea &
Febiger. p. 377-393.


APPENDICES
Appendix 1. Dorsal Radiogram of an Ascorbic-Acid-Derived Trout after 26 Weeks from Time of Initiation of Experiment.
Appendix 2. Lateral Radiogram of an Ascorbic-Acid-Perfused Trout after 26 Weeks from Time of Initiation of Experiment.