

NUTRITIONAL REQUIREMENTS OF TROUT

A Thesis

by

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Submitted to the Faculty of Graduate
Studies and Research in partial ful-
filment of the requirements for the
degree of Doctor of Philosophy.

McGill University

May, 1944

ACKNOWLEDGEMENTS

The author expresses gratitude to Dr. W. D. McFarlane of Macdonald College for kindly criticism and advice during the course of this investigation. Helpful suggestions were also given by Dr. W. A. DeLong, Dr. F. O. Morrison, and Dr. E. W. Crampton of Macdonald College. Interest and cooperation shown by the members of the Staff of the Institute of Parasitology, Macdonald College, was much appreciated; in particular that of Dr. T. W. M. Cameron, Dr. W. Swales, Dr. B. Collier, and Miss Muriel Cameron, and of Mr. B. W. Taylor of McGill University. Appreciation is expressed to the Biological Board of Canada for the facilities for carrying out practical feeding trials at Antigonish, Nova Scotia. The Quebec Department of Fisheries very kindly provided a grant-in-aid for experiments on the nutritional requirements of trout.

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NUTRITIONAL REQUIREMENTS OF TROUT

I. INTRODUCTION

A. GENERAL CONSIDERATIONS.

Between 1860 and 1870 an American, Seth Green, established the first fish-rearing station somewhere in the Eastern States. From this small beginning, pisciculture has become an industry of national importance. McCay of Cornell University and Davis of the United States Bureau of Fisheries have clarified many problems pertaining to fish culture and nutrition.

The food of wild trout varies with the locality, the season, and the age of the fish, but it consists mainly of the larvae of various insects such as the dragonfly or caddis fly and crayfish (1). Species' differences play a part in food selection; for example, 15% of the stomach contents of rainbow trout consists of algae (2), while green vegetation is eaten in negligible amounts by other species of trout. Perhaps this fact may account for the difficulty which is sometimes experienced in raising rainbow trout in captivity.

The food available for hatchery feeding may be divided into three classes: (a) fresh meat, such as liver, heart, et cetera; (b) dried animal products such as meat or fish meal, and dried skim-milk; (c) vegetable products

such as wheat middlings and cottonseed meal.

Liver was one of the first substances to be used and has continued to be one of the most successful of artificial trout foods. However, there are certain disadvantages in using liver as a trout food. For example, when scattered on the surface of the water, it imparts a milkiness due to solution of its soluble and valuable constituents. Furthermore, as liver contains a considerable quantity of connective tissue which is not digested by the trout, an appreciable part of it is without value. As liver is an extremely perishable food, adequate refrigeration facilities must be provided to insure a steady supply of a fresh product. In addition, liver itself is one of the most expensive of trout foods. For these reasons, it is greatly to be desired that a satisfactory substitute for liver be found.

Some authorities consider beef heart or a mixture of beef heart and beef liver, to be the most satisfactory food for young trout during the first six weeks of feeding. The physical consistency of a mixture of beef heart and beef liver is superior to liver alone, and they may supplement each other to give a better food.

Recent developments in trout nutrition have involved replacing a part of the liver in the diet by dried animal or vegetable products, the so-called "dry feed mixtures".

After the trout have passed the fry and fingerling stage, the selection of their food is much easier. They

can be fed larger particles, so that there is less danger of loss through the dissolving of soluble nutrients. The liver of different animals, beef, swine, sheep, and horse all seem to give equally good growth in young trout, although horse liver has a better physical consistency. Dried horse liver gave an excellent growth response with young fingerling trout, although when the fish were able to eat larger particles of food, the ones fed beef liver grew at a faster rate than those fed dried horse liver (3).

Donaldson and Foster (4) reported on the use of fresh beef blood as a substitute for liver in feeding salmonoids. Beef blood has been used as a fish food for many years and has been fed in the fresh, cooked, or dried form. In the trial cited, the fresh beef blood was mixed with salmon carcass meal and fed to young Chinook salmon. The results of the experiment indicated that beef blood was not equal in food value to beef liver, but its availability at packing plants and its low price resulted in reduced food costs in relation to the weight of fish produced.

The palatability and physical consistency of the food is one of the most important questions to be considered in choosing substitutes for an all meat diet (5). To insure rapid growth, a food must be readily eaten. Hunger may force the trout to eat unpalatable foods, but such circumstances will not produce strong, healthy trout. In certain combinations of meat and dry feed mixtures the fish will pick out the meat and leave the meal. Consequently,

every effort must be made to have a mixture which will be eaten readily by the trout with as little waste as possible. The above facts are of particular importance with fingerling trout, so it has been customary to delay the introduction of the dry feed mixture until the fingerlings are one and one-half inches long. Liver can be replaced by dry meals in amounts up to 50% of the diet of young trout (6).

There are several ways in which these dry meals can be fed. They may be fed alone, but it will be necessary to substitute liver every fifth day so as to provide sufficient "Factor H" for rapid growth, or the meat and meal may be mixed together and fed as a combination. The best method of feeding these dry feed mixtures is to mix the spleen and dry feed mixture in equal proportions, or incorporated at a 25% level with a similar amount of liver and then mixed with the dry feed. Ground frozen spleen and salt form an unusual combination which, when mixed together, coats the other materials and prevents separation in the water (6).

In choosing substitutes for fresh meat in trout foods, the ingredients most commonly used are cottonseed meal, wheat middlings, and skim-milk powder. Trout can utilize cottonseed meal up to one-half of the total calories ingested, and even at levels as high as 70%, there is absence of toxicity (7). Cottonseed meal seems to be the best of all available foodstuffs of plant origin; linseed meal, the poorest. Trout are very sensitive to linseed meal and die within a few weeks if

it constitutes a part of their food (8). Not all fish exhibit this sensitivity toward linseed meal (9) which has been shown to be due to hydrocyanic acid set free by enzyme action in the intestinal tract. Dried milk products are especially valuable as trout foods. Dried skim-milk and liver furnish a complete food, although dried milk itself is not rendered an adequate food by supplements of the B-vitamins, cod-liver oil, copper, iron, zinc, and iodine (10). Milk products seem to be well utilized even by the young fry. Meat and fish meals are valuable in feeding trout though these do not constitute a complete diet (11).

Trout seem to compare very favorably with swine in their efficiency in converting foodstuffs. Over periods of twelve to twenty-eight weeks it may require 2.7-5.3 units of dry food to produce a unit increase in body weight (12). Brown trout seem to be the most efficient converters of foodstuffs among the four varieties of trout studied. There are several other factors to be considered. For example, Cornelius (13) showed that, at low levels of oxygen pressure in the water, the efficiency of food conversion decreased.

Hayford and Davis (14) reported that the inclusion of 25% of dried material such as fish and meat meal in the food of yearling and two-year old trout caused little waste in feeding. The fish were of better color and were more uniform in size than they had been on foods

previously used. However, after some months on the above diet, the fish became anemic and on dissection showed lipoid degeneration of the liver; only rainbow trout were affected. Gottschalk (15) stated that the above diseased condition was caused by overfeeding or by the use of unsuitable foods. He stated that a deficiency of vitamins in the diet might be an important factor.

Davis (16) reported that cereals such as wheat flour, middlings, and oatmeal provided roughage and absorbed meat juices so that there was less waste in feeding. However, he stated that only a small percentage of these products were digested by the trout. In contrast, animal meals, such as fish, meat, salmon-egg, and dried skim-milk, were more easily digested and their inclusion in the diet made it possible to grow better fish at less expense. It was not advisable to include more than 50% of the dry meal in the diet as greater amounts were not well utilized. This inclusion of roughage in the diet was intended to overcome the danger of excessive feeding which resulted in degeneration of the liver and pancreas. The data indicated that bulk in the diet was of definite benefit, especially when food concentrates were used (17).

B. CARBOHYDRATE METABOLISM.

The part played by carbohydrates in the nutrition of fish is obscure. Geng (18) showed that carbohydrate might constitute 40% of the weight of the plankton which formed a part of the natural food of even carnivorous fish. A considerable portion of the food of fish consists of cellulose, and its fate in the digestive tract is uncertain. It may act solely as a roughage constituent, which Schaperclaus (19) claimed fish needed in their food. Under natural conditions this would be supplied by chitin as well as cellulose (20). At levels above 25% of cellulose in the diet, the growth rate was decreased; at 35%, it ceased (21). European workers (22) have obtained excellent growth using mixtures containing 25% sawdust. Foster et al (23) used apple flour to provide bulk in diets containing a large percentage of dry foods. They claimed that the inclusion of roughage in the diet cut down the development of fatty livers caused by overfeeding. Agar-agar did not improve trout diets as judged by growth rates (24).

In 1925, Krezincky (25) reviewed the evidence concerning the utilization of dissolved nutrients. Upon the basis of experiments with tadpoles he concluded that nutrients, such as glucose, could be absorbed from water. There were three possible routes for this absorption: the skin, the alimentary canal, and the gills. In fish this absorption seemed to take place through the gills,

while in tadpoles some absorption might have taken place through the skin. Krough (26) found that fish could utilize dissolved glucose to satisfy one-quarter of their energy requirements. In glucose solutions (1-5%) carp doubled their blood sugar in the course of 15 minutes (27).

McCay, in the course of experiments carried out at the Cortland Experimental Hatchery, found that trout digested and absorbed relatively large amounts of sucrose. The livers of trout fed considerable quantities of sucrose, cooked starch, or dextrin, became very large and rich in glycogen (6). This indicated that these carbohydrates were being digested and absorbed. On the other hand, trout fed raw starch or cellulose had livers comparable in size and glycogen content to those of trout fed raw meat (6). In later work carried out at the Cortland Experimental Hatchery, it was shown that the absorption of carbohydrates occurred at a much slower rate in cold-blooded animals than in warm-blooded ones such as the rat. Further, it was found that the inclusion of carbohydrates in the diet of fish had no apparent effect on the rate of growth (28). The rate of digestion of carbohydrates by trout has been shown to be in the following descending order: glucose, maltose, sucrose, lactose, cooked and raw starch (29).

C. PROTEIN REQUIREMENTS.

One of the most important questions to be considered in trout feeding is the source and amount of protein. In general, proteins of animal origin are better than those from vegetable sources. Growth curves indicate very little difference in utilization between the liver, heart, or spleen of all species of animals when used in combination with dried foods such as skim-milk powder or cottonseed meal (30). McCay showed that the protein level should be above 14%, and that the protein from such a source as dried skim-milk might supplement that of fresh meat to make a complete food.

The protein requirements of trout are difficult to determine since so little is known about their vitamin needs, and their carbohydrate and fat utilization (29). Embury and Gordon (31), in comparing the composition of natural and artificial feeds, found that about half of the naturally consumed feedstuffs were protein.

Knauthe (32) demonstrated that carp possessed the enzymes essential for the digestion of the common classes of feedstuffs. He discovered a strong trypsin which digested fibrin quite readily. In addition, he found that the nitrogenous portions of meat were about 90% digested, and about 80-90% of the cereal proteins were digested by enzymes from carp bodies in tests conducted in vitro.

The level of protein in trout diets is very important.

If casein is used as the source of protein, it must exceed 10% of the total calories in the diet or the trout cannot grow (33). At 20-25% levels moderate growth results (34). If liver is used as the source of protein, 14% is sufficient to give rapid growth (35).

D. FAT METABOLISM.

The physiology of fat digestion and absorption in fish has been studied very little in spite of the fact that fat plays an important part in the diet of most animal species.

van Herwenden (36) investigated the gastric processes in fish. He demonstrated the presence of active lipotropic enzymes in gastric mucosa, and showed that the fat droplets normally occurring in stomach epithelia were absent from fasting fish.

Greene (37) showed that the pyloric division of the stomach of the King salmon was much more active in fat absorption than the cardiac division. He found that fat absorption in the stomach decreased with increasing age of the fish.

Lovern (38) pointed out that the problem of fat metabolism in fish is somewhat different from those encountered in plants or animals. Fish fats are more unsaturated and of higher molecular weight than those characteristic of the other groups. Two points may be of importance: (a) cold-blooded animals lay down a more unsaturated type of fat than warm-blooded ones; and (b) fish are chiefly carnivorous, and their fat composition will be decided largely by the type of fat in their food, since little fat will be synthesized from carbohydrate.

Tsujimoto (39) established the fact that marine flora contained poly-unsaturated acids of high molecular weight. Lovern (40) showed that permeability played an important role in the deposition of fat in certain species. This may explain the fact that in the Salmonidae Family the lower molecular weight fatty acids are more readily utilized than those of high molecular weight.

Channon and Saby (41) found that in the herring the gonad-fat was very highly unsaturated just prior to spawning as shown by a change in the Iodine Number from 137 to 200. The gonad-fat must be formed from the depot-fat and the change in the degree of unsaturation indicated that a part of the depot-fat must be selectively removed and passed on to the ova. An alternative explanation would be that the gonad-fat was synthesized from carbohydrate sources. This seems improbable since the carbohydrate reserves of fish are small, and in the case of the mammal, there is evidence that the fats synthesized from carbohydrates are of a relatively simple type; and are not to be compared to the complex unsaturated type of fat found in fish gonads.

Lovern (38) pointed out that marine and fresh-water fish fats differed in several ways:

- (1) In the fat of fresh-water fish the amount of oleic-linoleic acid was high.
- (2) The C_{20} and C_{22} were present in smaller amounts.
- (3) Palmitoleic acid was a very important constituent.

He examined the fatty-acid composition of salmon parr, smolts, and adult salmon. The results of the experiment indicated that salmon parr were typically fresh-water fish; adult salmon, a marine species; and smolts, at a transition stage between the other two. Lovern (42) next examined the fatty acid composition of plankton and crustacea and found that they could be divided into fresh-water or marine species by the characteristic differences in the composition of their fatty acids. He concluded that a simple deposition of ingested fat would account for the characteristic differences between fresh-water and marine fish. The metabolism of fat constitutes a unique problem in any species, such as the trout, that can survive only in cold water.

The mesentery fat of brook trout tends to reflect the food fat in respect to its Iodine Number (12). When cottonseed meal was fed as a supplement to the diet, the mesentery fat had an Iodine Number of 100 to 119; if hydrogenated oil was fed, the Iodine Number was 90 to 95.

In balance studies with brook trout, using cottonseed oil, salmon oil, and hydrogenated oil, the first two were about 90% utilized; while with the hydrogenated oil, 15-53% was recovered from the feces (12). In spite of low body temperature trout are able to utilize fats of relatively high melting points.

E. MINERAL REQUIREMENTS.

Fish are able to secure their mineral requirements from the water in which they live. Carp are known to have more calcium in their skeleton, as well as more ash, if they live in hard water. The source from which fish derive their calcium is a problem of much interest since many of the diets employed in trout hatcheries for many years have consisted largely of meat, especially liver. Upon these meat diets, which are poor in calcium and rich in phosphorus, one might expect poor skeletal development. Analyses of the entire body of trout reared on liver and on liver plus a source of extra calcium, such as skim-milk powder, were similar, indicating that the trout were obtaining sufficient calcium from the water (9, 12). It was shown that trout developed enlarged thyroid if kept in water deficient in iodine (49). All available evidence indicates that trout can remove from the water in which they live the essential materials which they need for their physical well-being.

F. VITAMIN REQUIREMENTS.

1. Vitamins D, B, and C.

Zuntz and Conheim (43) have investigated the vitamin needs of trout. They found that carp needed 25% of their food in the living form. They believed also that the crustacea eaten furnished essential enzymes that were needed to assist in the digestion of food. Davis and James (44) claimed that trout needed vitamins A, B, and C for proper growth.

Thompson (3) observed a condition called knot-head, a malformation of skull bones, prevalent in carp of a certain stream. He suggested that the malady might be similar to mammalian rickets. Vitamin D (33) was proved to be essential for goldfish. These workers claimed that fish fed vitamin D had larger, stronger skeletons than those without it.

In order to determine whether or not irradiation was responsible for the storage of vitamin D in the livers of fish, Bills (45) irradiated catfish. Although he did not alter the vitamin D content of the stored fat, he found that a few minutes irradiation for several days killed the fish. As the diets used in hatcheries are high in phosphorus and low in calcium, (exclusive of calcium present in the water), the conditions which were required for rachitic development were present. While various forms of bone malformation have been reported, no one has furnished any substantial

evidence that these were of dietary origin or were related to rickets.

In 1930, Drummond and Gunther (59) tested extracts of zoo- and phytoplankton and showed that vitamin D was probably absent from the phytoplankton, but present in the zooplankton since it cured rickets in rats. They suggested that the zooplankton might derive the vitamin from irradiation while in surface waters.

Belloc (60) found that sterols obtained by saponification of the oils extracted from copepods were anti-rachitically active without irradiation. These observations are in accord with the statement by Russel (61) that copepods came up to the surface of the water during July, August, and September. Earlier in the season they were at a depth of 10-15 mm.

In recent feeding trials with channel catfish, fed on buttermilk powder, a high mortality, an extreme nervousness, and a loss of motor co-ordination were observed. This condition could be cured by adding yeast, cod-liver oil, and liver to the diet (46). Schneberger (47) reported that it was necessary to add fresh meat and vitamin B at regular intervals to trout foods in order to maintain health and vigor. He claimed that a B-vitamin deficiency was demonstrated among rainbow trout in 1939. The condition was produced experimentally and the fish were cured by adding to the diet vitamin B or dried brewer's yeast. Yeast appeared to have some pro-

protective action, as it prevented injury to the gills of trout fed a basal diet of autoclaved meats (48, 49). Jewell and co-workers (50) showed that goldfish receiving diets lacking vitamin B₂ were more susceptible to fungus growths than those receiving this vitamin in their food.

Recent studies on vitamin C distribution in fish indicate that it is high in roe, brain, and lens (98).

2. Vitamin A.

von Euler and Euler (52) demonstrated that predatory sea-birds and -fish could obtain little carotene in their diet and if it was given experimentally only traces of vitamin A appeared in the liver. The enormous fluctuations in the vitamin A content of halibut-liver oil (e. g. from 0.17-10%) cannot be readily correlated with the diet of the fish. The principal variable seems to be the age of the fish, old age going with increased vitamin concentration (53). This relationship supports the suggestion that the vitamin content of the liver is the result of a gradual accumulation, and militates against the theory of a synthesis by the fish itself (54).

Drummond and Hilditch (55) discussed the origin of vitamin A in the liver of fish, and stated that the common foods of cod were much poorer in vitamins A and D than had been suspected. As an explanation for the large reserves of vitamins A and D in cod liver, they suggest that even although the amount of these substances in the food of cod was

small, it might be retained so efficiently that by the time the fish was mature, the accumulation would be considerable. On the other hand, cod and other fish might be able to synthesize these substances.

Gillam (56) came to the conclusion that in marine fish carotene was usually ingested directly or indirectly from phytoplankton and then broken down into vitamin A. In mammals carotene ($C_{40}H_{56}$) is split into two molecules of vitamin A ($C_{20}H_{30}O$). Fresh water fish or some other animals on which they feed must be able to break down the carotene molecule into vitamin A₂ ($C_{22}H_{34}O$) since this homologue appears in the liver in greater concentrations than is normally met with in marine fish livers.

In another report Drummond and Zilva (57), searching for the ultimate source of vitamin A in nature, found that the marine diatom, *Nitzschia colostereum*, was capable of synthesizing a fat-soluble growth factor. Jamison, Drummond, and Coward (58) demonstrated that this diatom could synthesize the factor under controlled conditions in the laboratory.

MacWalter and Drummond (62), in experiments with young rainbow trout, believed that the fish, before they began to eat, could synthesize a carotenoid pigment (not carotene) which they converted partly to vitamin A.

Edisbury et al (63) found a high concentration of

vitamin A in oils prepared from tissues of the alimentary tract, especially from pyloric caeca, which they reported for cod to be 10-20 times more potent than an average cod-liver oil. These observations concerning the presence of vitamin A in the intestinal wall led to speculations concerning a possible role of the vitamin in the assimilation of fat from the intestinal tract.

Morton and Creed (64) showed that the feeding of carotene to fresh-water fish (perch and dace) gave rise to a considerable increase in the stores of both vitamins A and A₂ (A₂ is an isomer of vitamin A which is peculiar to fresh-water fish (65)).

Lovern et al (66, 67) showed that the deposits of vitamin A in fish were located in the mucosa and tunica propria of the intestinal tract. The distribution of fat in the intestine runs parallel to that of vitamin A. The mechanism of fat absorption is unusual, since it proceeds through the mucosal epithelium rather than by a lymphatic system. These two facts led to the hypothesis that vitamin A esters in combination with protein may assist in the dispersion of fat droplets accumulating in the tunica propria. This would aid in their removal through the continuous layer of connective tissue (Stratum compactum) and their transmission to the blood stream.

Edisbury et al (63) showed that fish viscera and notably the pyloric caeca and intestines, were often very rich in vitamin A. Large deposits of this vitamin

in the absorptive parts of the alimentary canal made it necessary to study the possibility that vitamin A played an essential role in food assimilation in some species. It is possible that some species accumulate large reserves of vitamin A in the same way as the experimental rat that is receiving carotene in large quantities. This may mean that the enormous reserves of vitamin A in the halibut are accidental and without function. However, it should be remembered that the diet of carnivorous fish consists mainly of fat and protein, and vitamin A may play an important role in the assimilation of one or both of these compounds. The distribution of vitamin A runs parallel to the sites of fat absorption. On the other hand, the comparative poverty of some species in respect to vitamin A suggests that it is not an essential for assimilation of either fat or protein.

Moore (68) showed that in rats restricted to a diet deficient in both vitamins A and E, the reserves of vitamin A were used up much more rapidly than in control animals given synthetic α -tocopherol acetate. It is possible that under physiological conditions vitamin E may protect vitamin A against oxidative waste.

McCay et al (29) stated that beef liver seemed to be the best source of vitamin A for trout, while cod-liver oil and carotene at moderate levels gave indications of poor assimilation. It was shown that in blood serum; for example, vitamin A or carotene was combined with protein (69) and could not be extracted by the usual solvents

unless the serum was treated with an equal volume of alcohol. In liver the vitamin A may be in some form of a physico-chemical combination with protein, and may be in a form which is particularly adapted to the needs of trout.

3. Other Growth Factors.

It has been shown that trout can grow at a moderate rate for periods up to three months on diets of casein, starch, fat, inorganic salts, and the usual vitamin supplements of the rat (70, 71). At the end of such periods they die unless special supplements of fresh meat or meat extracts are added to the diet (72). Similarly, trout could be reared on mixtures of dried skim-milk and fresh liver, while they died if the dried skim-milk were supplemented with such products as cod-liver oil, "Vivatos" or inorganic elements such as iron and copper (10). On the basis of such evidence, it was recognized that trout needed some thermolabile factor present in fresh meat and different from any of the recognized vitamins. This thermolabile factor was called "Factor H" by McCay et al (70, 71), and resembled that which Wulzen (73) had recognized in her studies with planaria. It was discovered that liver preserved in alcohol (74), dried under carbon dioxide (10) and hypochlorites (10) retained its potency. Heard (71) found that extracts of liver with alcohol, ether, and acetone were active for short feeding trials.

McCay et al (75) later reported that alcoholic

or aqueous extracts of liver contained little of the factor. They stated that brook trout, when fed a purified diet, required about 10% of fresh beef liver to satisfy their need for Factor H. In the 1939 report from the Cortland Experimental Hatchery (6), it was stated that lack of Factor H caused a severe anemia with low red blood cell count. In the Cortland Experimental Hatchery Report for 1940 (29), the authors stated that the anemia which developed in trout on synthetic diets could be cured by feeding fresh liver, and also by the liver extracts used in treating pernicious anemia in man. The maggots of the house-fly larvae were also potent.

The Effect of Various Substances in Checking the Anemia of Trout

<u>Extract Fed</u>	<u>Red Blood Cells</u> (Average of Six Counts in Thousands)			
	4 Weeks	6 Weeks	8 Weeks	13 Weeks
Fresh beef liver	973	1057	1130	1120
Chappel's P. A. fraction	861	851	1025	1010
Armour whole dried liver	840	926	978	1040
No supplement	671	651	656	660
Fly maggots	886	1056	1020	1290

In 1941 Simmons and Norris (76) reported on the effectiveness of xanthopterin in curing the anemia in fish fed synthetic diets. Further studies in this connection will now be reviewed.

G. PTERINS.

1. Relation of Pterins to Anemia.

Tschesche and Wolf (77) reported that goat's milk anemia in rats could be cured by the injection of 10 micrograms of xanthopterin. Previous to their experiments Rominger et al (78) had shown that weanling rats fed goat's milk developed an anemia not curable by the administration of iron and copper. The anemia was not due, as had been suggested by clinical observers, to a hemolytic action of unsaturated fatty acids of the milk fat, since increasing the fat content of the milk by the addition of separated goat's milk fat prevented or cured the anemia. Brouwer (79) and György (80) stated that infants reared on unsupplemented goat's milk developed an anemia different in type from, and more severe than, that caused by cow's milk. Kronacher et al (81) considered this fact to be true in the majority of cases investigated.

Trotter and Day (82) recently reported on the use of xanthopterin in curing the agranulocytosis and anemia which occurred in rats after sulfathiazole therapy. Wright and Welch (83) used "folic acid" and biotin to overcome the anemia produced under similar experimental conditions. In 1941, Hutchings et al (84) isolated a sub-

stance from spinach which they called folic acid and which was found to be necessary for the growth of Lactobacillus casei. On the basis of elementary analyses, molecular weights and properties, folic acid and xanthopterin were apparently very similar in composition.

2. Occurrence of Xanthopterin.

The pterins, of which xanthopterin is the best-known example, were first investigated by Hopkins (85). He isolated a substance from the wings of the common white butterfly which was very similar in certain properties to uric acid. Since 1925 intensive research on the pterins has been carried on in Germany. Xanthopterin occurs in alfalfa, grasses (86), and many of the organs of the human body (87). Koschara (87) isolated the substance from urine, and reported an increased excretion of xanthopterin in cases of pernicious anemia.

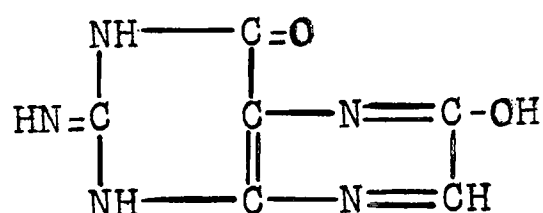
In 1939, Jacobson (88) reported that in most cases of pernicious anemia, the argentaffine cells of the gastro-intestinal tract were either absent or very few in number. These cells contain a pterin and are situated in the gastric mucosa similarly to the cells which excrete the principle active against pernicious anemia.

3. Properties of Xanthopterin.

Xanthopterin is lemon yellow in color and also forms characteristic yellow salts. It is precipitated by

ammoniacal silver nitrate and by mercury salts. It dissolves in acids and alkalies to give an intense yellow color. It is adsorbed on Fuller's earth from acid solution and is eluted by means of bases such as pyridine or ammonia. It gives the murexide reaction, thus showing its relationship to the purines. It is characterized by the formation of a yellow barium salt and by its fluorescence. The fluorescence in acid solution is yellow; in sodium carbonate at a pH of 11, green; and in 20% sulfuric acid solution, a faint and transient pink.

It was synthesized by Purrmann (89) in 1940; by Koschara (90), in 1943. Purrmann suggested the following structure for xanthopterin:



Other pterins occur in nature, the commonest of which is leucopterin, apparently a reduced form of xanthopterin. Erythropterin and guanopterin have been reported but very little is known of their constitution or biological significance (99).

H. SUMMARY.

Although the metabolism of trout has been studied for approximately half a century, little is known of their needs for the different classes of foodstuffs or vitamins.

The most commonly used and in many respects the most satisfactory food material has been found to be fresh meat, particularly liver. Dried animal and vegetable proteins have been found useful in supplementing fresh meats. Beef spleen is valuable due to the physical properties which it imparts when mixed with meat and meal combinations.

Trout can utilize carbohydrates to satisfy part of their energy requirements, but the level of carbohydrate fed has no influence on the rate of growth. Too high a proportion in the diet leads to the development of large livers rich in glycogen.

At least 14% of first quality protein must be present in trout diets for rapid growth. Diets consisting of dry feed mixtures permit excellent growth in trout for periods of three months. After that time the trout die due to a lack of Factor H.

Fish fats are more unsaturated and of higher molecular weight than those characteristic of either plants or animals. A simple deposition of ingested fat explains the characteristic differences in fat composition of marine and fresh-water species. Experimental evidence indicates that trout can utilize fats of relatively high melting points.

Very little work has been done on the vitamin requirements of trout. Thiamin and riboflavin seem to be necessary for general well-being, and vitamin D for good skeletal development.

The accumulation of vitamin A in fish livers seems to be the result of a gradual deposition. This vitamin in combination with protein may assist in the digestion of fat. Vitamin A as it exists in liver is well utilized, but that from cod-liver oil shows evidence of poor assimilation.

Trout need fresh meat to satisfy their requirements for Factor H. An anemia, which can be cured by meat, housefly maggots, and xanthopterin, develops in trout restricted to a dry feed mixture.

Xanthopterin has been used to cure the anemia which developed in experimental animals restricted to a diet of goat's milk. It is excreted in large amounts in the urine of patients suffering from pernicious anemia. The role of xanthopterin in the process of blood formation is unknown.

Nothing is known of the course of intermediary metabolism of food in trout. It is unlikely that there is any conversion of carbohydrate to fat, as under natural conditions, the amount of carbohydrate in trout diets is small. The end-products of protein metabolism in fish are ammonia derivatives, such as trimethylamine. The ordinary analytical methods of studying metabolic products in animals cannot be applied to trout. Up to the present time little progress has been made along these lines.

It is obvious that there are many difficulties to be contended with in feeding experiments which are not experienced with mammals. Perhaps the greatest of these difficulties is the variability in the rate of growth on the same diet, due no doubt to variations in food consumption. It is virtually impossible to control the food intake of fish. Amongst the results reported in the Cortland Hatchery Report (12) it is observed that in three groups of fifty fish of the same age, the weights varied from 24-100 grams, 8-44 grams, and 10-37 grams respectively. It is evident, therefore, that the variability to be expected within groups is large in comparison with other species.

II. EXPERIMENTAL

A. PRACTICAL FEEDING TRIALS.

The first experiment considered in the experimental section was designed to afford practical experience in feeding trials with large numbers of trout. Various combinations of meat and meal mixtures were tested with the object of lowering costs and producing larger fish. This led to laboratory experiments in which detailed studies were made of the "Factor H" and vitamin A requirements of trout.

During the summer of 1942, through the courtesy of the Department of Fisheries at Ottawa, arrangements were made for practical feeding trials to be carried out at the Antigonish Fish Hatchery, Frazer's Mills, Nova Scotia. These experiments were conducted during the months of July, August, and September and had as their objectives:

(1) To study the substitution of fish livers such as cod and mackerel for animal livers.

(2) To compare methods of feeding including broadcasting with a spoon as compared with a specially designed potato ricer.

(3) To study dry feed mixtures introduced at an earlier stage in the growth of the fingerlings than the one and one-half inch stage recommended.

(4) To study modifications in the composition of the standard dry fed mixture #353 (Table VI).

The experiments were conducted with 1000 fingerling trout in each trough. The hatchery water was 87-89% saturated with oxygen and the temperature varied between 60-72° F. The troughs were cleaned regularly each morning and were disinfected every week with copper sulfate. At the same time the fish were dipped for one and one-half minutes in a bath containing copper sulfate 1:2000, acetic acid 1:500, and sodium chloride 1:1000. When the hatchery water supply became warmer, the copper sulfate bath alone was used because acetic acid injures the fish at higher water temperatures. The fish were not fed immediately before or after dipping.

There was more fin-rot than usual in some of the experimental troughs due presumably to the low water level and high water temperatures which prevailed. However, when the dipping was done twice weekly, the number of deaths due to fin-rot was negligible.

The fish were fed three times daily, and the amount of food was controlled as much as possible to reduce waste and prevent contamination. In this connection it was noted that special care had to be exercised when cleaning the troughs where cod liver was fed because of its high oil content.

Many preliminary experiments were conducted with each diet to determine the best physical consistency which would permit distribution of the food so that each fish had an opportunity to eat and reduce waste. For example, it was found that a diet with the desired physical characteristics could not be prepared with cod liver and milk powder alone. The best mixture was found to be 25% cod liver, 25% spleen, and 50% milk powder. Diets containing liver were prepared as follows: the fresh meat was ground once, using a grinder plate with 2 mm. openings; the spleen and cod livers were macerated in the frozen condition. The meat pulp was mixed with the correct amount of the other constituents of the diet, and enough water was added so that the final mixture had the consistency of a heavy dough. A thinner mixture was used for the smaller fish. After they had become accustomed to the food, the amount of water was greatly reduced. Enough food was prepared to last for two days.

At bi-monthly intervals the number of fish in each trough was equalized, those which were to be discarded being taken from the lower end of the trough. In this way the number of fish per cubic foot of water in each trough was kept constant. At the end of each experimental period the fish were weighed and ten from each group were sent to MacDonald College for analysis. It was desirable that these fish should reach the College in as fresh a state as possible. Therefore, the first lot was sent to the Fisheries Research Station at Halifax where the fish were frozen and shipped by air-mail. However, the consignment arrived in very poor condition, so it was decided to use 10% formaldehyde as a preservative in further shipments.

Experiment I. Comparison of Cod Liver and Animal Livers.

Group 1. Hatchery diet (equal parts of beef liver, pork liver, and beef heart).

Group 2. Equal parts hatchery diet (same as Group 1) and cod liver.

Group 3. 25% Cod liver, 25% beef spleen, and 50% skim-milk powder.

This experiment was of very short duration. It was discontinued on August 11 due to a temporary shortage of cod liver. The results are summarized in Table I on the following page and are recorded in the form customary in hatchery reports.

Table I

Duration of Experiment (July 2-August 11)	Group 1	Group 2	Group 3
Wt. 1000 fish July (oz.)	26	24.75	25.25
Wt. per fish (oz.)	0.026	0.025	0.025
Mortality for period	47	20	76
Discard for period	175	202	146
No. of survivors	778	778	778
Total wt. of survivors (oz.)	43.75	43.75	26.00
Wt. per fish (oz.)	0.056	0.056	0.033
Increase per fish (oz.)	0.030	0.031	0.008
Total wt. of fish produced (oz.)	23.3	23.5	6.25
Wt. of food fed (lbs.)	9.5	11.0	12.0
Lbs. of food per lb. of trout	5.3	7.3	30.0
Cost of food per lb. (cents)	12	7	5.2
Cost per lb. of trout (cents)	64	51	156

Apparently the duration of the experiment was long enough to indicate that cod liver lacks some dietary factor which is contained in beef liver, but is not present in spleen or skim-milk. The fish in Group 3 ate well but did not gain in weight. A comparison of the growth rates of Groups 1 and 3 indicates that at least 50% of the hatchery meat diet can be replaced by cod liver.

The experiment with Group 2 was terminated on August 11, but Groups 1 and 3 were continued on the hatchery diet until August 22. The data in Table II show that Group 3 gained weight rapidly when changed to the hatchery diet. This would indicate that some nutrient required by trout is lacking in cod liver.

Table II

Duration of Experiment (August 11-22)	Group 1	Group 3
No. of fish	778	778
Total wt. (oz.)-August 11	43.75	26.00
Total wt. (oz.) August 22	47.75	32.75
Mortality	7	12

Experiment II. Comparison of Hatchery Meat Diet and Dry
Feed Mixture, and of Two Different Met-
hods of Feeding

Group 1. Hatchery diet fed with a spoon.

Group 2. Hatchery diet fed by ricer.

Group 3. Hatchery diet and dry feed mixture #353.

The proportion of dry feed was increased from 25% at the beginning to 50% after two weeks. Fed with a spoon.

Group 4. Same diet as Group 3, but the initial level of dry feed was 12.5% and this was increased to 50% after one month. Fed with a spoon.

Group 5. Same diet as Groups 3 and 4, but the dry feed was withheld for two weeks and then incorporated at an

initial level of 12.5% which was increased to 50% at the sixth week of the experiment. Fed with a spoon.

The data on this experiment are given in Table III.

Table III

Duration of Experiment (July 2-August 22)	Group 1	Group 2	Group 3	Group 4	Group 5
Wt. 1000 fish July (oz.)	26	24.75	25.25	24.75	26.2
Wt. per fish (oz.)	0.026	0.025	0.025	0.025	0.026
Mortality for period	54	237	70	52	25
Discard for period	215	21	199	217	244
No. of survivors	731	731	731	731	731
Total wt. survivors (oz.)	47.73	44.5	60.25	49.25	59.75
Wt. per fish (oz.)	0.068	0.061	0.082	0.067	0.082
Increase per fish (oz.)	0.042	0.036	0.057	0.042	0.054
Total wt. produced (oz.)	30.75	26.35	38.0	30.7	39.4
Wt. of food fed (lbs.)	10	10	12	12	12
Lbs. food per lb. fish	5.5	6.2	4.6	4.8	4.9
Cost of food per lb. (cents)	12	12	8	10	11
Cost per lb. trout (cents)	66	73	36.8	48	54

Comparison of the results obtained in Groups 1 and 2 shows a high mortality in Group 2, but there is not much difference in the growth rates. This might have been due to the ricker carrying infection from one feeding to another although particular care was taken to avoid this. The fact remains, however, that there was a considerable amount of fin-rot among

Group 2. A ricer cup with 2 mm. holes was used and the food was released into the water by pressing down on the plunger while holding the cup so that the bottom was about one inch below the surface of the water, and moving it about with a rotary motion. This procedure released the food in a form suitable for the fish to eat it off the bottom of the trough. An ordinary potato ricer was tried without any better results. One advantage of ricer feeding lies in the fact that a drier mixture can be used with less waste. It was concluded that this advantage was offset by the loss of time involved in cleaning the ricer. The food stuck in the crevices in the bottom making it difficult to clean and sterilize. It was observed that the ricer method did not produce as much turbidity in the water as broadcasting with a spoon.

Comparison of Groups 3, 4, and 5 with Group 1 shows that there is not much difference in the mortality with the exception of a slight reduction in Group 5 where the introduction of dry feed was delayed for two weeks. The growth of the fish on dry feed mixture was better than those on the hatchery diet. Apparently the inclusion of the dry feed at the earliest date and at the highest level (Group 3) gives the best rate of growth. The dry feed mixtures have been widely used in experiments at different hatcheries during the past two years. The results in this experiment indicate that the dry feed can be used to advantage earlier than the one and one-half inch fingerling stage.

Analyses (conducted by Charles Lusena at Macdonald College) were run on random lots of 10 fish from each of the groups under observation. The results are shown in Table IV; Group 3a was sampled August 11; Group 3b, August 22.

Table IV

Experiment I				
	Group 1	Group 2	Group 3a	Group 3b
<u>Weight in Grams</u>				
Minimum	1.15	0.82	0.53	0.56
Maximum	2.57	1.92	1.57	1.73
Average	1.70	1.53	1.18	1.52
<u>Length in Centimeters</u>				
Minimum	3.8	3.5	3.1	3.2
Maximum	5.4	4.9	4.2	4.5
Average	4.5	4.4	4.0	4.4
<u>% Dry Matter</u>				
Minimum	22.6	26.9	23.4	24.6
Maximum	23.5	27.5	27.4	25.5
Average	22.9	27.2	25.3	25.2
<u>% Ash in Dry Matter</u>				
Minimum	6.0	4.5	4.0	5.4
Maximum	6.5	5.1	5.1	5.6
Average	6.2	4.6	4.5	5.5

Table IV (Experiment II) is tabulated below.
The fish used for these tests were sampled on August 22.

Table IV

Experiment II					
	Group 1	Group 2	Group 3	Group 4	Group 5
<u>Weight in Grams</u>					
Minimum	0.88	1.02	0.85	1.31	1.43
Maximum	2.21	2.95	3.41	5.03	3.67
Average	1.90	2.20	1.92	2.86	2.40
<u>Length in Centimeters</u>					
Minimum	3.5	3.9	3.6	4.2	4.2
Maximum	5.1	5.6	5.8	6.6	6.2
Average	4.6	5.6	4.7	5.1	5.4
<u>% Dry Matter</u>					
Minimum	23.1	24.7	22.7	22.6	23.0
Maximum	24.8	25.9	23.0	29.5	31.7
Average	24.2	25.4	23.1	25.2	29.4
<u>% Ash in Dry Matter</u>					
Minimum	6.0	6.0	6.8	6.2	7.0
Maximum	6.2	6.2	7.4	7.8	8.0
Average	6.1	6.1	7.2	7.2	7.3

It will be seen that there is a great variation in weight, length, and dry matter content in individual fish in each group. The conclusions from Experiment II (Table III), relative to the use of dry feeds, are in the main substantiated by the results in Table IV. To reduce the errors resulting from individual variations on the same dietary regime, it would appear that a larger sample than 10 fish of this size would have to be taken for analyses.

Experiment III. Further Experiments Comparing the Feeding Value of Cod Liver and Beef Liver

Group 1. Equal parts of cod liver and dry feed mixture #353.

Group 2. Cod liver, 25%; beef spleen, 25%; and dry feed mixture #353, 50%.

Group 3. Beef liver, 100%.

The results of this experiment are shown in Table V on page 39. Mortality is lowest in Group 3, but this may not be significant. Apparently the mixture of cod liver and dry feed mixture #353 makes an excellent ration. The ingredients mixed well to give good physical consistency and the trout ate the feed readily. However, the duration of the experiment was extremely short, but previous experience has shown that the effects of an unsatisfactory diet would have been apparent in the same length of time. The cause of poor growth in Group 2 is uncertain. If it is assumed that cod liver supplies the essential dietary factors known to be pre-

sent in beef liver and absent from beef spleen, then 25% of cod liver may supply less than the optimum amount necessary for good growth.

Table V

Duration of Experiment (August 1-September 9)	Group 1	Group 2	Group 3
Wt. 1000 fish August (oz.)	43.5	43.5	44.25
Wt. per fish (oz.)	0.044	0.044	0.044
Mortality for period	112	89	59
Discard for period	58	81	111
No. of survivors	830	830	830
Total wt. of survivors (oz.)	94.00	67.25	90.75
Wt. per fish (oz.)	0.112	0.080	0.109
Increase per fish (oz.)	0.068	0.036	0.065
Total wt. fish produced (oz.)	56.5	29.8	54.0
Wt. of food fed (lbs.)	15	15	15
Lbs. of food per lb. of trout	4.3	8	4.4
Cost of food per lb. (cents)	3.5	5	16.3
Cost per lb. of trout (cents)	15.1	40	71

Experiment IV. Comparison of Several Modifications of Dry
Feed Mixture #353 with Beef Liver as a
Standard

The modifications of diet #353 used in this experiment are given in Table VI, which also includes the results of protein analyses which were done at Macdonald College.

Table Va

Experiment III			
	Group 1	Group 2	Group 3
<u>Weight in Grams</u>			
Minimum	2.92	2.09	1.39
Maximum	5.54	7.89	6.27
Average	4.49	4.49	3.64
<u>Length in Centimeters</u>			
Minimum	5.3	4.7	4.3
Maximum	6.7	7.4	7.6
Average	6.1	6.2	5.8
<u>% Dry Matter</u>			
Minimum	23.8	24.0	21.6
Maximum	26.2	24.8	22.9
Average	24.8	24.5	22.3
<u>% Ash in Dry Matter</u>			
Minimum	5.2	5.7	6.1
Maximum	7.8	6.0	8.6
Average	6.7	5.8	7.3

The results are considered to be sufficiently comparable to warrant the conclusion that there would be no significant difference in the protein level of the various diets.

Table VI

	Modifications in Cortland Hatchery Diet #353					
	353	353 A	353 C	353 D	353 E	353 F
Dried skim-milk	24			24	24	
Wheat middlings	24	40	37	30		24
Cottonseed meal	24	24	24		24	24
Fish meal	24	32	24	32	25	
Wheat germ				10		
Meat meal			11			24
Salmon-egg meal						24
Cod-liver meal					23	
Salt	4	4	4	4	4	4
Crude protein (N x 6.25)	41.6	40.8	37.1	41.4	42.5	42.4

The following mixtures were fed to the trout.

- Group 1. Beef liver, 100%.
- Group 2. Beef liver, 25%; beef spleen, 25%; #353, 50%.
- Group 3. Beef liver, 25%; beef spleen, 25%; #353 A, 50%.
- Group 4. Beef liver, 25%; beef spleen, 25%; #353 D, 50%.
- Group 5. Beef liver, 25%; beef spleen, 25%; #353 E, 50%.
- Group 6. Beef liver, 25%; beef spleen, 25%; #353 F, 50%.

The results are shown in Table VII on pages 42 and 43.

Table VII

Duration of Experiment (August 1-September 9)	Group 1	Group 2	Group 3
Wt. 1000 fish August (oz.)	44.25	47.5	43.25
Wt. per fish (oz.)	0.044	0.047	0.043
Mortality for period	59	79	31
Discard for period	111	91	139
No. of survivors	830	830	830
Total wt. of survivors (oz.)	90.75	100	86.5
Wt. per fish (oz.)	0.109	0.120	0.104
Increase per fish (oz.)	0.066	0.073	0.061
Total wt. of fish produced (oz.)	54.0	59.8	50.65
Wt. of food fed (lbs.)	15	15	15
Lbs. of food per lb. of trout	4.4	4.1	4.7
Cost of food per lb. (cents)	16.3	7.6	7.0
Cost per lb. of trout (cents)	71	31	33

Table VII is continued on page 43.

Table VII

Duration of Experiment (August 1-September 9)	Group 4	Group 5	Group 6
Wt. 1000 fish August (oz.)	41.75	46.25	43.00
Wt. per fish (oz.)	0.042	0.046	0.043
Mortality for period	101	78	170
Discard for period	69	92	0
No. of survivors	830	830	830
Total wt. of survivors (oz.)	71.25	94.75	79.75
Wt. per fish (oz.)	0.085	0.114	0.096
Increase per fish (oz.)	0.043	0.068	0.053
Total wt. of fish produced (oz.)	35.7	56.5	44.0
Wt. of food fed (lbs.)	15	15	15
Lbs. of food per lb. of trout	6.9	4.3	5.4
Cost of food per lb. (cents)	7.7	7.5	6.8
Cost per lb. of trout (cents)	53.1	32.3	34.6

It will be seen from Table VII that the highest incidence of mortality, and the poorest growth, was in Groups 4 and 6. Fin-rot was prevalent in these two groups, but particularly in Group 4, and is probably responsible for the high mortality. This may also have affected the growth, but it was thought that these diets were inferior. The diet of Group 2, which contained the unmodified dry feed mixture #353, is apparently as good as the straight liver diet. There is no evidence that any modification of this dry feed constitutes an improvement. The growth with Group 5 receiving cod-liver

Table VIIa

Experiment IV

Group 1 Group 2 Group 3 Group 4 Group 5

Weight in Grams

Minimum	1.39	3.03	1.98	2.26	2.75
Maximum	6.27	8.85	5.46	4.92	6.55
Average	3.64	4.44	3.74	3.22	5.16

Length in Centimeters

Minimum	4.4	5.6	4.8	4.9	5.3
Maximum	7.6	8.0	6.8	6.7	6.9
Average	5.8	6.2	5.8	5.6	6.6

% Dry Matter

Minimum	21.6	22.5	21.7	20.1	20.8
Maximum	22.9	24.0	22.9	22.1	21.1
Average	22.3	22.9	22.1	21.2	21.0

% Ash in Dry Matter

Minimum	6.1	5.7	6.7	5.7	6.6
Maximum	8.6	7.0	7.7	7.2	6.8
Average	7.3	6.4	7.2	6.4	6.7

Table IX is continued on page 47.

residue in the dry feed mixture was very good, but it is not favoured because of its poor physical consistency and resulting waste.

Experiment V. Comparison of Beef Liver and Beef Liver Mixed with #353 A, #353 C, and #353 D

The dry feed mixtures #353 A, C, and D used in this experiment were the same as those referred to in Table VI. The composition of the rations was:

Group 1. Beef liver, 100%.

Group 2. Beef liver, 50%; #353 A, 50%.

Group 3. Beef liver, 50%; #353 C, 50%.

Group 4. Beef liver, 50%; #353 D, 50%.

The results of this experiment are given in Table VIII on page 45. It would appear that dry feed mixtures, at least in the case of #353 A, can replace 50% of the beef liver without noticeable effect on the rate of growth. However, these diets without spleen do not have as good physical properties as the diets with spleen in Experiment IV. These results are in agreement with those obtained by Tunison and McCay, who found that 50% of the beef liver could be replaced by dry feed mixtures. No significance is attached to the difference in the results with Groups 3 and 4, because there was a fairly high incidence of fin-rot.

Table VIII

Duration of Experiment (August 1-September 9)	Group 1	Group 2	Group 3	Group 4
Wt. 1000 fish August (oz.)	44.25	44.74	41.75	42.25
Wt. per fish (oz.)	0.044	0.045	0.042	0.042
Mortality for period	59	39	111	38
Discard for period	111	131	59	132
No. of survivors	830	830	830	830
Total wt. of survivors (oz.)	90.75	90.25	77.5	82.5
Wt. per fish (oz.)	0.109	0.108	0.094	0.099
Increase per fish (oz.)	0.065	0.063	0.052	0.057
Total wt. of fish produced (oz.)	54.0	53.0	43.2	47.3
Wt. of food fed (lbs.)	15	15	15	15
Lbs. of food per lb. of trout	4.4	4.5	5.5	5.0
Cost of food per lb. (cents)	16.3	9.3	8.8	9.3
Cost per lb. of trout (cents)	71	41.8	48.4	46.5

Table IX

Experiment V				
	Group 1	Group 2	Group 3	Group 4
<u>Weight in Grams</u>				
Minimum	1.39	1.90	1.57	2.16
Maximum	6.27	6.20	5.48	3.42
Average	3.64	3.42	4.16	2.76
<u>Length in Centimeters</u>				
Minimum	4.3	4.6	4.3	5.1
Maximum	7.6	6.9	6.9	5.7
Average	5.8	5.8	5.3	5.4
<u>% Dry Matter</u>				
Minimum	21.6	22.1	22.5	21.6
Maximum	22.9	24.9	23.8	22.3
Average	22.3	23.7	23.2	22.0
<u>% Ash in Dry Matter</u>				
Minimum	6.1	7.3	7.5	8.4
Maximum	8.6	8.7	8.6	8.6
Average	7.3	8.2	7.6	8.5

Va, VIIa and

Table IX presents the same data for Experiments III, IV, and V as Table IV gives for Experiments I and II. The results of these analyses confirm the previous conclusion that a random sample of 10 fish is not representative. Samples should consist of at least 10% of the total number of fish used in the feeding trials. The data also show that the variability among the younger fingerlings (Table IV) is much less than among the older fingerlings (Table IX).

M. W. Smith examined a sample of the water taken from the Antigonish Hatchery for any natural trout food that was present. He was able to identify only the chadocerans and copepods. The species identified were:

Chadocera

Eurycercus lanellatus

Chydorus sphaericus

Alona rectangula

Pleuroxus provurcatus

Acroperus harpae

Chydorus sp.

Copepods

Macrocyclus albidus

Eucyclops agilis

A number of immature specimens were noted, but not identified.

B. STUDIES ON PTERINS AND ANEMIA.

The experience gained in the practical feeding studies suggested that nutritional work with trout under rigidly controlled conditions might help to solve some of the problems that have been puzzling trout culturists for many years.

Two points seem to be of importance:

(1) The possible role that xanthopterin plays in curing the anemia of trout as well as of other experimental animals.

(2) The assimilation of vitamin A from different foodstuffs.

The first phase of this investigation was an examination of Wu's (91) method for determining hemoglobin and adapting it to a photoelectric colorimeter. The benzidine test for blood is very sensitive and can be carried out with small samples. It depends on the oxidation of benzidine by hydrogen peroxide under the catalytic influence of hemoglobin.

Bing and Baker (92), in their investigation of the blood-benzidine reaction, stressed the importance of the purity of the reagent. They dissolved the benzidine in alcohol, decolorized with charcoal, and recrystallized from dilute alcohol.

McFarlane and Hamilton (93) purified the benzidine by converting it to the hydrochloride, decolorizing with charcoal, and then decomposing the hydrochloride by the addition of ammonia. The free base was recrystallized from alcohol

and dried in a vacuum desiccator.

Lieboff (94) has used the dihydrochloride in routine work, and as this compound is much more stable than the free base, it was decided to employ it in this investigation.

The dihydrochloride was prepared as outlined by McFarlane and Hamilton (93) up to the point where decolorization by Norite A was complete. The colorless filtrate was concentrated until, on cooling slightly, crystals of benzidine dihydrochloride separated out. These were filtered off and dried in a vacuum desiccator over sulfuric acid. They were well-formed and colorless. A second crop of crystals was obtained by treating the mother liquor with Norite A and then concentrating it.

The benzidine solution was freshly prepared just before carrying out the test. The benzidine dihydrochloride was dissolved, without the aid of heat, in sufficient 20% acetic acid to give a 2% solution which was filtered and stored in a glass-stoppered bottle.

To carry out the test, 2 cc. of the benzidine solution, 1 cc. of a blood solution of appropriate dilution, and 1 cc. of 1% hydrogen peroxide (freshly prepared from Merck's Superoxyl) were mixed in a 10 cc. graduated cylinder. At the same time, a blank was prepared containing 1 cc. of 20% acetic acid in place of the blood solution. The color was allowed to develop for one hour (93). The cylinders were preferably kept in the ice-box during this time, otherwise

the blank became too highly colored. The solutions were made up to volume with 20% acetic acid and allowed to stand at room temperature for fifteen minutes. The percentage of light transmitted was determined in an Evelyn photo-electric colorimeter, filter 520. The blank tube was used to obtain the "centre-setting" for the instrument.

For preliminary work a 2.5% solution of "Difco" dried hemoglobin was prepared. This solution was dialyzed in a cellophane membrane against distilled water for twenty-four hours. It was transferred to a glass-stoppered bottle, a few grains of methiolate added as a preservative, and was stored in the refrigerator until needed.

One cc. of the above hemoglobin solution was diluted to 1000 cc. with 20% acetic acid. This solution constituted the standard for the preliminary work. Dilutions of 1,2---7% of the standard were prepared as needed. To study the stability of the color produced in the test, a series of tubes was prepared as outlined above and the light transmission measured at regular intervals. The data are tabulated in Table X on page 52.

Table X

The Stability of the Color Produced in the Blood-Benzidine Test

% Dilution	Galvanometer Readings		
	After 15 Min.	After 60 Min.	After 240 Min.
Blank	74	79	92
1	79	78	79
2	61	61.5	63
3	47	47	49
4	35.5	35.5	37
5	26	26.5	29
6	19	18.75	20
7	14	14.5	16

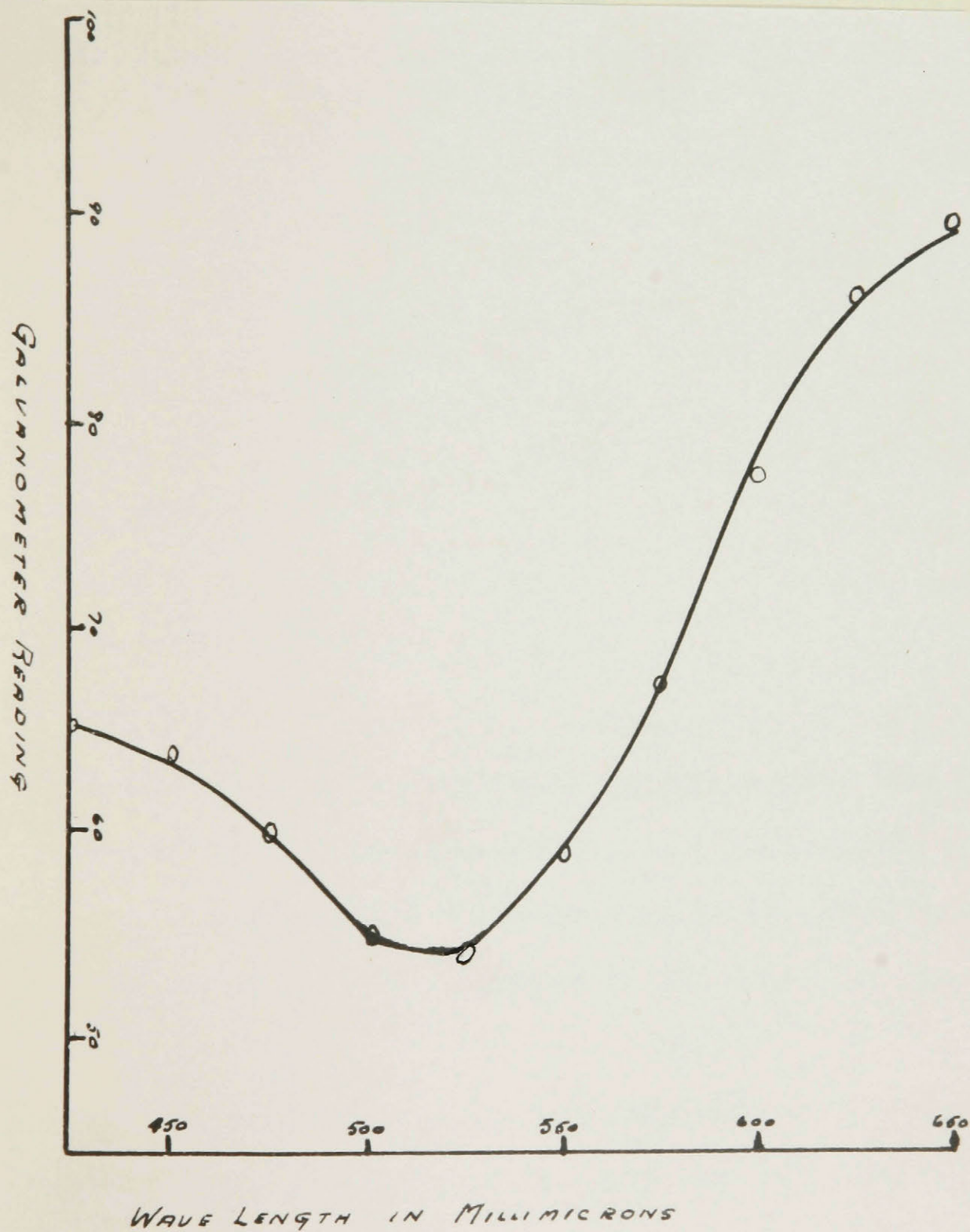
The results expressed in Table X show that there was a pronounced increase in the color of the blank on standing four hours, while the color of the test solutions decreased slightly.

The absorption maximum for the red color produced in the blood-benzidine reaction, as measured in a Coleman spectrophotometer, was found to be at 525 millimicrons. Andrews and Brooks (96) carried out their measurements at 660 millimicrons. An absorption curve is shown in Figure I on page 53.

The influence of the strength of the hydrogen peroxide used was next investigated. The results are shown in Table XI on page 54. Previous workers (92, 93) have found that superoxyl diluted to 0.6-1.5% was preferable as commercial

Figure I

Absorption Curve for Blood-Benzidine Reaction



3% hydrogen peroxide contains preservatives which may interfere with the reaction.

Table XI

The Influence of Hydrogen Peroxide Concentration on the
Blood-Benzidine Reaction

Concentration of H_2O_2	Galvanometer Readings	Remarks	
10.00%	44	Pronounced Turbidity	
7.5 %	34.5	"	"
5.0 %	35.5	"	"
2.5 %	26.25	Slight	Turbidity
1.5 %	26	"	"
1.0 %	26	Clear	Solution
0.75%	28	"	"

Since a 1% solution of hydrogen peroxide gave the best results, that concentration was chosen for all subsequent work.

The agreement between duplicate determinations on the above series of standard solutions is shown in Table XII on page 55.

To prepare a standard reference curve for subsequent work, a sample of oxalated horse blood was obtained and 1 cc. diluted to 1000 cc. with 20% acetic acid. A series of standards containing 2-22% of this stock solution was prepared, and the benzidine test run on 1 cc. aliquots. The results are expressed in Table XIII on page 55.

Table XII

Reproducibility of the Blood-Benzidine Reaction

% Dilution	Galvanometer Readings	
	Test 1	Test 2
Blank	72.5	71
1	78.25	78
2	60	60
3	44.5	44
4	33	33
5	24	24
6	17.5	18.5

Table XIII

Galvanometer Readings and "K" Values of Standard Solutions of Horse-Blood in the Blood-Benzidine Test

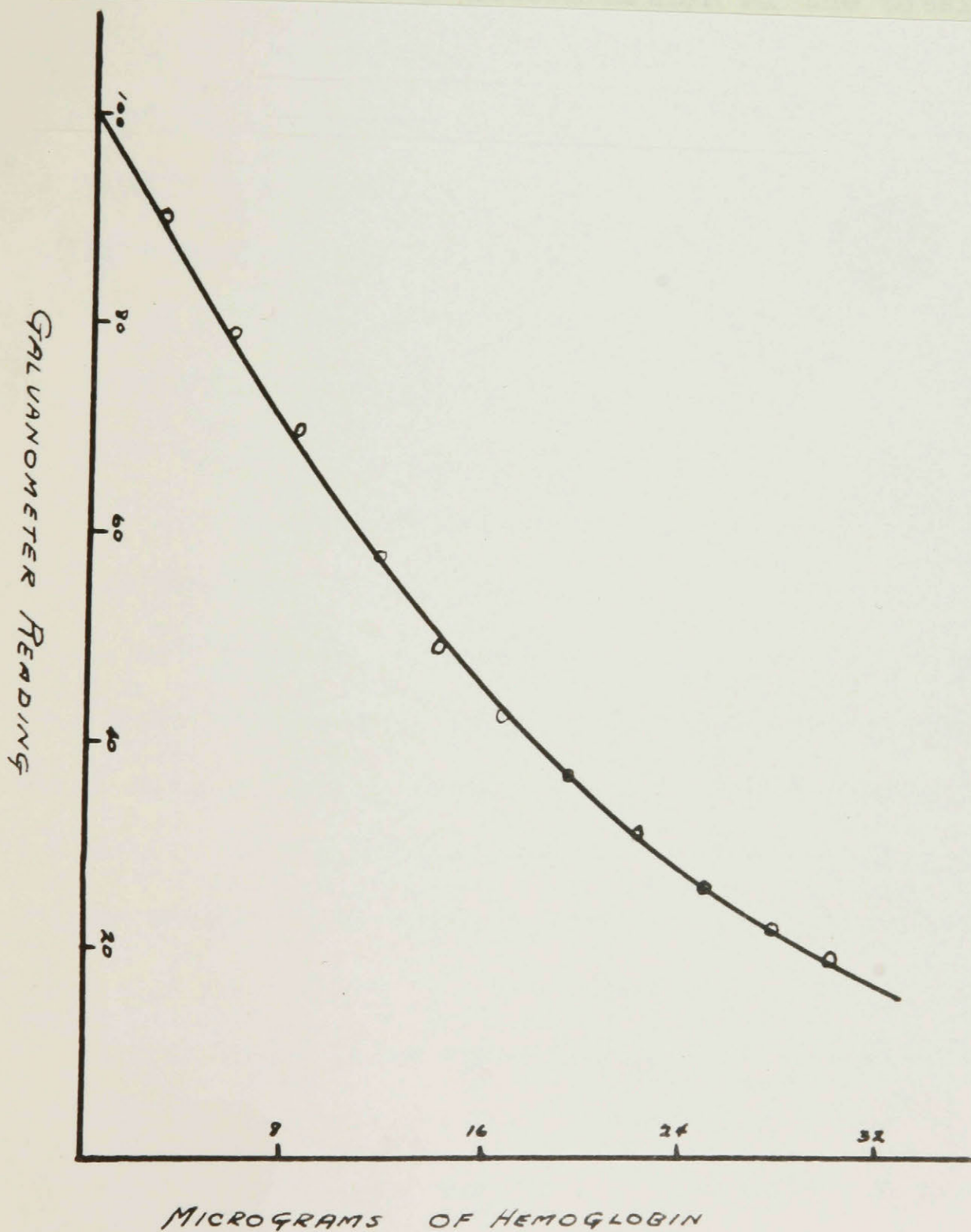
% Dilution	Galvanometer Readings	"K" Values
2	89.5	0.241
4	77.0	0.284
6	66.5	0.297
8	59.5	0.282
10	48.75	0.312
12	43.0	0.305
14	35.5	0.321
16	29.75	0.329
18	26.75	0.321
20	24.75	0.303
22	18.75	0.330

The hemoglobin content of the blood was checked by determining its iron content. Duplicate samples (2 cc.) of the blood were digested in 100 cc. Kjeldahl flasks with 1 cc. of sulfuric acid and 0.5 cc. of 72% perchloric acid until clear. The solutions were allowed to cool slightly, 5 cc. of distilled water added, and the mixture heated to boiling. They were then allowed to cool and transferred to 25 cc. graduated cylinders and made to volume with 25% ammonium acetate. Aliquots of these solutions were transferred to 10 cc. graduated cylinders, 0.5 cc. of 0.1% α, α' -dipyridyl in 10% acetic acid and 0.5 cc. of 0.2% p-hydroxyphenylglycine in 0.4 N sulfuric acid were added. The solutions were made to volume with 25% ammonium acetate. The red color of the ferrous dipyridyl developed immediately and its intensity was measured in an Evelyn photoelectric colorimeter using filter 520. The amount of iron in the sample was determined from a standard reference curve.

The hemoglobin content of the oxalated blood was 13.73%, calculated on the basis that hemoglobin contains 0.335% iron (recent work (95) has shown that the percentage is 0.340). The figures reported in Table XIII are expressed graphically in Figure II on page 57 in which the galvanometer readings in the blood-benzidine reaction are plotted against micrograms of blood used.

Figure II

Reference Curve for Blood-Benzidine Reaction



It is difficult to obtain from trout of less than 6 grams in weight even the small amount of blood needed in the benzidine method. The procedure outlined above was therefore adapted to the determination of the total peroxidase systems in the trout body. Hemoglobin contains both thermostable and thermolabile peroxidase systems. The assumption underlying the determination of total peroxidase is that, in cases of anemia, hemoglobin is decreased with no compensating increase in other peroxidases. Consequently, any changes in hemoglobin content should be reflected in the total peroxidase systems.

The following procedure was used. The trout were killed by severing the spinal cord. The bodies, freed of external moisture and slime by wiping carefully with filter paper, were weighed. Each entire body was ground with acid-digested sand or solid carbon dioxide and the ground mass transferred to 50 cc. centrifuge tubes. Ten cc. of glacial acetic acid were added, and the contents of the tubes heated just to boiling on a sand bath. The tubes were centrifuged and the clear supernatant liquid decanted into 50 cc. volumetric flasks. The residue was extracted five times using 5 cc. of glacial acetic acid for each extraction. The extracts were made to volume with glacial acetic acid, mixed, and 1 cc. aliquots used in the benzidine test.

Alternatively, the trout were killed as above, cleansed, weighed, and dried to constant weight in an electric oven at 105° C. The bodies were ground in a mortar and weighed

samples of the dried and ground tissue were used for the extraction of the thermostable peroxidase systems by the following method. Samples of dried tissue (0.2-0.4 g.) were extracted with 5 cc. of glacial acetic acid as previously outlined. The clear supernatant liquid was decanted into 25 cc. volumetric flasks, and the extraction was repeated five times.

In these preliminary analyses the trout varied in weight from 0.6-5.0 g., and were non-feeding except for possible cannibalism. The results of the analyses are shown in Table XIV as milligrams of hemoglobin per gram of wet tissue.

Table XIV

A. Reproducibility of Results on Dried Tissue

Sample Number	Weight of Trout	Mg. Hemoglobin per Gm.
1	1.1265	1.72 1.74
2	1.1315	2.40 2.37
3	1.2613	4.45 4.48
4	1.4450	3.19 2.92
5	1.7004	2.86 2.72
6	2.3488	1.58 1.60

Table XIV is continued on page 60.

B. Hemoglobin Determinations on Fingerling Trout Bodies
(dried to constant weight)

Sample Number	Weight of Trout	Mg. Hemoglobin per Gm.
1	0.8156	1.19
2	0.9168	1.76
3	1.0008	1.09
4	1.0576	1.27
5	1.1848	1.38
6	1.2508	1.17

C. Hemoglobin Determinations on Fresh Tissue

Sample Number	Weight of Trout	Mg. Hemoglobin per Gm.
1	0.6921	7.9
2	0.7652	6.0
3	0.9862	5.2
4	1.0407	6.8
5	1.0919	7.2
6	1.1008	4.8
7	1.1657	5.4
8	1.1648	5.3
9	1.4441	4.7
10	2.5997	4.9
11	5.3192	3.8

The results shown in Table XIV C include both the thermostable and thermolabile peroxidases systems, and it is evident that about two-thirds of the total is destroyed on drying.

The variation in hemoglobin content between different trout is evidently very great. However, the determinations on fresh tissue indicate a very high level of hemoglobin in trout of less than 1 g. in weight in comparison to corresponding analyses on the dried tissue. The ratio is approximately 5:1. There is also a tendency for the relative amount of hemoglobin to decrease as the weight of the trout increases.

As iron is an essential constituent of the hemoglobin molecule, the total amount present in the trout body and the relationship between the total iron and hemoglobin contents was next investigated. The results are shown in Table XV.

Table XV

Relationship between Hemoglobin and Iron Contents of Trout Bodies

Sample Number	Weight of Trout	Total Iron (Micrograms)	Total Hemoglobin (Milligrams)	Ratio Hb:Fe
1	0.9977	138.8	6.28	1:22
2	1.0738	83.4	3.88	1:21.5
3	1.2763	120.0	4.6	1:27
4	4.9547	152.5	6.1	1:25

The ratio of total iron to hemoglobin content is nearly constant, but the individual values vary widely.

The above analyses were performed on starved trout. However, as they varied in size, it was difficult to prevent cannibalism. Therefore the remaining trout were divided into

two groups of 100 each. Group 1 was fed beef liver; Group 2, a dry feed mixture of the following composition:

Casein	20%
Starch	29%
Dextrin	29%
Yeast	5%
Mineral Mixture	4%
Cod-liver Oil	3%
Lard	10%

The above mixture, exclusive of lard, is the anemia-producing diet used by McCay (6). The lard was incorporated in the mixture to coat the particles and thus prevent their too easy solution in water. The mixture was prepared in the following way. The dry ingredients were mixed together. The lard was melted on a steam bath and cooled to just above the solidification point, the cod-liver oil added, and the melted fats mixed intimately with the other ingredients. The cod-liver oil used contained 3600 I. U. of vitamin A per gram. This diet was made up at weekly intervals.

The trout were fed three times a day during the experiment. The dry feed mixture was scattered on the surface of the water, but the trout did not take this mixture readily. It was approximately ten days after feeding was begun before they were eating any quantity of it. The liver was mixed with a small amount of water and broadcast by means of a spoon. It was eaten readily and the trout in Group 1 grew much more quickly than those in Group 2.

Hemoglobin and iron determinations were carried out on trout from each group at intervals during the feeding trials. The results are shown in Table XVI.

Table XVI

The Results Obtained in Feeding Trials with Trout

Date of Analysis	Weight of Trout	Hemoglobin (Mg. per Gm.)	Total Iron (Micrograms)
<u>Group 1</u>			
Jan. 3	1.9322	1.03	197.2
	2.8463	0.70	170.7
Jan. 19	2.5829	0.45	
Feb. 3	3.6828	0.87	49.0
	5.4999	0.42	27.0
	3.0926	0.63	29.5
<u>Group 2</u>			
Jan. 3	3.0201	0.49	108.1
	1.8861	0.65	53.8
Jan. 19	2.0291	0.65	
	1.6411	0.42	94.9
	1.5288	0.53	
	2.4552	0.60	
Feb. 3	2.0880	0.97	109.0
	1.7966	0.94	109.0
	2.4325	0.45	104.0
	4.2324	0.49	90.0

The hemoglobin results are again very variable. With the increase in the fat content of the trout body, it was not possible to obtain a clear extract for the benzidine test. Therefore, the procedure for extracting the peroxidases was changed. The trout bodies were dried in an electric oven, ground, and extracted with petroleum ether for 16 hours in a Johnson extractor. The residue furnished a clear extract for the benzidine test. The petroleum ether solution of the fat was evaporated to dryness and the fat content of the trout body determined. The analytical results are given in Table XVIIa for Group 1 fed liver and Table XVIIb for Group 2 fed dry feed mixture.

Table XVIIa

Analyses of Trout Bodies under Different Feeding Conditions

Date of Analysis	Weight of Trout	% Dry Matter	% Fat	Hemoglobin (Mg. per Gm.)	Total Iron (Micrograms)
<u>Group 1</u>					
Feb. 16	2.7080	21.55	22.2	0.54	119.4
	4.2340	22.42	26.6	0.54	106.0
	4.0405	22.79	27.0	0.44	83.2
	4.9760	22.34	23.3	0.76	96.6
Feb. 23	6.2846	23.56	25.7	0.63	123.0
	23.0941	27.35	28.5	0.36	123.0
	7.5547	21.42	26.3	0.42	94.8
	11.0414	25.18		0.38	110.4

Table XVII is continued on page 65.

Table XVIIb

Date of Analysis	Weight of Trout	% Dry Matter	% Fat	Hemoglobin (Mg. per Gm.)	Total Iron (Micrograms)
<u>Group 2</u>					
Feb. 16	3.4350	31.19	17.5	0.62	115.1
	3.9649	30.10	16.4	0.78	80.0
	3.8269	24.09	25.5	0.67	127.5
	3.4036	23.50	27.5	0.51	165.1
Feb. 23	15.4016	25.98	23.1	0.63	163.0
	12.2476	21.63	17.9	0.36	158.0
	24.2274	24.02	24.9	0.58	138.0
	8.3417	24.14	27.0	0.42	157.0

From a survey of the hemoglobin figures reported above, it is evident that the benzidine test cannot be relied upon to give any indication of anemia. The variations among trout are too great to permit any comparison of the results of these feeding trials.

As the trout were somewhat larger than at the beginning of this work, it was possible to obtain blood from the posterior vena cava for the benzidine reaction. The trout were dried with filter paper, washed free of slime with alcohol and ether, and the tails cut off below the anal opening by means of a sharp scalpel. One drop of blood was caught in a weighed amount of 20% acetic acid contained in a small glass-stoppered vial. The vial was re-weighed and the amount of hemoglobin in 1 cc. of the

solution was determined. As the trout bled freely from the posterior vena cava, enough blood was obtained to check the hemoglobin determinations by red blood cell counts. The results of the experiment (Table XVIII) show no evidence of anemia in the trout receiving the anemia-producing diet.

Table XVIII

Iron and Blood Analyses on Trout Bodies

Date of Analysis	Micrograms Iron		Hemoglobin Gm. per 100 cc.	Red Blood Cell Count
	per Gm. Wet	Wt. Gm. per 100 cc.		
	<u>Total Available</u>			
	<u>Group 1</u>			
March 9	133.7	74.0	13.4	1,265,000
	131.5	56.5		1,710,000
	125.0	54.3	14.1	1,360,000
	89.8	52.0		1,385,000
March 16	116.1	80.0	15.1	1,405,000
	121.1	86.2	13.4	1,420,000
	137.1	88.6	17.0	1,795,000
	130.3	60.5	12.7	1,050,000
	<u>Group 2</u>			
March 9	123.4	72.0		1,420,000
	117.7	62.5	13.6	1,290,000
	128.4	74.0	14.5	1,180,000
	98.5	50.0	16.5	1,160,000
March 16	97.7	57.0	10.2	1,055,000
	99.5	50.0	10.3	1,185,000
	87.5	48.0	16.2	1,635,000
	93.0	54.7	12.5	1,060,000

While the results are not definite, the trout on the liver diet show an increase in total iron, while those receiving the McCay diet show a decrease. The available iron is approximately 50% of the total and parallels it. The cell count on the blood of the last trout in Group 2 may be in error as a small clot was present.

As it was found difficult under these experimental conditions to produce a Factor H deficiency in trout, it was decided to test the efficiency of xanthopterin preparations in the prevention of anemia in weanling rats fed solely on cows' milk.

Male rats, 21 days old, were divided into 8 groups of 5 rats each by random selection. They were placed in individual galvanized-iron wire cages free from rust (97). The cages were cleaned frequently. Glass feeding dishes, used in the trials, were washed in soapy water and rinsed in redistilled water before being filled with milk. The milk was obtained from the Macdonald College Dairy and was milked directly into an enamelled bucket and transferred immediately to bottles for distribution. These bottles had previously been rinsed with redistilled water.

The rats were given fresh milk ad libitum twice a day. Redistilled water was also provided.

Two xanthopterin fractions were tested:

(1) Xanthopterin isolated by Koschara's (87) procedure from liver residue, a by-product in the preparation of the anti-pernicious anemia factor.

(2) Xanthopterin synthesized according to Purrmann's (89) method.

The groups were fed supplements by means of a medicine dropper as follows:

Group 1. No supplement.

Group 2. 2 mg. FeCl_3 and 1 mg. CuSO_4 daily per rat.

Group 3. 2 mg. FeCl_3 daily per rat.

Group 4. 1 mg. CuSO_4 daily per rat.

Group 5. Synthetic xanthopterin, 50 micrograms daily per rat.

Group 6. Xanthopterin isolated from liver residue, 50 micrograms daily per rat.

Group 7. 2 mg. FeCl_3 and 50 micrograms synthetic xanthopterin daily per rat.

Group 8. 1 mg. CuSO_4 and 50 micrograms synthetic xanthopterin daily per rat.

The ferric chloride was prepared from iron wire (analytical grade) by dissolving the required amount in concentrated hydrochloric acid and then adding ammonium hydroxide to precipitate the iron and filtering. The material was purified by repeated solution in hydrochloric acid and re-precipitation as the hydroxide. The ferric chloride was made up to such a volume that 2 drops supplied 2 mg.

The cupric sulfate solution was made from C. P. crystals which had been recrystallized from redistilled water. One drop of the final solution contained 1 mg. of cupric sulfate.

The xanthopterin preparations were dissolved in redistilled water with the aid of hydrochloric acid. Fresh solutions were made up weekly.

The rats were weighed weekly and on the same date a determination of hemoglobin was made on a sample of the blood. The results are shown in Tables XIX and XX on page 70.

The blood pipette employed was made from capillary tubing and was calibrated by means of mercury. It contained 0.0049 cc. of blood. The rats were bled by cutting off the tip of the tail by means of a sharp scalpel. A measured amount of freely flowing blood was taken up in a micro-pipette and transferred into 10 cc. of 20% acetic acid. Appropriate aliquots were used for the benzidine test. The rats' tails were cauterized with a hot spatula. Blood analyses were carried out at the beginning of the test. Two weeks later other blood samples were taken, and thereafter hemoglobin determinations were done weekly.

The results (Table XIX) show that Group 2 made the best growth, while Group 7 was second. The rats in

Table XIX

Weekly Gain in Weight

Group	Initial Weight (Grams)	<u>Average Gain</u>			
		First Week	Second Week	Third Week	Fourth Week
1	34	15	16	10	3
2	33	18	23	23	21
3	34	15	22	10	10
4	35	16	18	17	11
5	37	9	12	13	5
6	33	16	19	12	5
7	37	13	22	20	16
8	36	17	16	14	4

Table XX

Hemoglobin Content of Rat Blood in Grams per 100 cc.
(Initial Level: 6.1-7.4)

Group	<u>Hemoglobin</u>		
	Second Week	Third Week	Fourth Week
1	6.3	5.3	4.2
2	9.9	10.8	11.1
3	9.2	8.6	8.1
4	7.9	6.9	5.6
5	7.9	7.4	5.0
6	6.2	6.1	4.6
7	7.0	10.1	10.5
8	6.2	7.6	6.3

these two groups were healthy and active. Those in Group 8 were inferior to the preceding two groups, but superior to all the others. The rats in the remaining groups were not active, and as the experiment proceeded, had a greatly diminished appetite.

The results also show a progressive increase in the hemoglobin level in Group 2, until at the end of four weeks a value of 11.1 g. per 100 cc. of blood was reached. The only other group to approach this figure was Group 7 which had a hemoglobin value of 10.5.

This experiment also shows that rats fed cows' milk ad libitum, together with iron and copper, grew at a faster rate than those fed any of the other supplements. These rats gained on the average 21 g. weekly over a period of four weeks. The rats receiving milk and supplements of iron and xanthopterin gained on an average 18 g. weekly. The rats in all the other groups showed, over the period of the experiment, progressively smaller gains.

There are several possible mechanisms by which xanthopterin may apparently substitute for copper in the formation of hemoglobin:

(1) Xanthopterin may make the utilization of copper in the formation of hemoglobin more efficient, thus the small amount of copper in cows' milk may be sufficient.

(2) Xanthopterin may replace copper in the formation of hemoglobin by taking its place in some oxidation-reduction system within the body.

It must be remembered that Tschesche and Wolf (77) found that xanthopterin was able to cure goats' milk anemia without benefit of added iron. Goats' milk, however, contains on an average four times the amount of iron present in cows' milk. These authors advanced an hypothesis of increased economy in the utilization of iron through supplementary addition of xanthopterin.

C. VITAMIN A STUDIES.

1. Preliminary Experiments on Vitamin A Requirements of Trout.

The trout which had been fed on a modified McCay diet for about three months became pop-eyed. There are several forms of this disease due to different causes, of which one is brought about by an infestation of trematode worm. As it is known that the vitamin A from cod-liver oil is poorly assimilated by trout, it was thought that this condition might be a form of xerophthalmia. On dissection the trout showed evidence of internal hemorrhage, but no parasites were found. Therefore, a study of vitamin A utilization was undertaken in the hope that it might yield information on this disease. The first experiments were of a preliminary nature designed to establish suitable conditions for subsequent work.

All the trout were raised under the following experimental hatchery conditions. Eight troughs 7' 8" long, 1' 3" wide, and 6.5" deep were provided. These were cleaned thoroughly each week. The water supply was pumped from an artesian well, warmed to a temperature of 62° C by a steam heater regulated by hand, and aerated by allowing it to flow over a series of three slightly inclined washboards. By this means the oxygen content of the water was raised from 3.2 to 5.6 ml. of oxygen per liter as determined by the Winkler method.

On June 11, 1942, 700 spring fry (average weight, 0.29 g.) and 116 yearlings (average weight, 20 g.) were received. The yearlings were extremely pop-eyed, and the mortality in both groups was very high for the first two weeks. The yearlings were divided into two groups, Group 1 was fed beef liver; Group 2, beef heart with 6% of cod-liver oil added. The vitamin A content of this oil was 3600 I. U. per gram. Two fish were taken from each group every week, starved for thirty-six hours, and the vitamin A content of the whole body determined.

The trout were killed by severing the spinal cord and then weighed. The body was placed in a flask, 5-10 cc. of 50% potassium hydroxide and 30-75 cc. of 95% ethyl alcohol, depending on the weight of the fish, were added. The contents of the flask were refluxed on a water bath for thirty minutes and then cooled. The vitamin A and carotene were extracted with petroleum ether. This extract was purified by alternate washings with water and dilute potassium hydroxide solution, and dried over anhydrous sodium sulfate. The petroleum ether was distilled off in vacuo and the residue dissolved in 10 cc. of chloroform. The vitamin A content of this solution was determined by the Carr-Price reaction.

After July 29 one fish was removed weekly and the vitamin A contents of the liver, caecum, and the remainder of the body separately determined. The total vitamin A content of the entire body was calculated from the data in Table XXI on page 75 and Figure III on page 76.

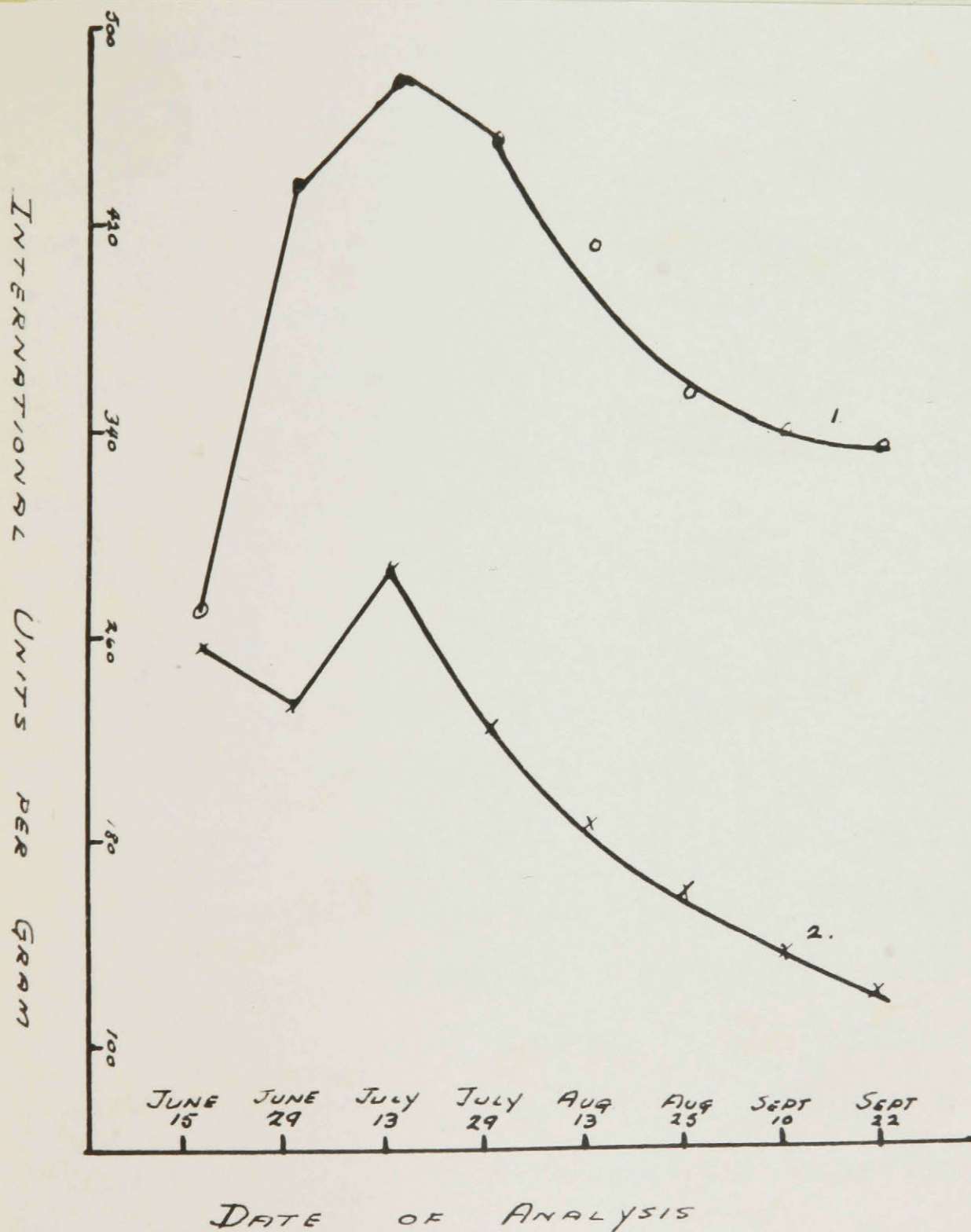
Table XXI

The Vitamin A Contents of Various Tissues of Trout Bodies
under Different Feeding Conditions

		Vitamin A Content (I. U. per g. Wet Tissue)		
Date of Analysis	Weight of Trout	Caecum	Liver	Whole Fish
<u>Group 1</u>				
June 15	21.1			273
June 29	24.7			438
July 13	27.1			480
July 29	42.4			453
Aug. 13	44.3	2880	6499	399
Aug. 25	55.2	2630	6358	358
Sept. 10	60.6	3260	5397	342
Sept. 22	84.4	4297	3692	338
<u>Group 2</u>				
June 15	22.7			257
June 29	26.7			235
July 13	17.3			288
July 29	29.0			226
Aug. 13	44.2	2867	3641	187
Aug. 25	50.0	1425	3733	165
Sept. 10	67.5	1350	3964	135
Sept. 22	61.2	2241	4216	121

Figure III

Vitamin A Content of Trout on Different Diets



1. Group 1

2. Group 2

The preceding data show that the vitamin A level in the group fed beef heart, even with the added cod-liver oil, is considerably below that of the group fed beef liver. The decline in the level of vitamin A in the latter group may be attributed to rapid growth.

During the period from June 28 to July 13, Group 2 was fed liver in an attempt to reduce the mortality. This explains the rise in vitamin A content during that time. After July 13, liver was discontinued and there was an immediate decrease in the vitamin A content of the whole body.

The small fingerlings were fed on beef liver until August 31, then they were divided into two groups: one continued to receive beef liver; the other, fly larvae. At regular intervals the vitamin A content of the whole body was determined on two fingerlings. The domestic fly larvae were produced as required and as described in the Cortland Hatchery Report (29). These larvae were freed from the culture media by flotation with 10% saline solution, and were stored in a refrigerator. On analysis they were found to contain only traces of carotene and no vitamin A. The vitamin A content of the trout fed fly larvae decreased rapidly. The results of this experiment are summarized in Table XXII on page 78 and Figure IV on page 79. The analyses were carried out by Charles Lusena at Macdonald College.

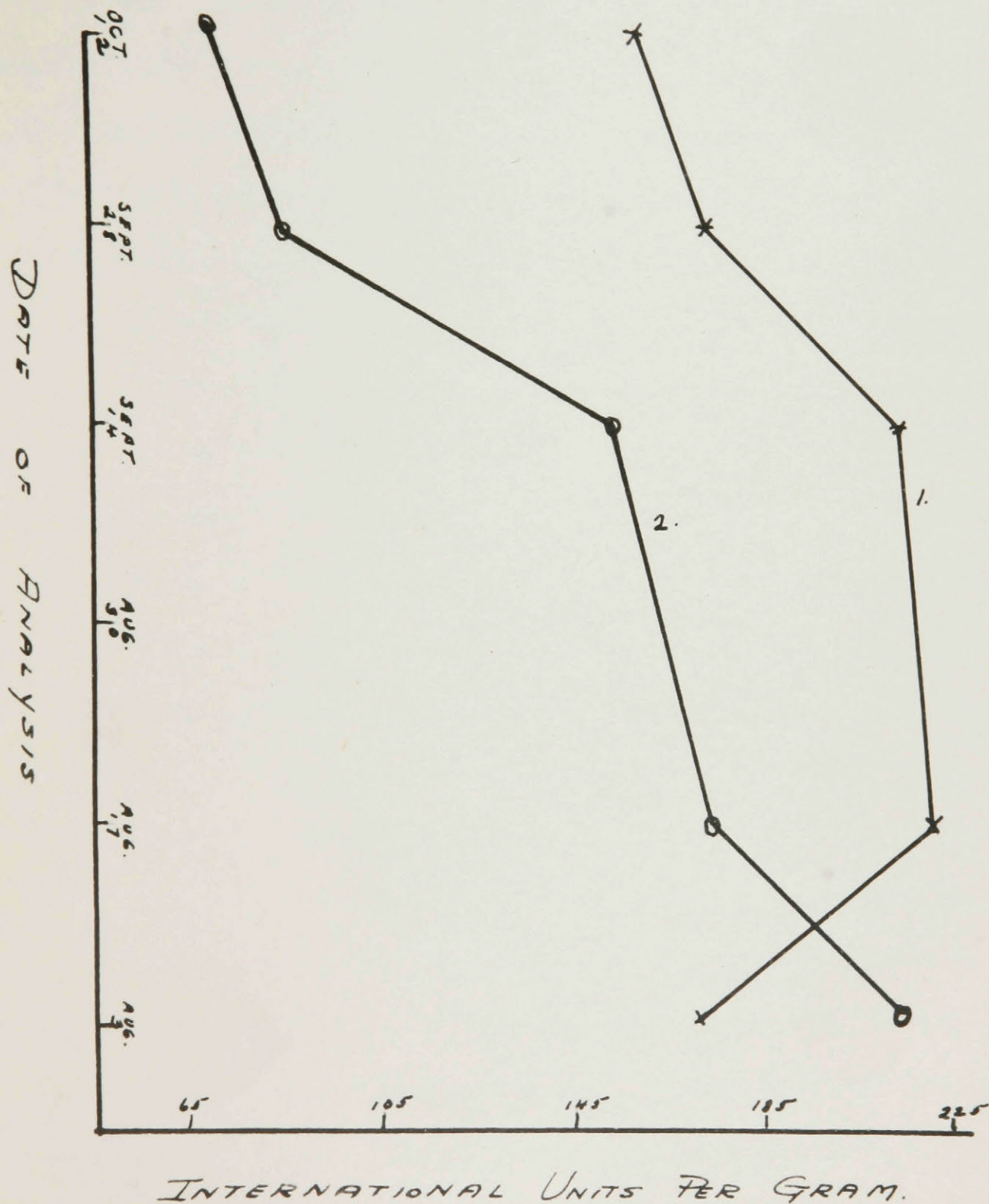
Table XXII

The Vitamin A Content of Trout Fed Beef Liver and Fly Larvae

Date of Analysis	<u>Fish Fed Beef Liver</u>		<u>Fish Fed Fly Larvae from August 31</u>	
	Weight of Trout	Vitamin A (I.U. per g.)	Weight of Trout	Vitamin A (I.U. per g.)
Aug. 3	2.139	172	1.575	226
	2.354	176	1.860	210
Aug. 17	2.263	236	2.067	176
	1.565	209	2.540	171
Sept. 14	8.812	211	2.223	116
	4.231	214	8.843	187
Sept. 28	5.040	145	10.566	84
	5.073	173	9.388	86
Oct. 12	11.633	114	5.355	43
	9.541	224	6.202	98
	7.932	144		

Figure IV

Vitamin A Content of Trout on Different Diets



1. Group 1

2. Group 2

2. Preparation of Vitamin A-Gelatin Complex.

The preliminary work showed that vitamin A deficiency could be produced in trout, and that the vitamin from liver was much better utilized than that from cod-liver oil. Further attempts were made to study its assimilation, particularly the effect of the vitamin in combination with protein. Such a complex may exist in liver, but in order to eliminate other factors which might interfere with the interpretation of the results, the simpler system, vitamin A-gelatin, was chosen. The materials studied were prepared by the following methods.

Four grams of a dogfish-liver oil containing 10,000 I. U. of vitamin A per gram were saponified by refluxing on a hot water bath for thirty minutes with 30 cc. of ethyl alcohol and 12 cc. of 50% potassium hydroxide. The unsaponifiable fraction was extracted with petroleum ether and purified by alternate washings with water and 0.16 N potassium hydroxide until the aqueous phase gave no precipitate upon acidification with 10% hydrochloric acid.

Meanwhile 300 cc. of a 1.5% gelatin solution was prepared, cooled, and transferred to a liter flask. The petroleum ether solution of vitamin A was added to it, and the solvent distilled off in vacuo. The semi-liquid vitamin A preparation was stored in glass-stoppered bottles in the refrigerator. The following complexes were prepared with different antioxidants and the recovery of vitamin A determined in each case by extraction with alcohol and ether and estimation of the Carr-Price blue value.

(1) The vitamin A was stabilized by the addition of 0.1% of citric acid. The acid was dissolved in 20 cc. of acetone and added to the petroleum ether solution of the vitamin before mixing the latter with the gelatin solution. Recovery of vitamin A, 85.7%.

(2) Wheat germ oil (0.5%) was added as an antioxidant. The requisite amount of oil was dissolved in 20 cc. of acetone and added to the petroleum ether solution as in (1). Recovery of vitamin A, 71.3%.

(3) No antioxidant or stabilizer was used. Recovery of vitamin A, 70.0%.

(4) The three preceding preparations were semi-liquid; a solid material more suitable for feeding trials was also prepared. A complex was made as described under (2), and the vitamin A-protein precipitated with ammonium sulfate, dried in a vacuum desiccator, and ground to a fine powder. The recovery of vitamin A was 57.0%, but the preparation deteriorated rapidly in storage. After thirty days in a corked glass tube, kept in the dark, only 22.0% of the vitamin was recovered.

Appropriate tests indicated that the first three preparations retained their vitamin potencies for a period of ten days if the material was stored in a cool place.

3. Feeding Trials with Vitamin A-Protein Complexes.

The fish used in the following experiments were received from St. Faustin in October, 1942. They were

spring fry, extremely variable in size. They were divided into groups of 100 each and fed the following diets.

Group 1. Beef heart.

Group 2. Beef heart and vitamin A-gelatin complex.

Group 3. Beef heart and vitamin A-gelatin complex containing 0.5% wheat germ oil.

The vitamin A-gelatin complexes were thoroughly mixed with beef heart at a level sufficient to give each trout 25 I. U. per day and broadcast on the water with a spoon. No vitamin A or carotene could be detected in the beef heart by chemical analyses. On saponification with alcoholic potassium hydroxide, a deep yellow substance was extracted with petroleum ether. This may have been a sterol, for it gave a strong Liebermann-Burchard test.

The fish were fed twice daily, receiving the vitamin supplements at the morning feeding. It was difficult to control the intake of vitamin A because of the solubility of the complex in water. The fish were starved for thirty-six hours before being taken for analysis. Vitamin A was determined in the entire body as described on page 74. The results are shown in Table XXIII on page 83.

Table XXIII

The Effect of Different Vitamin A Supplements on the Vitamin A Content of the Trout Body

Date of Analysis	Vitamin A (I.U. per g.)		
	Group 1	Group 2	Group 3
Nov. 9	163	217	
Nov. 16	172	268	
Nov. 23	117	157	205
Dec. 1	136	194	191

The results indicate that trout can store vitamin A when it is combined with protein. Therefore, a second experiment was carried out. Five different diets were used and 100 trout were included in each group. The composition of the diets was:

Group 1. Beef heart.

Group 2. Beef liver.

Group 3. Beef heart and vitamin A-gelatin complex.

Group 4. Beef heart and vitamin A-gelatin complex with 0.5% wheat germ oil.

Group 5. Beef heart and vitamin A-gelatin complex with α -tocopherol acetate and vitamin C.

The vitamin A-gelatin complex in the diet for Group 5 was prepared in the same way as that for Group 4. The analytical results for this experiment are given in Table XXIV on page 84.

Table XXIV

The Effect of Different Vitamin A Supplements on the Vitamin A Content of the Trout Body

Date of Analysis	Vitamin A (I.U. per g.)				
	Group 1	Group 2	Group 3	Group 4	Group 5
Dec. 21	71.4	226.6	113.3	90.3	88.2
Dec. 28	75.6	285.6	116.5	120.8	132.3
Jan. 4	79.8	388.5	71.4	84.1	117.7
Jan. 11	73.5	306.6	71.4	65.0	94.3

The carotene reserves of the trout in the above experiment were also investigated. The procedure followed was identical with that for vitamin A except that the intensity of the yellow color was measured in the chloroform solution of the unsaponifiable extract. The concentration of carotene was determined from a standard reference curve. No attempt was made to fractionate the different carotenoid pigments, as preliminary tests showed that only a very small fraction was xanthophyll. The results, shown in Table XXV on page 85, are calculated as (a) total carotene in micrograms per trout and (b) micrograms carotene per gram of trout.

Table XXV

The Carotene Reserves of Trout Fed Different Supplements

Date of Analysis	Carotene (Micrograms)									
	Group 1		Group 2		Group 3		Group 4		Group 5	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Dec. 21	50.0	0.97	60.0	1.56	49.2	0.93	40.0	0.88	48.0	0.97
Dec. 28	49.5	0.99	45.6	1.56	45.6	1.11	45.6	1.00	55.2	1.20
Jan. 4	50.4	0.93	84.6	1.64	70.0	0.83	70.0	1.00	70.0	1.40

Examination of Table XXV shows that carotene expressed as micrograms per gram of trout (b) is much more constant than total carotene per trout (a). The data in Tables XXIV and XXV indicate that the trout receiving the vitamin A supplement with vitamins C and E have the highest reserves of vitamin A and carotene.

One serious difficulty in this work is the large sampling error. Two fish taken from a group of 100 is not representative, but if the number of fish taken for analysis is increased to 5, the difficulty in the saponification of the fish bodies is greatly increased, and the volume of the saponified extract becomes too large to be handled easily. McCay (29) has shown that 90% of the vitamin A in the fish is present in the pyloric caeca, liver, and intestine. Therefore, by taking only the intestinal tract of the fish for analysis, more accurate results should be obtained.

To test this theory the trout were killed by severing the spinal cord, the body cavity opened, and the intestinal

tract entirely removed, weighed, and saponified as previously described. The results are shown in Table XXVI.

Table XXVI

The Vitamin A and Carotene Reserves in the Intestinal Tract of Speckled Trout

Date of Analysis	Group 1 (a) (b)		Group 2 (a) (b)		Group 3 (a) (b)		Group 4 (a) (b)		Group 5 (a) (b)	
Jan. 14	647	0.84	2562	1.25	350	0.53	372	0.65	777	0.92
Jan. 21	392	2.00	2163	1.37	463	1.10	430	0.90	758	0.90
Jan. 28	420	0.89	1995	0.80	420	0.73	441	0.82	557	0.99

(a) Vitamin A in I. U. per gram

(b) Carotene in micrograms per gram

The results again indicate that vitamin A is better utilized by trout in the presence of vitamins C and E, but the differences are small. Possibly a large part of the vitamin A-gelatin complex dissolved in the water.

Preliminary tests showed that only a fraction of the total vitamin A present in the trout body could be extracted with ethyl ether. This suggested that the vitamin might be present in some form of combination with other material. To study the distribution of the two forms of vitamin A in the trout body, 2 fish from each of the five groups receiving different supplements were analyzed in the following manner. The bodies were weighed, cut into small pieces, placed in a Soxhlet cylinder, and extracted with

ethyl ether for twenty-one hours. The residue was removed from the cylinder and saponified with alcoholic potassium hydroxide in the usual manner. The hydrolysate was extracted with petroleum ether and the extract analyzed for vitamin A and carotene. The ethyl ether extract was likewise analyzed for the same constituents. The results are shown in Table XXVII.

Table XXVII

The Relationship between Free and Combined Vitamin A and Carotene

Group	Vitamin A (I. U.)		Carotene (Micrograms)	
	<u>Total</u>	<u>% Extracted</u>	<u>Total</u>	<u>% Extracted</u>
1	24.6	63.7	15.2	54.3
2	176.4	79.5	16.5	64.2
3	56.7	75.0	13.6	67.0
4	21.0	54.4	10.3	49.0
5	52.9	73.6	26.0	63.4

In Groups 2, 3, and 5 most of the vitamin A and carotene is in the free form. In Groups 1 and 4 more than 50% of the carotene and 35% of the vitamin A is in the combined form. It is impossible to give any explanation for the variation from the normal values in Group 4, since the vitamin A supplement which these trout received should be as good as or better than that of Group 3.

Since it was difficult to prevent the solution of

the vitamin A-gelatin complex previously used, it was decided to feed the vitamin supplement in the form of a dry powder. The preparation of this material is described on page 81. Four groups of 50 trout each were used; the following diets were fed.

Group 1. Beef heart and 3% of cod-liver oil containing 3600 I. U. per gram. This supplied 25 I. U. per trout.

Group 2. Beef heart and vitamin A-gelatin complex together with α -tocopherol acetate and vitamin C. The dry vitamin A complex was mixed with the meat in an amount sufficient to supply 25 I. U. per trout.

Group 3. Beef heart, skim-milk powder, and vitamin A. The skim-milk powder-vitamin A preparation was obtained from R. A. Chapman, Macdonald College, and contained 450 I. U. of vitamin A per gram. It was prepared by adding cod-liver oil to skim-milk and drying by the roller process. It was mixed with the meat in an amount sufficient to supply 25 I. U. of vitamin A per trout.

Group 4. Beef heart and carotene-gelatin complex. This material was prepared in the same manner as the vitamin A complexes. The preparation was mixed with the meat in an amount sufficient to supply 25 I. U. per trout on the assumption that 0.6 micrograms of carotene is equivalent to 1 I. U. of vitamin A.

The results of this experiment are presented in Table XXVIII on page 89 and in Figure V on page 90.

Table XXVIII

The Vitamin A and Carotene Contents of Speckled Trout Fed
Different Supplements

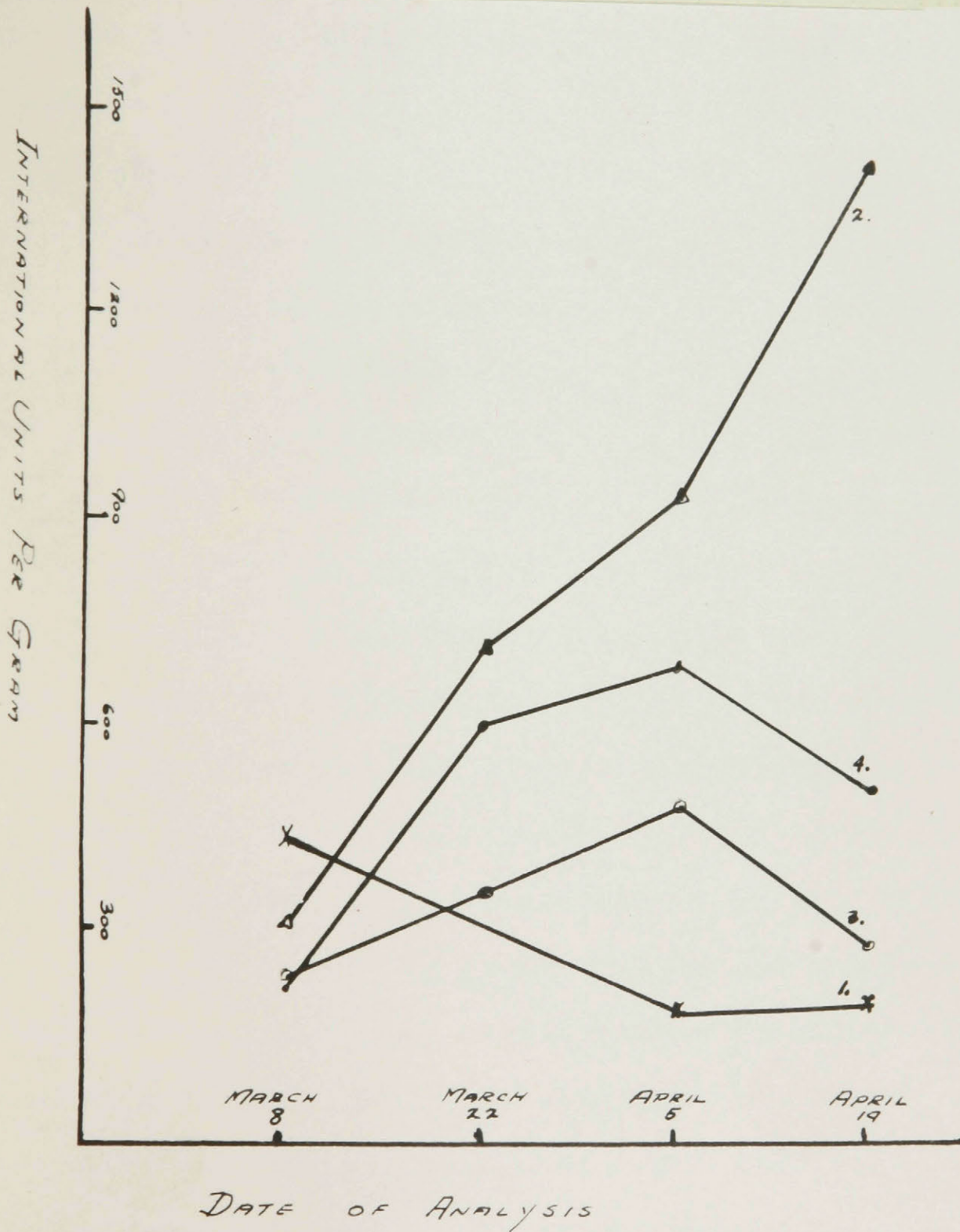
Date of Analysis	Group 1		Group 2		Group 3		Group 4	
	(<u>a</u>)	(<u>b</u>)	(<u>a</u>)	(<u>b</u>)	(<u>a</u>)	(<u>b</u>)	(<u>a</u>)	(<u>b</u>)
March 8	443	0.58	317	0.50	237	0.37	212	0.36
March 22			735	1.90	358	1.00	609	1.05
April 5	184	0.45	956	0.80	483	0.85	714	0.91
April 19	200	0.80	1449	1.00	273	0.50	514	0.70

- (a) Vitamin A in I. U. per gram
(b) Carotene in micrograms per gram

The vitamin A reserves in Groups 2, 3, and 4 are above that of Group 1. Of the three supplements tested, the second gave the best results. The high storage of vitamin A in Group 2 is also interesting in view of the fact that vitamin E has been reported to facilitate the absorption of vitamin A in other species. Although the trout in Group 4 were fed a carotene-gelatin complex as the source of vitamin A, their carotene reserves were lower than those in Group 2. This indicates that trout must have the same power as mammals to convert carotene to vitamin A.

Figure V

Vitamin A Content of Trout on Different Diets



1. Group 1

3. Group 3

2. Group 2

4. Group 4

D. DISCUSSION.

The experimental feeding trials carried out at Antigonish, Nova Scotia, indicate that dry feed mixtures can be fed to fingerling trout before they are one and one-half inches long (Table III). Of the various diets tried #353 gave the best results, as none of the modifications used improved upon this mixture (Table VII).

Fresh cod liver can be used with these dry feed mixtures to give excellent growth in short term experiments (Table V), but in combination with spleen and dry skim-milk it is worthless (Table I). One great drawback in feeding cod liver lies in the fouling of the tanks from its residue.

Feeding by broadcasting the food with a spoon is best. The ricer releases the food in a very satisfactory form, but its disadvantage lies in the difficulty experienced in cleaning it.

Weight and length measurements on the trout indicate clearly the need of taking large numbers for analyses in order to overcome the individual variations in the fish (Tables IV, IX). This fact is even more apparent when analyses for various constituents of trout bodies are required. For example, hemoglobin levels in trout on the same diet have been shown to vary as much as 100%. Consequently, to obtain results to which statistical methods could be applied would require more fish for each analysis than a few rearing troughs could supply. Since trout can survive for long periods

without food and thus lose their reserves of essential constituents very slowly all experiments with trout should be of several months' duration. The above facts apply not only to the practical feeding trials but also to the hemoglobin and vitamin A experiments which were carried out at Macdonald College.

Wu's (91) method for determining hemoglobin has been adapted for use in the photoelectric colorimeter. Following Lieboff's (94) suggestion, benzidine dihydrochloride was used and found to be very satisfactory. The method of carrying out the test was essentially as outlined by McFarlane and Hamilton (93), except that the tubes were stored in the cold during color development. A reference curve (Figure II) was prepared using a sample of oxalated horse blood as the standard.

In attempting to determine a lowered hemoglobin value in trout fed on an anemia-producing diet by a determination of either the total or thermostable peroxidases, it was found that individual differences in the fish were far greater than the accuracy of the method (Table XV). However, when red blood cell counts and direct hemoglobin determinations were done on the test fish, no evidence of anemia was found. No valid explanation can be advanced for the failure of the fish to develop anemia. Lard was incorporated in the McCay anemia-producing diet, but no evidence has ever been obtained that trout anemia is tied up with a lack of unsaturated fatty acids. Xanthopterin has been found to be the curative factor,

and the efficiency of fresh meats such as liver, fly maggots, and liver extracts is likely due to the xanthopterin content of these substances.

Since an experimental trout anemia was not produced, the effectiveness of xanthopterin in preventing the development of anemia in weanling rats on a cows' milk diet was tested. Contrary to the results of Tschesche and Wolf (77) who used goats' milk as the anemia-producing diet, it was found that xanthopterin alone was ineffective, but xanthopterin plus iron fed to rats on a cows' milk diet provided suitable conditions for normal growth and hemoglobin development. However, food tables show that goats' milk has about four times the amount of iron that cows' milk contains.

The hypotheses have been advanced that xanthopterin may take the place of copper in some enzyme systems essential for the production of hemoglobin; that xanthopterin may increase the efficiency of the utilization of the small amounts of copper necessary for this production, or that it may have a stimulating action upon some other, as yet, unknown systems. The growth and hemoglobin levels of rats receiving xanthopterin plus iron were only slightly below those of the positive control and far above those of the negative control group (Tables XIX, XX).

The peculiarities exhibited by trout in the assimilation of vitamin A raises some interesting questions. It has been shown that vitamin A from liver is well-utilized, but that from cod-liver oil is poorly assimilated (29). Palmer (69)

has shown that the carotenoids in blood serum are combined with protein since they cannot be extracted with the usual solvents. Ethyl alcohol must be used to break up the complexes after which the carotenoids can be extracted in the usual way.

On the assumption that the above form of vitamin A was better suited to the needs of trout, vitamin A and carotene were combined with protein and fed as supplements with beef heart. When the complexes were fed in the gel form, their ready solubility in water permitted much loss of vitamin content. If the supplements, as dry preparations, were incorporated with beef heart, the loss due to solubility in water was minimized and the trout were able to build up reserves of vitamin A to a level equal to the ones fed liver.

The trout receiving the supplements containing vitamin E in addition to vitamin A seemed to be able to effect better storage of vitamin A (Tables XXIV-XXVIII). This result is in line with the experiments of Moore (68), who found that vitamin A was better utilized by rats in the presence of vitamin E.

The trout receiving the carotene-protein supplement converted the carotene to vitamin A since the carotene reserves were no higher than those of the other groups (Table XVIII). Gillam (56) stated that in marine fish carotene was ingested from phytoplankton and broken down into vitamin A. Morton and Creed (64) have made similar observations on fresh-water fish.

The vitamin A and carotene present in the trout body could not be completely extracted with ethyl ether (Table XXVII). This indicates that some complex form of vitamin A or carotene must be present in the trout. The trout receiving vitamin A in the form of a complex with gelatin had a larger percentage of the total body vitamin A and carotene in the free form than those receiving a supplement of cod-liver oil containing an equivalent amount of the vitamin.

E. SUMMARY.

(1) Large numbers of trout must be taken for analyses if statistically significant results are to be obtained.

(2) Dry feed mixtures can be introduced into trout diets at an earlier time than the one and one-half inch fingerling stage.

(3) Cod liver lacks some essential food constituent contained in beef liver.

(4) Xanthopterin and iron are effective in preventing the development of anemia in weanling rats on a cows' milk diet.

(5) Trout can utilize and store vitamin A from vitamin A- or carotene-protein complexes.

(6) Carotene is largely converted to vitamin A and stored as such by trout.

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