

THE BIOLOGICAL ASSAY OF FISH OILS
FOR VITAMIN A

by

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THE BIOLOGICAL ASSAY OF FISH OILS FOR VITAMIN A.Introduction.

Domestic animals must be furnished with a ration that is complete in carbohydrates, fats, proteins, vitamins, and minerals if normal health, reproduction, and growth are to be maintained. A deficiency in any nutrient or vitamin may cause retardation of growth in young animals, or decreased output in mature animals of such products as milk or eggs. Furthermore deficiencies in diet may result in diminished health or vigor as evidenced by such diseases as xerophthalmia, nutritional roup, rickets, pellagra, anaimia, or goiter.

The causes of the most common of these deficiency diseases have been traced, in recent years, to a lack of some specific vitamin. Of the many different vitamins known to exist today, Vitamin A has received considerable attention. Many investigators have shown that this vitamin is indispensable for the normal growth of animals although the actual amount needed is very small. Of this small total relatively moderate quantities are required for maintenance, somewhat larger quantities for growth, and relatively large quantities for the production of eggs or milk.

Since Beach (12) in 1923 showed that Vitamin A was essential for normal poultry nutrition, a great deal of work has been done by other investigators to determine the

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quantitative requirements of chicks for this vitamin.

Ordinarily, Vitamin A is supplied through the feeding of yellow corn, dehydrated alfalfa, or green feed. These feeds, as a rule, must be supplied in comparatively large amounts to meet the Vitamin A requirements of growing chicks and particularly of laying birds. In recent years fish oils have become widely utilized as sources of Vitamin A for poultry because they are very potent sources of this vitamin. According to Fraps and Treichler (83) a good grade of Cod Liver Oil contains 600-1000 units of Vitamin A per gram, while a good grade of yellow corn contains only 5 units of Vitamin A per gram and dry alfalfa 12 units per gram. It will be readily seen, therefore, that the Vitamin A content of a ration can be readily fortified and controlled by the mere addition of a small quantity of a Vitamin A potent fish oil.

Amongst the fish oils, Cod Liver Oil has hitherto occupied a pre-éminent position as a source of Vitamin A in both human and animal nutrition. In recent years it has been shown, however, that many other fish oils are just as rich sources of Vitamin A.

Amongst the various fish oils, BRITISH COLUMBIA PILCHARD OIL is already extensively used as a source of Vitamin D. On account of this, it was deemed advisable to carry out at The University of British Columbia investigations regarding the Vitamin A potency of this oil.

The purpose of these investigations were not only to throw light on the Vitamin A content of Pilchard Oil, but to

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determine the Vitamin A requirements of growing chicks as well. At the same time, it appeared that a satisfactory method should be developed, if possible, for the biological and chemical assay of Vitamin A in fish oils.

The present investigation consists of three distinct experiments. (a) Estimating the Vitamin A content of British Columbia Pilchard Oil in terms of International Units. (b) Developing a suitable basal ration free from Vitamin A for the biological assay of Vitamin A in fish oils, using the chick as the test animal. (c₁) Comparing the biological and chemical methods of assaying British Columbia Pilchard Oil. (c₂) Determining the Vitamin A content of Pilchard Oils produced at different periods of the fishing season.

No reference is made in this investigation to the storage of Vitamin A in the livers of chicks fed varying amounts of Vitamin A. Work on this phase of the problem is in progress.

DISCOVERY OF VITAMIN A.

The specific growth-promoting property which is now associated with the occurrence of Vitamin A was first observed by McCollum and Davis (142) in 1913, who found that an ether extract of butter or egg yolk in a synthetic ration had a stimulating action on growth which was not possessed by other fats such as lard or olive oil.

These workers pointed out that certain mixtures of fats of animal origin, as butter fat, egg fat, and the fats extracted from the internal organs, eg. the kidney, liver, etc., contain something which is absolutely indispensable for either maintenance or growth, and that this substance is not found in vegetable oils or fats and in but very small and inadequate amounts in the bodyfats of animals.

They showed that when the diet is inadequate in its content of this substance which they designated as fat-soluble A, the animals become emaciated and suffer from edema of the eyes. Blindness results if the animals are permitted to go without this dietary essential or with an inadequate supply for a sufficient time.

Very shortly thereafter Osborne and Mendel (171) published experiments in which it was pointed out that their milk rations had special dietary properties not found in their milk-free rations and that this characteristic seemed to be true of rations carrying an equivalent amount of butter as well. Later

(172) they obtained uniform success by substituting cod liver oil for a portion of the lard in their standard diets.

Stephenson (238) in 1920 found that a crude alcohol-light-petroleum extract of dried carrot when added to a fat not containing vitamin confers upon it the growth promoting property and the power of protecting the animal from or curing it of keratomalacia. His work showed that the substance or substances in the extract, responsible for these properties was not carotene. Other experiments that he conducted demonstrated that the colouring matter of butter fat may be completely removed or destroyed by filtration through charcoal without in the least affecting the vitamin content of the butter.

Zilva (267) in 1919 found that the fat soluble A factor in butter became inactivated when the butter was exposed to ultra violet light for eight hours.

Drummond and Coward (60) in 1920 decided that no hard and fast line could be drawn between the animal and the vegetable oils and fats when their value as a source of Vitamin A was being considered. These investigators also found that the animal fats, taken as a class, possessed a growth-promoting power superior to that of the vegetable oils. This superior growth promoting power of the animal oil or fat appeared to be influenced considerably by the diet of the animal.

In 1919, Haring, Beach and Jaffa (26) reported a study of several outbreaks of a disease occurring in flocks of pullets in California, which, so far as could be determined by a search

of the literature, had not been previously described. The symptoms resembled those of "roup" more than of any other disease of fowls known at that time, but differed enough so that a differential diagnosis was readily made. Later on Beach (12) definitely showed that the disease with which he was dealing in poultry was due to a lack of Vitamin A.

4. VITAMIN A IN THE PLANT KINGDOM

Vitamin A Content of Plants.

Steenbock, Kent and Gross (226) in 1918, discovered that barley contained an abundance of the water soluble vitamin but was deficient in the fat soluble vitamin.

Osborne and Mendel (173) in 1919, were the first investigators to note that green vegetables supplied an important addition to the diet of man because the staples such as cereals, meats, potatoes, fats and sugar furnished too small an amount of the vitamin to meet fully the requirements of an adequate dietary.

As early as 1919 Steenbock and Gross (228) demonstrated the high fat-soluble vitamin content of carrots and yellow sweet potatoes as compared with other roots. A year later, Steenbock and Boutwell (229) showed that yellow corn contained enough of the fat-soluble vitamin to allow growth at the normal rate to take place in the rat. White corn on the other hand did not contain any demonstrated amount of the fat-soluble vitamin.

In 1920 Steenbock and Gross (230) found that 5 % of clover or alfalfa as the sole source of fat-soluble vitamin in a ration, when other dietary requirements were met, allowed normal growth and the rearing of some young.

Steenbock, Sell and Boutwell (234) worked with peas to find their fat-soluble vitamin content in relation to their pigmentation. The results of this investigation demonstrated

that in ripe peas, those of a green colour, also carrying considerable yellow pigment, were far richer in their fat-soluble vitamin content than yellow peas which contained much less yellow pigment.

About the same time as these investigations were going on Coward and Drummond (35) were investigating nuts as a source of Vitamin A. They found that the various nuts contained a large percentage of fat but possessed little or no vitamin A. Their results further substantiate the theory that Vitamin A is formed in the green part of the living plant and is not stored to any appreciable extent as such in the seed and other resting tissues.

Willimont (259) showed that 5 c.c. of navel orange juice contained sufficient Vitamin A for growth and well-being in the rat.

The Vitamin A content of barley was intensively studied by Hughes (124). He noted that when growth was used as a measure of the Vitamin A content of barley yellow corn, white corn and barley, the indications were that the Vitamin A content of barley lay somewhere between that of yellow corn and white corn. When growth and vaginal smears were used as a measure of the Vitamin A content of barley, it was shown that the quantity in barley was low, in fact, much lower than that of yellow corn but higher than that of white corn. The experiment definitely showed that barley, as the only source of Vitamin A in the diet did not produce normal growth in rats. In conclusion he stated that barley contained less than one sixth as much Vitamin A as

did yellow corn.

Extensive tests of the Vitamin A potency of alfalfa were carried out by Miller and Bearse (155a.). They found that a sample of commercial dehydrated alfalfa contained twice as much Vitamin A as a sample of commercial sun-cured alfalfa. After a year's storage the potency of the two samples was still the same. They also found that the dehydrated alfalfas they used were thirty times more potent than samples of yellow corn.

Hauge (106) found that the Vitamin A activity of freshly cut young alfalfa was preserved when the enzymes were destroyed immediately before drying. A direct correlation was found between the effect of temperature on the Vitamin A and on the enzyme activity. The influence of the sun's rays on the destruction of the Vitamin A was thought to be due to the production of temperature which accelerated enzyme activity.

Guilbert (98) observed no loss on drying but considerable loss on autoclaving and during sun-drying, most of which was due to the photochemical action of the sun, though in slow drying, enzyme and bacterial action also played a part. Temperature was the most important factor influencing loss in storage; below 5⁰ c. no loss was detected in 6 months but the rate of loss increased very rapidly with rise of temperature.

Recent work on alfalfa hay by Vail, Tobisha and Douglass (252) showed how Vitamin A may be lost or conserved according to methods of handling.

1. Loss or inactivation of Vitamin A in alfalfa hay

results from:

- (a) Usual practice of curing and stocking.
- (b) Exposure to ultra-violet light in presence of moisture.
- (c) Storage of alfalfa meal in cloth sacks.

2. Conservation of Vitamin A in alfalfa hay results from:

- (a) Curing indoors.
- (b) Curing by rapid artificial drying.
- (c) Curing by crushing and rapid drying.
- (d) Storage in the bale.
- (e) Storage of alfalfa meal in paper sacks.

The Vitamin A in alfalfa hay tended to become more inactive the longer it was stored.

Woods et. al. (266 a) found that third cutting alfalfa hay, cock cured and in fresh condition, contained 308 ± 13 Sherman units of Vitamin A activity per gram (leaves 483 ± 34 , stems 121 ± 7 units). A sample of the same hay ground and stored in diffused light for 4 months, contained only 233 ± 20 units. Hay cured in the swathe for 3 days, then cock cured and sweated in the stack contained 116 ± 9 units per gram compared with 144 ± 10 units for similar hay which had been exposed in the swathe only one day before cooking. Sweating in the stack caused a significant reduction in the Vitamin A activity from 233 ± 20 Sherman rat units for cock cured, $2\frac{1}{2}$ months old unstacked hay to 144 ± 10 units for similar hay sweated in the stack 49 days.

Fraps, Treichler and Kemmerer (84 a) showed that alfalfa products containing from 7.3 to 63.5 micrograms of carotene had a Vitamin A potency of from 13 to 77 Sherman-Munsell units (15.6 to 92.4 international units) per gram, with an average value of 1.4 units (1.6 international units) per microgram of carotene. They also observed that one microgram of carotene in the international standard had a value of 1.4 Sherman-Munsell units, and 1 microgram of purified carotene had the same value.

Synthesis of Vitamin A in Plants.

Wilson (260) in 1922 found that either etiolated or green wheat sprouts furnished an adequate amount of Vitamin A when the dried sprouts made up 5 % of the diet of white rats. He drew the conclusion that Vitamin A is produced in the growing plant with or without any accompanying photosynthesis.

Coward (37) in 1923 pointed out that light is necessary for the formation of Vitamin A in plant tissues. This process, however, can be carried out in the absence of carbon dioxide and of oxygen in the surrounding atmosphere. The production is also independent of the ultra-violet rays of the spectrum (or such of those as are held back by 2 plates of window glass) and can be carried on under the influence of electric light in the absence of sunlight. On the other hand the presence of chloroform in the atmosphere prevents the formation of the Vitamin.

In 1925 Coward (39) further showed that Vitamin A is not used in any process carried on by living plant tissue in the dark. The Vitamin A appears to increase when a leaf loses its green color and becomes yellow; but is completely destroyed when the leaf dries up and dies.

The same investigator (40) later presented data to show that the light from a quartz mercury vapor lamp was effective in accelerating the formation of Vitamin A in living plant tissues. These short ultra-violet rays from such a lamp, used in conjunction with the visible rays, did not have any influence on the ultimate amounts of Vitamin A contained

in the tissues. The investigation also showed that the amount of Vitamin A in etiolated shoots is an inverse function of the temperature at which they have been grown, this having influenced markedly the rate of growth.

Moore (159) corroborated the work of both (260) and (40). He further showed that rats fed for some months on etiolated wheat shoots appeared lean and rough-coated when compared with similar rats receiving a liberal diet.

The year after the above evidence had been published Moore (160) came out with the opposite idea. He fed etiolated wheat shoots to rats under conditions involving the minimum of red light illumination consistent with the feeding and handling of the animals. They were effective as a source of Vitamin A, thus supporting his conclusion that light is not essential during any stage of the formation of the Vitamin from seed.

Dye, Medlock and Crist (72) observed that leaf lettuce exceeded head lettuce in the promotion of growth in rats that had ceased to gain on a diet deficient in Vitamin A. The outside green leaves of head lettuce were far superior to the inside yellow leaves in furnishing the Vitamin.

Heller and St. Julian (113) found that Vitamin A was formed and stored chiefly in that portion of the plant exposed to light and that it did not migrate to other portions of the plant.

Luce and MacLean (141) stated that Vitamin A is synthesized by the yeast cell and the synthesis may be brought

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about in the absence of sunlight.

According to Goode (91) the Vitamin A content of white corn sprouts was increased by irradiation and the Vitamin was transferred in part from the sprouts to the grain during the irradiation of the growing process.

Plant Carotinoids And Vitamin A.

Palmer and Kennedy (174) in 1921 showed that carotinoids and fat-soluble Vitamin are not even quantitatively associated in the plant tissues in which both are presumably synthesized.

Two years later Coward (38) stated that the chief point in the formation of the Vitamin which was apparent in her experiments was that some lipochrome (generally carotene) was always associated with the Vitamin in plant tissues; and that where carotene was found, particularly carotene exposed to sunlight, there the Vitamin may be expected to be present also.

Smith and Morgan (223) later found that chlorophyll was not a necessary intermediary in situ for the formation of carotene, lycopene, or any other possible precursors of Vitamin A. Fruits which develop carotene and Vitamin A activity under normal light conditions do so also under glass and in the dark although usually in slightly smaller amounts. They also noted that a constant relation exists between the level of daily intake of carotene by Vitamin A free rats and their growth per unit of total carotene ingested. This relation illustrates the "Law of Diminishing Returns."

Norris (169) discovered that the exposure of etiolated plants to artificial light resulted in the formation of chlorophyll and xanthophyll in constant proportions. Carotene decreased at first, but after 4--5 hours increased more rapidly than the chlorophyll and xanthophyll. With increasing oxygen

in the atmosphere carotene developed still more rapidly, but formation of chlorophyll and xanthophyll reached a maximum with 20% oxygen. The proportion of all pigments increased with rising carbon dioxide in the atmosphere up to 3-5%, but subsequently declined.

Indications were obtained by Macwalter and Drummond (149) that the lipochrome content of young fish may be increased when they are fed on a diet containing a typical green alga.

Examination of the lipochromes of *Fucus Vesiculosus* by Heilbron and Phipers (111) showed that whereas the dead material contained beta carotene and zeaxanthine, the living plant contained beta carotene and fucoxanthine. No trace of xanthophylls usually associated with the higher plants could be found and zeaxanthine was probably a post mortem product of fucoxanthine.

Heilbron, Parry and Phipers (112) found that in the unsaponifiable matter from extracts of *Cladophora, sauteri*, *Nitella opaca*, *Oedogonium* and *Rhodomenia palmata*, various sterols could be identified and also the lipochrome pigments, lutein, zeaxanthine and beta carotene. Alfa carotene was observed only in *Oedogonium*.

Extractability of Carotinoids from Plants.

In 1920 Steenbock and Boutwell (232) found that when carrots were saturated with lard or corn oil and then extracted with ether, none or little of the fat-soluble vitamin was removed. The lard preparation gave no evidence of containing the vitamin, but the corn oil preparation contained it in small but persistent amounts. They also noted that ether had little solvent properties for the fat-soluble vitamin as found in carrots; chloroform and carbon disulphide removed some of it; while alcohol and benzene removed considerable amounts of it. While the vitamin is not extracted from corn by ether, alcohol removed it quantitatively and with little if any, destruction. They give their procedure for fractionating an extract from alfalfa.

These same two investigators (229) later demonstrated that the fat-soluble vitamin as found in the plant kingdom in a grain, in leaf and stem tissue, in fleshy roots and in a cucurbitous vegetable was comparatively stable at a high temperature.

VITAMIN A IN THE ANIMAL KINGDOM.Synthesis of Vitamin A in Fish.

In 1922 Jameson, Drummond and Coward (125) demonstrated that pure cultures of a common marine diatom grown in Miguels' solution or sterilised sea water synthesised large amounts of Vitamin A. These investigators draw a parallel between the dependence of land animals on fresh green leaves and that of marine animals on the synthetic activity of the marine flora for their supplies of Vitamin A.

Working with brown trout, Coward and Drummond (36) found that the ova of these trout normally contain relatively large amounts of Vitamin A. If in the post-larval period or even before the yolk sac is completely absorbed, the fish are given food rich in Vitamin A their growth and development are satisfactory and they appear able to store that factor in their tissues.

Drummond and Zilva (63) concluded from their work that the ultimate origin of the Vitamin A found in the oils derived from fish, and particularly fish liver oils, to be chiefly the unicellular marine plants. Except very occasionally these organisms are not consumed, directly by the fish.

These investigators also noted that the extraordinary rise in the number of marine plants which begins as soon as the intensity and duration of sunlight increase, early in the year, is followed by a rapid rise in organisms largely copepods and larval decopods and molluscs, whose growth and development are

dependent on their food supply which consists of minute plants. These minute animals, which form a large proportion of plankton contain relatively large quantities of Vitamin A presumably derived from the deatoms on which they have thriven.

The plankton forms the staple food of innumerable species of marine animals from small fish to some whales. This no doubt accounts for the presence of Vitamin A in the tissues or fat depots of these animals.

Two years later Zilva, Drummond and Graham (268) demonstrated that the sexual condition and age of the cod did not influence the Vitamin A potency of the liver oil. Bailey (6) later corroborated this work while working with Ling Cod of the Pacific Coast.

Finn (82) in 1931 published data to show that pilchard oil contained a substance which promoted the growth of rats which had failed to grow on a Vitamin A deficient diet. Biely and Chalmers (16) working with chicks further demonstrated the Vitamin A potency of pilchard oil.

Truesdail and Boynton (246) undertook a study of the Vitamin A content of body oils of 5 species of Pacific Coast Salmon. They found all 5 were decidedly inferior to the sample of high-grade medicinal cod liver oil with which they were compared. The Vitamin A potency was found to be proportional to the intensity of the natural yellow colour of the oil.

While working with halibut liver oil, Lavern, Edisbury and Morton (139) obtained very interesting results. No parall-

ism could be traced between the Vitamin A and Vitamin D potencies. In certain species, the Vitamin A content of the liver oil was found to increase with the age and or size of the fish, the total Vitamin A reserve increasing more rapidly than the oil potency. They believe that halibut liver oil is by far the richest known natural source of Vitamin A available in quantity. It has been found, however, that the oil varies in potency over a wider range than any other source. No correlation has emerged between the immediate diet of the halibut and the oil potency. Their work also showed that halibut liver oils exhibit well-marked seasonal fluctuations in Vitamin A concentration which cannot be attributed to changes in the oil content of the liver occasioned by spawning. The best oils from the standpoint of Vitamin A content are obtained from large halibut caught in northern waters in the late spring or early summer, and in the autumn. Very rich oils at other times of the year are exceptional.

Working along similar lines Lavern and Sharp (140) found that the diet of the halibut was of a general nature, with no outstanding rich source of Vitamin A to account for the high potency of halibut liver oil. Taking the glycogen content of the liver as a criterion of intensity of feeding, they found that no correlation could be established between intensity of feeding and the Vitamin A potency of the oil. They obtained further evidence that in general the livers of older fish afforded a more potent oil than those of younger fish.

Weekly records were taken of the fat, Vitamin A and Vitamin D contents of the livers of halibuts (caught near Seattle) by Bills, Imboden and Wallenmeyer (18). The oil content increased slowly from January (12%) to June, there was then a sudden rise to a maximum value (25%) in August, followed by a slow decline during the rest of the year. The Vitamin A content (January 240,000, August 35,000 international units per g.) and Vitamin D content (January 1,400, August 900 international units per g.) moved inversely with the fat content although in the case of Vitamin A the fluctuation was wider than in the case of Vitamin D.

Bills et. al. (19) conducted a toxonomic study of the distribution of the Vitamins A and D in many species of fish. They found that for a given species, no relationship could be established between the Vitamin A and D potencies of the liver or body oils. In general, but with many exceptions, liver oils rich in the other, and the potency tended to vary inversely with the oil content of the liver.

The Vitamin A Content of Animal Products.

In 1917 McCollum, Simmonds and Steenbock (143) stated that fat free milk, when included in a diet consisting otherwise of purified food substances, promoted growth and prevented decline of animals in a manner which indicated it still contained the fat-soluble essential in appreciable amounts. This led them to suspect that the factor was appreciably soluble in water. They demonstrated that when butter fat was melted and thoroughly agitated with twenty successive portions of water it no longer contained the fat-soluble factor.

Continuing along this line of thought, Steenbock, Boutwell and Kent (227) found that while the vitamin was removed from the washed butter fat the washings did not contain it. They were convinced that somewhere in the course of the manipulations to which the butter fat had been subjected, factors had been introduced which were responsible for the vitamin destruction. They then realized that heat alone in the absence of water or in the absence of conditions designed to bring about intimate contact with air was responsible for the vitamin destruction observed in the early experiment.

Hopkins (122) found that the fat-soluble A substance in butter, which displaying marked resistance to heat alone at temperatures up to 120^o was readily destroyed by simultaneous aeration of the fat, presumably because it is a substance prone to oxidation by atmospheric nitrogen.

Working along the same lines as Hopkins (122), were

Drummond and Coward (61). They noted that the destruction of the Vitamin present in butter fat occurred on heating in the presence of air. They concluded that the loss was due to changes of an oxidative nature. They also noted that the destruction took place rapidly at high temperatures but provided the exposure to air is extensive, considerable loss of nutritive value takes place at temperatures as low as 37°.

Steenbock, Sell and Buell (233) found that butter fat showed a seasonal variation in the fat-soluble vitamin content when obtained from stall fed cows during the winter and pastured in the summer. They note that the fat-soluble vitamin content of butter fat does not run closely parallel to the yellow pigment; yet in general, due to determination by their content in the feed, butter highly pigmented are rich in the vitamin; butter low in pigment should be looked upon with suspicion. In beef fats the relations are somewhat similar; those most pigmented are also generally richest in their fat-soluble vitamin content. These investigators demonstrated that the fat-soluble vitamin withstood severe methods of saponification. This indicated that it was not a fat and probably not an ester.

The inactivating action of some fats on Vitamin A in other fats was studied by Fridericia (85) in 1925. He reported that rats did not grow on an apparently adequate diet when the butter fat yielding the Vitamin A of the diet was mixed (after melting) with a brand of hydrogenated whale oil. This hydrogenated whale oil, had no toxic action on the growth of

rats but had an inactivating action on the Vitamin A of butter fat when the two fats were mixed after melting at a low temperature. Two hydrogenated vegetable oils (hydrogenerated coconut oil, and hydrogenated hemp-seed oil) and a non hydrogenated vegetable oil (cocoanut oil) showed neither any toxic effect on the growth of rats nor any inactivating action on the Vitamin A of butter fat, this action accordingly not being regularly connected with the process of hydrogenating. Untreated abdominal pig fat did not inactivate the Vitamin A of butter fat. After being heated in thin layers to 102 to 105 °C. for 24 hours on exposure to air, the same pig fat acquired an inactivating action on the Vitamin A of butter fat when the two fats were mixed after melting. This inactivating action is supposed to depend on the generation of peroxides in the aerated heated fat, the Vitamin A of butter fat being destroyed by these peroxides through oxidation.

Sjorslen (219) showed that butter fat gave a reaction with sulphuric acid but not in such low concentrations as cod liver oil and colour indices do not attain so high a value. Lard did not give a reaction with sulphuric acid. A mixture of equal parts butter fat and non-heated lard gave a reaction with sulphuric acid. When butter fat was heated 4 hours in the air its ability for a typical sulphuric acid reaction disappeared.

Gillam (88) found that on the average, the ratio of carotene to xanthophyll in butter is 14 to 1 by weight.

Many investigators have studied the vitamin content of

cow's milk. Kennedy and Dutcher (131) noted that the presence of Vitamin A in cow's milk is entirely dependent upon its occurrence in the ration. Stall fed cows will produce a milk rich in vitamins provided their ration consists of a proper combination of grains and leafy foods. Russel et. al., (195) found that in fresh milk the vitamin content may vary as a result of change in lactation period and of variations of provitamin A and the Vitamin A content of the ration when the assay extends over a period of months. They show that the percentage output of vitamin expresses the relationship between intake and output but does not take into consideration the possibility that some of the Vitamin A in the milk may come from the body stores. The percentage of the factor which appears in the milk decreases as the amount of it in the ration is increased and that the increase in Vitamin A content of the milk is not proportional to the increased consumption. In a comparison of Ayshire and Guernsey butters, Wilbur, Hilton and Hauge (255) found that, although the Ayshire butter contained 1.8mg carotin per 100 gr. of butter and the Guernsey 4.0mg. per 100gr. of butter, they were identical in Vitamin A activity. Hathaway and Davis (103) demonstrated that the Vitamin A content of milk is closely associated with the butterfat content. They show that skim milk or separated milk containing only a small quantity of butterfat contains also only a small amount of Vitamin A. They also noted that Holstein cream is more potent in Vitamin A than is Jersey cream. As the percentage of fat in the Holstein and Jersey

milk approach each other the difference in Vitamin A content of the cream separated from them will disappear. In further tests Hathaway and Davis (104) showed that there is little difference if any in sour and sweet cream butter. Margarine on the other hand are poor sources of Vitamin A.

Investigating the Vitamin A content of butter, Fraps, Copeland and Treichler (84) demonstrated that cows receiving feeds which were low in Vitamin A content produced butter low in Vitamin A potency. The Vitamin A content of butter depends both upon the Vitamin A potency of the feed and the length of time the cow has been fed upon it. When a cow is on a feed supplying insufficient quantities of Vitamin A, the Vitamin A in the butter fat decreases with the period of time the cow has been on the feed or the stage of lactation on account of the depletion of the reserve of Vitamin A stored by the cow at the beginning of lactation. In the cow it requires approximately eleven units of Vitamin A to give one unit of Vitamin A in the butter. The cow uses Vitamin A much less efficiently than poultry.

According to Moore (163) carotene when supplied to cows undergoes conversion to Vitamin A.

Guilbert and Hart (96) found that carotene being the principle pigment of beef fat, could be withdrawn from the adipose tissue during Vitamin A privation without a coincident reduction of fat reserve. They showed that the carotene in the fat of cattle constituted a significant part of the total Vit-

amin A reserve.

These same investigators noted that the storage of Vitamin A in the liver of newborn calves is relatively low regardless of the storage in the dam.

The Vitamin A content of the livers of normally fed oxen, guineapigs, rabbits, rats and dogs was tested biologically by Simonnet, Busson and Asselin (218) and found to vary considerably. The liver of the ox was the most potent while that of the guinea pig was the least potent. Using a dog, these same investigators (217) fed liberal daily doses of carotene for a month. It was then killed and the lungs, liver, kidneys, brain and part of the body fat were tested for Vitamin A. They found that only the liver and kidneys contained Vitamin A and these in approximately equal amounts.

Ahmad and Malik (5) showed that animals differ in their ability to synthesize Vitamin A from carotene, as judged by the Vitamin A content of the liver. With the ability of rats to synthesize Vitamin A rated at 100, chickens give a rating of only 24, while rabbits give a rating of only 16 and cats zero. They conclude that the Vitamin A potency of feeds may vary for different animals according to the relative proportion of the Vitamin A and carotene present.

Localisation of Vitamin A in tissues by a fluorescent microscope was noted by Querner (178). In the cells of the liver and other organs a fluorescent material was present which was rapidly destroyed by exposure to ultra violet light . This

"Leuchtstoff" was found to correspond to the amount of Vitamin A present.

The nutritive value of lard was extensively studied by Drummond et. al. (62) in 1920. They concluded that the pig was able to store up supplies of Vitamin A in the body fat when fed a diet containing ample supplies of the factor. When the diet was deficient in Vitamin A no appreciable amounts of the dietary factor could be found in the body fat. The process employed in the manufacture of lard causes a very marked destruction of the vitamin present in pig fat.

Investigating this problem of the Vitamin A content of lard further, Mallon and Clark (151) concluded that lard made from leaves and back fat of hogs whose diet contained Vitamin A, did not contain an adequate supply of this vitamin to prevent xerophthalmia even when fed in large amounts.

Storage of Vitamin A in the Body.

The storage of Vitamin A in the body was reported by Goldblatt and Soames (90) in England and Steenbock, Sell and Nelson (235) in America in 1923. Both groups of investigators came to the same conclusion that Vitamin A is stored in the liver and that it is stored in rough proportion to the amount contained in the diet.

Continuing this work further, Sherman and Cammack(207) showed that by feeding diets graded in their content of Vitamin A, the richer the diet in this vitamin, the greater is the amount stored in the body. The attainment of the maximum store of Vitamin A is a process of gradual accumulation which is relatively rapid in its earlier stages and becomes slower as the maximum is approached. A rapid storage of the entire maximum amount is not possible.

McCoord and Clausen (145) demonstrated that when 1 drop of halibut liver oil was added to the diet of the rat and the animal killed 24 hours later, there was a definite increase of Vitamin A in the liver and body fat. When 4 drops were so added, marked increase was found in a number of other organs and tissues, particularly the adrenals.

Working on the problem quantitatively Baumann, Riising and Steenbock (10) found that 95 % of the Vitamin A in the body was stored in the liver. The remainder was located in lung and kidney tissue. The minimum daily dose of Vitamin A necessary to produce storage in the liver was between 25 and 50 blue units. When Vitamin A was fed in the form of halibut

liver oil, the amount stored in the liver was found to parallel the amount administered, but only 10 to 20 % of the Vitamin could be accounted for. When equal amounts of Vitamin A were fed to normal and to Vitamin A depleted rats, the liver storage was greatest in the normal animals. When equal amounts of Vitamin A were fed to animals in various stages of depletion, the amount stored was inversely proportional to the state of depletion. The absorption and storage of Vitamin A to a large extent takes place within 6 hours after ingestion of the Vitamin.

The elimination of Vitamin A from the livers of rats was studied by Davies and Moore (49). Female rats 18 months old, which had finished breeding and had been fed a diet well supplied with Vitamin A, received for 12 weeks, in addition, a large amount of a Vitamin A concentrate. The mean Vitamin A concentration in the liver at the end of this time was 18,000 blue units per gram. The surviving animals then received a diet deficient in Vitamin A and the concentration in the liver fell rapidly, being reduced in 4 weeks to an average of 2,700 blue units per gram; after 12 weeks it fell further to about 400 units and was still at that level after 24 weeks. The rate of depletion of these enormous reserves of Vitamin A was far more rapid than a rate representing the rats' daily requirement.

Transmission of Vitamin A From Parents to Young.

The problem of Vitamin A transmission from the mother to the foetus has been extensively studied by Dann (44) and (46). He showed that Vitamin A is normally found in the liver of the rat and rabbit at birth, but only in small amounts which cannot be increased by giving the mother a diet rich in carotene during gestation. The store of Vitamin A in the liver of the young rat increases 2 or 3 fold during suckling, but the increase may be greater when the lactating parent receives a diet rich in the factor. There is a limit to the amount of this increase, due in turn to a limitation of the amount of Vitamin A which can pass into the milk.

Working with chicks, however, Bearse and Miller (13) have shown that the hen's diet markedly controls the amount of Vitamin A found in baby chicks.

The work of Hale (101) is interesting in that he produced 3 litters of pigs born without eyeballs or with very serious eye defects when the mother had received a diet deficient in Vitamin A before mating and during the first 30 days of gestation.

Conversion of Carotinoids Into Vitamin A.

The transformation of carotene into Vitamin A was studied by Copper (30) in 1930. He used the absorption spectra of rat liver oils. He found that the band at 325μ generally attributed to Vitamin A was absent from the absorption spectra of the liver oils of rats suffering from Vitamin A deficiency, but was shown by the liver oils of similar rats which had been subsequently cured by massive doses of carotene. The 325μ band was absent from the absorption spectrum of carotene, therefore, he concluded, the substance responsible for the exhibition of this band by the liver oils of carotene-treated rats, had been synthesised in vivo from the carotene.

Moore (161) noted that the conversion of carotene into Vitamin A took place in the liver, but the efficiency of the conversion was by no means quantitative.

The absorption of carotene from the intestinal tract was investigated by Ahmad (3) who observed that it may be considerably affected by the composition of the diet.

Studying the isomers of carotene Karrer, Euler and Hellstrom (128) showed that the blue values given by alpha and beta isomerides in the Sb.Cl_3 reaction were identical. Rat growth tests for Vitamin A activity however, demonstrated that the beta is more active than the alpha isomeride.

The curative effects of 5 to 10 y of pure carotene on rats suffering from symptoms of Vitamin A deficiency was definitely established by Glanzmann (89). He found that the blood platelets of the rats fall in numbers rapidly when

diets deficient in Vitamin A are given, and rise rapidly when carotene is supplied.

The Vitamin A activity of palm oils was found to be in accord with their carotene content by Ahmad (4). After hydrogenation, however, all the activity was lost.

Rea and Drummond (180) investigated the formation of Vitamin A from carotene in the animal organism. In the incubation of the liver of rats and cats with solutions of carotene, they failed to demonstrate the formation of Vitamin A. The intravenous injection of colloidal suspensions of carotene into the livers of cats which had received a Vitamin A deficient diet, produced no detectable increase in the Vitamin A content of the livers as shown by spectrographic examination of the non-saponifiable fractions before injection, and 4 to 72 hours after injection.

Conversion of carotene into Vitamin A in the animal body was studied by Skarzynski (220). He made spectroscopic and colorimetric examinations of extracts from the caecum, spleen, liver and serum of young rats which had developed xerophthalmia on a Vitamin A deficient diet and which had died or been cured with carotene (4 mg. in 36 days). In the extracts from the deficient group which had died, no trace of Vitamin A or carotene could be detected. In the group which received carotene, Vitamin A was found in the liver and serum, and carotene in the caecum and probably in the liver; the spleen contained neither. The author suggests that transformation of carotene does not take place in

the gut as supposed by Ahmad (4), but in the liver from which it passes into the blood.

The Vitamin A content of rat liver after feeding alpha, beta and gamma carotene was reported by Brockmann and Tecklenburg (22). They examined the unsaponifiable fraction of the liver oil with SbCl_3 and found that beta carotene produced 2 or 3 times as much Vitamin A as the other two.

Moore (164) reported in 1933 that carotene is utilised in the body as efficiently as preformed Vitamin A at levels approaching the minimum dose.

Using a spectrophotometer method, Drummond and MacWalter (68) observed that after injecting carotene into the portal circulation of rabbits no increase in the Vitamin A in the liver takes place until eight days after the experiment. They suggest the possibility that formation of the vitamin occurred within much shorter periods but either it was utilised and converted into another substance or that the amounts were too small to be detected by their technique.

The fates of carotene introduced into the circulation was investigated by Drummond, Gilding and MacWalter (69). These investigators injected intravenously into anaesthetised cats a colloidal suspension of carotene in isotonic glucose solution. Blood samples were taken in the ensuing 15 minutes and after death, the remaining blood and organs were examined for carotene. No carotene was found in any of the blood samples and, of the organs, only in the lungs, liver and spleen. In the lungs the

carotene appeared to be trapped in the capillaries. The greater part was absorbed selectively by the liver and was chiefly located in the Kupffer cells.

Working with rats, Drummond and MacWalter (70) reported that Vitamin A and carotene were evenly distributed through the three lobes of the animals liver,

Pure crystalline xanthophyll was shown not to be identical with Vitamin A by Willimott and Moore (257) in 1927. A few years later Kuhn, et. al. (134) showed that the xanthophylls, lutein, zeaxanthin and violaxanthin were negative as well as azafurin methylester, dihydrocrocin dimethylester and chlorophyll.

Certain microorganisms were found by Baumann, Steenbock and Ingraham (9) to synthesize carotene. Whenever an organism showed Vitamin A activity as determined by feeding experiments, enough carotene was found to be present to account for that activity. The Vitamin A activity of microorganisms did not appear to be affected by the presence of yellow pigments other than carotene. They concluded that since spectrographic determination failed to reveal any absorption band at 328 M^{u} it is exceedingly improbable that Vitamin A as such is generally present in bacteria. Attempts, however, to effect the transformation of carotene into Vitamin A by microorganisms failed.

VITAMIN A THERAPYIn Colds

It is generally recognized that a lack or deficiency of Vitamin A in the Ration results in the greater susceptibility of animals as well as man to various Respiratory infections. In view of the wide prevalence of simple colds in man, considerable emphasis has been laid on the inclusion of optimum amounts of Vitamin A in the diet. As a matter of fact, direct evidence that Vitamin A plays the role of an anti-infective agent, as has been commonly accepted, is lacking.

In this connection it is interesting to note Cameron's work (27), (28) and (29), on Vitamin A Therapy in colds. In an experiment (27) conducted at the University of West Virginia, he used five groups of women students. The members of one group took a tablespoonful of cod liver oil daily from November to March 1932-33, and the other groups an equivalent dose of a Vitamin A concentrate, an inert tablet, a cod liver oil residue in tablet form, and no medication. Records were made twice a week during the experimental period on the presence and duration of colds. At the end of the experiment it was found that the number of days with colds reported from the group taking no medication was considerably higher than from any of the other groups. The Vitamin A concentrate and the cod liver oil residue tablets appeared to give slightly better results than the cod liver oil.

The following is a quotation from the result of the above experiment.

"No conclusion is as yet justified from these results, but the volunteers participating in the experiment were unanimous in reporting subjective improvement from cod liver oil and its concentrates. This improvement took the form of increased appetite, weight, or endurance, or relief from skin eruptions or from sinus trouble, as well as in reduction in number and duration of colds. These changes can probably be attributed to the improved nutrition resulting from an abundance of Vitamin A, and this improved nutrition is worth while in itself whatever final conclusions are reached with reference to Vitamin A Therapy against common colds".

In his second experiment (28) the effect of increasing the Vitamin A intake of men and women students, either by increasing the amount of foods rich in Vitamin A, or by direct dosing with cod liver oil, haliver oil, cod liver oil tablets or carotene solution, was studied in regard to the number and severity of colds during a period of two years. The control groups received no medication or a lactose supplement. Statistical examination of the results indicated that increase in the Vitamin A intake did not decrease the number of colds, but did tend to lessen the duration and severity of the infection. Approximately fifty percent of the subjects of the test reported improvement in powers of endurance, in appetite and in decrease of skin eruptions. He concludes, however,

that Vitamin A Therapy should not be considered as a specific against colds.

From his third investigation (29) he gave the following conclusion:

"Whether the effect of Vitamin A upon colds, therefore, rests upon its ability to maintain intact a barrier of healthy mucous membrane to act as a local defense against invasion by virus or bacteria, or whether its effect is due also to its influence on circulation and nervous mechanisms which weather and dietary imbalance undoubtedly play a part, it has been shown to be a factor in the duration of colds in adults and worth a trial in all susceptible individuals."

In the Dressing of Wounds

The local use of Vitamin A in the treatment of wounds has been observed by many workers. Horn and Sandor (123) observed that dressings of a concentrate of Vitamin A, containing two thousand international units in 1 ml. of an oily medium, influenced favorably the healing of flesh wounds and of infected tissues. Zoltan (269) obtained good results when purulent wounds were treated with an ointment containing cod liver oil, carotene and irradiated ergosterol. Direct application of crude cod liver oil was found by Steel (225) to be most successful in the treatment of severe burns and ulcers. The use of the cod liver oil prevented the formation of scar tissue and pigmentation following healing.

Drigalski (55) made wounds of a measured size with scissors in the skin of guinea pigs and dressed them with a cod liver oil ointment "Norguentolan", or with the same ointment through which air had been bubbled at 130° C. for thirty-six hours. Biological tests on rats showed the destruction of Vitamin A by this procedure to be complete. Healing was considered to be more favorable and more rapid in wounds treated with unoxidized "Unguentolan", whether on the same or on different animals, even when the wounds were experimentally infected with staphylococci or streptococci. Shortly after the above observations had been made "Strauss (239) reported that over one hundred twenty cases of wounds, burns, etc., were satisfactorily treated with "Unguentolan". He stated that pain was reduced since the wound seldom needed redressing and drains were unnecessary. Using this same ointment "Unguentolan", Dreyfus (53) observed that is application to burns of the second and third degrees resulted in rapid and complete healing. Similar results were obtained in purulent and gangrenous ulcers, but little improvement was obtained in erythrodermia ichthyosiforma. He suggests that the healing might be due to Vitamin A or D, or the Phosphorus or Iodine content, or the protective effects of the preparation or a combination of these factors.

An extensive study of the effect of ointments containing Vitamins on the healing of wounds was conducted by Lauber

and Rocholl (135). Surface wounds on white mice were treated with various ointments containing Vitamins "H", "B", "C", "A", "D", and with control ointments without Vitamins. The wounds treated with vogan (Vitamin A) in a base containing Cholesterol healed best; vogan in a vaseline-lanoline base was considerably less effective, and in large doses even tended to inhibit healing. Vitamin "H" had some beneficial effect and Vitamin "B", very little, while Vitamins "C" and "D" appeared to hinder the healing process. Drigalski (56) disagreed with the above results and stated that they would go no further than the authors' own findings.

The effect of Vitamins on the growth of tissues and transplanted tumors was studied by Gordonoff and Ludwig (92). They noted that the growth of fibroblasts from embryo chicken heart, or of small transplants of mouse tumors, was very much less in plasma from rats which had been kept on diets deficient in Vitamin A, than in plasma from rats which had received large doses of vogan. The growth of normal and pathological tissues seemed also to be inhibited in plasma from rats deprived of the Vitamin "B" complex, but not in plasma from rats receiving doses of yeast. There was no difference in plasma from rats on McCollum's rachitogenic diet 3143, whether the animals received Vitamin "D" or not. The presence of Vitamin "C" seemed to have an inhibiting effect, as shown by cultures in plasma from scorbutic and normal guinea pigs.

The work of Turner and Loew (249), shows that carotene is protective against bacterial invasion of the upper respiratory tract and the development of the pathologic systemic conditions accompanying Vitamin A deficiency in rats. Carotene therapy reduces the number of spontaneous suppurative lesions in the upper respiratory tract occurring in animals deprived of Vitamin A; Xerophthalmia is cured in one hundred percent, and normal health regained in seventy-four percent. The percentage incidence of bacteria in the nasal cavities and middle ear in animals fed active carotene is noticeably less than that encountered in animals given faded carotene or that in animals receiving no source of Vitamin A. In another investigation (247), they noted that the withdrawal of Vitamin A from the monkey does not develop a characteristic susceptibility toward infection of the upper respiratory tract. They also observed while working with rats (248), that the age of the animal, the previous storage of Vitamin A, the season, had no marked effect on the bacterial flora.

In reviewing the researches on the value of cod liver oil plastus in the healing of burns and wounds, Gortzen (93), points out that cod liver oil does not inhibit the growth of bacteria in cultures. He claims its healing effect cannot, therefore, be explained by any bactericidal action on infected tissue. Hayaski (107) suggests that it is the lipoid content of cod liver oil that has the beneficial effect on infected

wounds and not Vitamins "A" and "D".

Antagonism Between Vitamins "A" and "C" and Vitamin "A" and Thyroxin

Wendt and Schroeder (253) studied the antagonism between Vitamins "A" and "C". When guinea pigs were given large simultaneous, daily doses of Vitamin "A" (1.5 ml. vogan) and Vitamin "C" (60 mg. Cebain Merck), they showed no signs of hypervitaminosis "A". If only 0.25 to 0.5 ml. daily of vogan was given together with 10 mg. Cebain Merck, there was not even retardation of growth. The Vitamin "A" content of the livers of the guinea pigs receiving both Vitamins was lower than that of others having Vitamin "A" only, but the Vitamin "C" content of the organs was not affected by the presence or absence of Vitamin "A".

While studying the problem of hyperthyroidism, Schneider and Windmann (201) noted that when rats were fully depleted of Vitamin "A", oral administration of carotene for ten days did not cause deposition of Vitamin "A" in their livers, although administration of the Vitamin itself caused storage in large amounts. When the livers were depleted of glycogen by injecting the animals with the thyrotropic principle of the pituitary, failure of deposition of Vitamin "A" after administration of carotene also occurred. This failure is attributed to an increased demand for Vitamin "A" rather than to inability of the liver to convert carotene into the

Vitamin. They suggest that the metabolism of glycogen is closely bound up with that of Vitamin "A".

Wendt (254) observed that an excess of thyroxine increases the rate at which Vitamin "A" is metabolised. In hypothyroidism the capacity to convert carotene to Vitamin "A" and to store carotene and Vitamin "A" in the liver are reduced.

Further studies by Sherwood and Luchner (216) dealt with the histological effect of cod liver oil on the thyroid gland. They found that excessive daily doses of carotene, "haliver" oil or cod liver oil, over a period of several weeks, resulted in a depletion of the colloid and an increase in the height of the epithelial cells in the thyroid glands of young albino rats. Carotene and "haliver" oil produced in addition a marked increase in stroma. Administration of potassium iodide, in amount approximately equivalent to the iodine in 0.3 ml. daily of cod liver oil, had no effect up to the eighteenth day. There was then an increase in colloid and the acini were distended, with extremely low epithelium. They concluded that the iodine of cod liver oil was less available for metabolic activity than that of potassium iodide, possibly being lost by excretion of the oil, and that such changes in the gland as were produced by cod liver oil were due to Vitamin "A", rather than to iodine.

The work of Abelin (1) noted that the ingestion of a Vitamin "A" preparation by rats, diminished the rise in basal

metabolism resulting from thyroid administration. The action of excess of doses of thyroid in lowering the growth rate was also partially counteracted by administration of Vitamin "A", which was shown to increase the glycogen content of muscles. He suggests that Thyroxine and Vitamin "A" are antagonistic because of their opposite influence on lipoid and carbohydrate metabolism.

In spite of the well recognized importance of Vitamins in nutrition, practically no work has been done on the associative action of the various Vitamins. While in the case of poultry and other domestic animals the minimum and optimum amounts have been fairly accurately determined, no information is available as to the most satisfactory combination of any two or more Vitamins to promote normal growth, health and reproduction.

BIOLOGICAL ASSAY OF VITAMIN A USING
THE RAT AS THE TEST ANIMAL.

Feeding Technique.

The technique used in carrying out the Vitamin A assays is described in great detail by the United States Pharmacopoeia (see appendix 1). These recommendations have arisen as the result of very careful work conducted during the past decade by several investigators of whom Coward of Great Britain has been the leading investigator.

In 1920 Drummond and Coward (59) stated that in testing foodstuffs for the presence of the fat-soluble vitamin the greatest care should be devoted to ensuring that the basal dietary is rendered as free from that vitamin as possible. They give details for the preparation of a highly purified ration. They conclude that the failure to work with a sufficiently pure diet may lead to conflicting and misleading results.

Steenbock, Nelson and Black (236) noted that the absence of growth on suitably constituted rations cannot be taken as an indication of the absence of Vitamin A unless the antirachitic factor is supplied.

Steenbock and Coward (237) advocated the use of the incidence of ophthalmia as a sign of exhaustion of the animals' store of Vitamin A in preference to cessation of growth. The two are often simultaneous, but the use of the former criterion prevents loss of animals through the very sudden and rapid decline that may ensue while waiting to become certain that growth

has really ceased. Growth ceases during the worst stages of ophthalmia and is only resumed when definite improvement in the animals' condition is observable.

In the biological assay for Vitamin A according to Javillier and Emerique (126) it is necessary to observe certain precautions with regard to: 1. An adequate supply of Vitamin D in the diet of the experimental animals; 2. The period of the test proper which should begin only after there has been a loss in weight of 10%; 3. The large number of animals (10, 20, 30) to be used in the tests of each dose; 4. The similar sex of all animals compared; 5. The manner of administration of the substance tested. The Javillier unit of Vitamin A is defined as "the quality of the vitamin which, when added to the minimal dose of maintenance (per 100g. of rat's body weight under the specified conditions) will induce a growth response lasting at least 30 days, the angle of growth being 30° and the gain in weight about 30%."

Sherman and Batchelder (208) claim on statistical grounds that with levels of Vitamin A feeding which induce a gain in weight of about 3 grams a week, a decrease of about 25% or an increase of 33% in the value of the daily intake of Vitamin A can undoubtedly be measured by this method.

According to Honeywell, Dutcher and Ely (121) the response to administration of Vitamin A of rats depleted of this factor is influenced by the type of yeast used in the diet. This influence is thought to be a function of the Vitamin B complex. The authors suggest that Vitamin A

consists of two factors, one possessing anti-xerophthalmic properties, concerned in maintaining the normality of the cornea and other epithelial tissues, and another with a purely growth-promoting function.

Nelson, Walker and Jones (167) noted that when single doses of material containing Vitamin A were given to rats deprived of the factor, the growth response and subsequent duration of life of the animal were directly proportional to the amount of Vitamin A given.

The investigators Polak and Stokvis (175) found that daily doses of 0.5 to 1.0 gamma of carotene were sufficient to prevent and cure xerophthalmia in rats on a diet deficient in Vitamin A.

Working along similar lines too(175) were Baumann, Riising and Steenbock (11). A solution of carotene in cottonseed oil in which no deterioration could be detected spectroscopically throughout the experiment, was administered for 8 weeks to rats on diets deficient in Vitamin A. A weekly dosage of 3 to 5 gamma of carotene did not cure or prevent xerophthalmia but 10 gamma did so and also promoted moderate growth; amounts larger than 20 gamma weekly did not enhance growth. They found it impossible to maintain rats at a growth rate of 3 grams per week, and also emphasise the danger of using a standard of animal response rather than the international standard substance for reference.

Coward and Key (42) found that the mean weight increase of groups of rats receiving 7 mg. of cod liver oil

weekly, after depletion of Vitamin A, was the same whether 1 mg. was administered daily, or 4 mg. on Thursday and 3 mg. on Monday. The high degree of statistical probability that the result is valid is worked out.

The determination of Vitamin A values by a method of single feedings was reported by Sherman and Todhunter (209). When male or female rats, depleted of Vitamin A received single large doses of 28, 56 or 112 gamma of carotene, the weight increase and survival time were proportional to the dose for each sex. The value best expressing this relationship was obtained by plotting weight increase and subsequent decline against the time in days and estimating the area, bounded on the upper side by this curve and on the lower side by a base line drawn parallel to the abscissae through the point representing the average weight loss of a group of untreated controls, at the end of the first week from beginning of dosage. This method is specially recommended for perishable or unstable materials.

Results of Vitamin A Deficiency in Rats.

In studying avitaminosis A of rats, McCollum, Simmonds and Becker (144) found that the feeding of excessive amounts of any one element or ion did not induce ophthalmia.

The histological changes are very well described by Tyson and Smith (250) who say "The characteristic histological changes found in rats fed a diet lacking Vitamin A are the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, paraocular glands and the genito-urinary tract."

Replacement of the normal epithelium of the body organs with keratinized cells was observed in mice maintained on Vitamin A free diet by Wolfe and Salter (265). Xerophthalmia developed in from 25 to 120 days although metaplastic changes in the respiratory tract were found previously to this.

Sure and Smith (242) noted that Vitamin A deficiency in rats did not alter the true blood sugar, the alkaline reserve of the blood or the glycogen content of the liver. The reducing non-sugars, however were frequently increased, thus raising the apparent blood sugars.

Pathologic changes in the tissues during early stages of Vitamin A deficiency were investigated by Thatcher and Sure (244). Their results showed metaplasia in the posterior portion of the tongue, in the salivary glands, and in the respiratory and urinary tracts in the majority of cases of early Vitamin A deficiency, although in many individuals there were as yet no external symptoms such as cessation of growth, persistence of the

cornified cell stage of the oestrous cycle or incipient ophthalmia.

The effect of Vitamin A on metabolism was recorded by Chevallier and Baert (33). Young rats with typical signs of Vitamin A depletion showed a basal metabolic rate 13 per cent higher than that of similar normal rats. Rats receiving daily 2000 U.S.P. units of Vitamin A and a guineapig receiving daily 10,000 such units, showed a rate lowered by 10 to 15 percent.

According to Malmberg (152) increase in weight and in tail length slackens simultaneously in young rats in process of depletion of Vitamin A.

From Tornblom's (245) experiments no significant difference could be detected in the oxygen consumption of rats developing well marked symptoms of deficiency, on a diet deprived of Vitamin A, and rats maintained in health on the same diet, by the daily addition of 10 gamma of carotene.

Hematopoietic function in deficiency diseases was investigated by Sure, et. al. (241). From their work they concluded that after Vitamin A deficiency has progressed to the ophthalmic stage inanition complicates the blood picture, so that the high figures of hemoglobin and erythrocytes may be an expression of anhydremia, indicated by the concentration of total blood solids of the pathological animals as compared with normal animals of the same age and weight. In the pre-ophthalmic stage they found a suggestion of an anemia characterized by reduction in either hemoglobin or erythrocytes. No

connection could be established between avitaminosis and pernicious anemia.

Batchelder (7) found that urinary calculi were present in rats receiving small quantities of Vitamin A but were not present in rats receiving no Vitamin A. He also noted that the general breakdown resulting in death, though accompanied by similar symptoms, was postponed to successively later stages as the Vitamin A content was increased.

Sutton, Sutterfield and Krauss (243) demonstrated that nerve degeneration occurred about the same time as ophthalmia and became extensive before external symptoms of paralysis appear. Working with a polarizing microscope on formalin-fixed frozen peripheral tissues, they showed that after external symptoms of paralysis appear, adequate amounts of Vitamin A do not relieve them but nerve degeneration is arrested.

The data published by Evans (80), demonstrated that inadequate Vitamin A injures the female reproductive system so that fertilization and implantation often fail. In this respect it differs radically from the reproductive impairment due to low Vitamin E content, where, typically the eggs are always healthy and implantation takes place but resorption follows.

A study of the effects on the male genital organs was carried out by Sampson and Korenchevsky (196). They observed that in most rats on a Vitamin A deficient diet the weights of the testes and especially of the penis and of the prostate with seminal vesicles were greater than those of rats kept on a

paired complete diet. The testes of rats on the deficient diet were oedematous. The decreased food intake produced no noticeable effect on the penis, decreased the weight of the prostate with seminal vesicles and in some cases slightly increased the weight of the testes.

Assimilation of Vitamin A in the Presence of Mineral Oil.

Rowntree (191) in 1931 undertook a study of the effect of the use of mineral oil upon the absorption of Vitamin A. When rats previously deprived of Vitamin A were given small doses of cod liver oil sufficient only to allow of slow growth, the addition of mineral oil to the diet in amounts equivalent to the human therapeutic dose cause the animals to lose weight and die. When, however, the dose of cod was an adequate one, the ingestion of the mineral oil did not appear to interfere with the absorption or utilization of the vitamin.

The assimilation of carotene and Vitamin A in the presence of mineral oil was studied by Dutcher et. al. (71) in 1934. Rats depleted of Vitamin A recovered when receiving 40 mg. of butter daily, but not when they also received 100 mg. of mineral oil, with or without hydroquinone. A comparable result was obtained with carotene but the adverse effect was overcome if the carotene was considerably increased relative to the mineral oil. Mineral oil exercised scarcely any unfavorable effect when administered with cod liver oil or a Vitamin A concentrate. When carotene was fed with mineral oil, it could be recovered from the faeces in an amount proportional to that fed, indicating that the action of the oil was to inhibit the absorption of carotene from the gut.

The influence of Vitamin A on fat metabolism was first investigated by Drummond (58) in 1919. He found that rats were able to absorb large amounts of fatty acids, and presumably synthesise these into fats, in the absence of Vitamin A in the

diet. Several years later, Green (95) worked along similar lines. From his work no evidence was obtained that a diet very rich in fat accelerates the utilization of Vitamin A in the body. The diminuation in the total crude fat of the whole body is not significantly different in the Vitamin A deficient rat from that in the rat on a complete diet whose food intake is restricted to that of the deficient animal. A rise in the iodine value of the fatty acids of the liver, coincident with a fall in the percentage amount of fat in the liver, occurs in the end stages of Vitamin A deficiency. The rise is of a similar loss of liver fat has been produced by a restriction of food intake or by infection.

Working on the esterase content of the blood-serum Green (94) reported that Vitamin A deficiency in the rat produced a large and progressive decrease of this enzyme. Relatively large amounts of cod liver oil in the diet, however, produce a rise in the serum esterase content well above normal.

Influence of Vitamin A on Fat Metabolism.

The toxic effects due to large doses of Vitamin A was studied by Drigalski (54) in 1933. He noted that rats given bread, oats and water together with 20,000 or 40,000 rat units of Vitamin A daily in the form of a concentrated commercial preparation died in 5 to 19 days with loss of weight and the development of conjunctivitis and hemorrhagic rhinitis. Control rats, given the same concentrates after the destruction of the Vitamin A by ultra violet irradiation, remained in normal health.

In the same year Domagk and Dobeneck (52) made a detailed histological examination of rats of 80 gram weight, which had received 100,000 "units" of Vitamin A in 1c.c. of sesame oil daily for a week. Kupffer's star cells of the liver, the pulp cells of the spleen, endothelial cells in the kidney and epithelial cells of the stomach were filled with fat, as compared with those of controls, receiving sesame oil only. The cells of the gastric epithelium showed abnormally rapid multiplication.

A year later Davies and Moore (48) fed massive doses of a Vitamin A distillate to young rats. They observed that their doses proved toxic, causing emaciation, haemorrhagic rhinitis, loss of hair round the mouth and in one rat gross lung lesions. Their attempts to induce hypervitaminosis A by feeding carotene failed on account of its limited solubility in fats.

Lewis and Reti (136) studied the effects of large doses of carotene when fed to rats. They noted that when young rats were given large amounts of a Vitamin A concentrate (vogan) they declined in weight, and died within 10-14 days, with the

usual symptoms of hypervitaminosis A (emaciation, conjunctivitis skin lesions on the face and paresis of the hind legs). When they fed 40 mg. of pure carotene dissolved in an innocuous oil there were no ill effects.

Practically no work has been reported on the toxic effects if any, of Vitamin A in domestic animals. The majority of investigations to date have been concerned with determining the minimum and optimum requirements of domestic animals for Vitamin A.

CHEMICAL AND PHYSICAL PROPERTIES OF VITAMIN A.

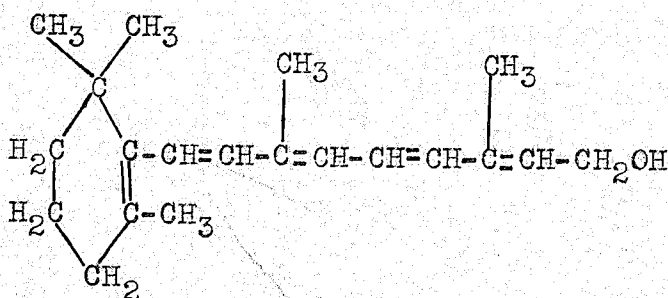
The Chemical Structure of Vitamin A.

A great deal of work has been done on the Structure and Chemical composition of Vitamin A and carotene.

Smith (222) interpreted his observations that carotene contained 9 easily saturated double bonds in a conjugated series, further conjugated with 2 other unsaturated linkages, either double bonds or cyclopropane linkages. He suggested that this system was responsible for the colour of the carotene molecule.

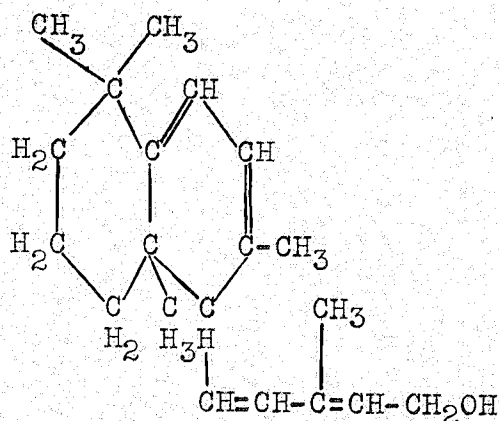
Bruins, Overhoff and Wolff (23) calculated the ratio of the molecular weights of carotene and Vitamin A and from this they deduced a molecular weight of about 330 for Vitamin A. From this value they concluded that the assumption of a simple chemical relation between Vitamin A and carotene to appear improbable.

In 1931 Karrer, Morf and Schopp(129) suggested the following formula for Vitamin A, (C₂₀H₃₀O), which is now the "provisional formula."

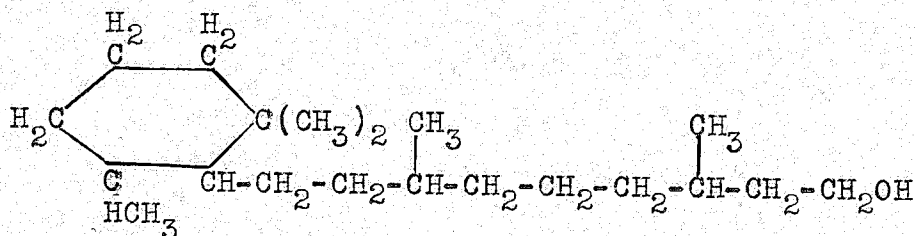


A year later, Heillbron, Morton and Webster (110)

suggested the previous formula and also the following:



The next year Karrer, Morf and Schopp (130) synthesised perhydro-vitamin A, ($C_{20}H_{40}O$) with the following structure:



The identity of the substance thus synthesised with that prepared by the reduction of the natural Vitamin A was established by comparing the boiling point, density and molecular refraction.

According to Drummond (67) the formulae for alfa and beta carotene are as follows:

He also summarized and compared the properties of Vitamin A and carotene.

Carotene

Synthesised in plant
Orange red
Antimony trichloride
gives steel blue colour,
band at 5900 Å.

Vitamin A.

Stored in animal
Almost colourless
Band in U.V. at 3280 Å⁰
Royal blue colour with
antimony trichloride,
band at 5720 and 6060 Å⁰.

The Effect of Chemical and Physical Agents on Vitamin A

Observations upon the chemical nature and properties of Vitamin A were reported by Drummond (57) in 1919. He noted that the fat soluble accessory food factor A was readily destroyed by short exposures (one hour to a temperature of 100°). Destruction was less rapid at temperatures of 50° to 100° . He believed at this time that the destruction was not due to oxidation or hydrolysis. The factor was not extracted from oils by water or dilute acid but was soluble in alcohol. The fat soluble A factor could not be identified with any of the recognized components of fats, such as glycerol, saturated or unsaturated fatty acids, cholesterol, lecithin, phosphatides or the lipochromes. In conclusion he suggested that in view of the low temperature at which destruction occurs, fat soluble A may be a liable substance of ill-defined constitution.

Working with whale oils Delf (50) found that the temperature used for extracting the oil played an important part in the potency of the oil. In his work he also found that the sperm whale gave from its head an oil richer in Vitamin A than from the blubber.

Emmett and Luros (77) showed definitely that benzine or acetone did not extract from the pancreas, thymus and suprarenal glands a fat that contained the fat soluble A Vitamin.

Drummond, Channon and Coward (64) prepared a concentrate of Vitamin A from Cod Liver Oil and examined its chemical properties. They found it contained no detectable traces of iodine or nitrogen, so that these elements are not related to the physiological action of the oil in promoting growth. Their results showed that approximately 50% of the unsaponifiable matter from Cod Liver Oil is cholesterol, which may be removed quantitatively without loss of vitamin activity. Vitamin A was found on distillation to pass over at 180-220° at 2-3 m.m. A chemical examination of the active distillate indicated the presence of (a) a saturated solid alcohol, (b) the unsaturated hydrocarbon spinacene, (c) one or more than one unsaturated alcohol, boiling about 200° at 2-3 m.m. Spinacene and the solid alcohol were without Vitamin A action.

Cody and Luck (25) studied the effect of different gases upon Vitamin A. They discovered that SO₂ rapidly destroyed the active principle of Cod Liver Oil. Alfalfa and spinach, when sulphured in the dry and green conditions, experienced no loss of Vitamin A activity. Phosphorous pentachloride, chlorine, acetyl chloride, nitrous fumes and Benedicts' alkaline copper reagent destroyed the active principle in Cod Liver Oil. Prolonged treatment with sodium bisulphite had the same effect. Hydrogen sulphide, ethylene, ammonia and Benedicts' reagent after neutralization exhibited no destructive action. Formaldehyde had little effect.

Hydrogen peroxide brought about a partial loss. They concluded that Vitamin A activity is the property of a specific atomic grouping rather than of a specific molecule, and that the active principle of Cod Liver Oil possesses aldehyde properties.

The destruction of Vitamin A by ultra-violet radiations was studied by Norris (168). He found that the curve of destruction of Vitamin A in Cod Liver Oil by ultra-violet irradiation, determined by means of biological experiments, differed in several particulars from that obtained by use of the color test. The first showed no disappearance of the Vitamin up to one hour's exposure, thereafter there was a rapid destruction, only 1.5% of the original content remaining after four hours irradiation. The color test, on the other hand, indicated that destruction started at once and proceeded slowly, the curve following that of a bimolecular reaction. Forty-four percent of the original chromogenic value still remained after sixteen hours and thirty percent after thirty-two hours irradiations. This suggested that Vitamin A and the chromogenic substance were separate entities.

Marcus (153) found the storage of the ether-soluble portion of the unsaponifiable fraction of Cod Liver Oil with finely divided solids, such as lactose granulate, ferric sulphate etc., in the presence of either air or carbon dioxide caused a progressive destruction of Vitamin A, often complete in fifteen days. Destruction also occurred when the concentrate

was stored with "nuchar" charcoal from which all traces of air had been removed, showing that in this instance oxidation was not the destructive agent. The presence of hydroquinone or water as ten percent of the total material delayed, but did not prevent, the destruction of the Vitamin.

From the work of Monaghan and Schmitt (158), carotene, the precursor of Vitamin A in the animal body, greatly inhibits the oxygen uptake of linoleic acid. Oxidized carotene, on the other hand, slightly accelerates the oxygen uptake of this acid. Vitamin A in small concentration may completely inhibit the oxygen uptake of linoleic acid for some hours. This inhibition wears off, as in the case of carotene, when the vitamin is destroyed by oxidation. They suggest the possibility that Vitamin A may be concerned with phospholipid metabolism.

Measurements made in a Warburg apparatus by Euler and Ahlstrom (79) showed that the rate of oxygen uptake of a series of fish-liver oils and Vitamin A concentrates increased with increasing "blue value" and growth-promoting power. Liver sections from rats showed a higher rate of oxygen uptake in blood than in Ringer's solution. They suggest that Vitamin A plays an important part in oxidation processes in the body.

Davies (47) in his work with the absorption spectrum observed that at room temperature, Vitamin A deteriorated less rapidly in liver specimens treated with potash than in untreated

tissues kept without preservative treatment. In the case of specimens of liver transferred immediately post mortem to potash solution and then stored at room temperature, no serious decrease in Vitamin A content was to be anticipated if the assay was carried out within fourteen days after death.

The production of high grade feeding oil from pilchards and similar fish was investigated by Brocklesby and Bailey (21). A dry, acid-free pilchard oil stored in a cool dark place and having a minimum access to air will remain unoxidized for a very long period. Samples kept in tightly stoppered glass and tin containers and stored in a dark cupboard for five years showed but a trace of oxidation. An oil with 0.001% maleic acid dissolved in it will resist oxidative rancidity twice as long as an untreated oil.

Gutteridge (100) at the Central Experimental Farm, Ottawa, showed from an examination of the free fatty acid and nitrogen content of several varieties of Cod Liver Oil, that, in general, oils with a high fatty acid content also had a high nitrogen content. Such oils abnormally high in these constituents were either manufactured from stale livers or improperly processed. One sample of Cod Liver Oil was deaminised and compared with the untreated oil by feeding experiments. The deaminised oil was superior to the untreated oil in that it had the effect of equalising the rate of growth and lowering the mortality in growing chicks, while

also rendering more efficient utilisation of food for egg production.

Reti (184) proved the invariable existence of Vitamin A in esterified form in the liver.

According to Miller (155) the Vitamin A potency of Cod Liver Oil in a feed mixture was preserved by mixing the oil with cottonseed meal before incorporating it in the feed.

The chemical and physical constants of Cod Liver Oil was extensively studied by Lindholm (138). Fifteen samples of Cod Liver Oil showed remarkable constancy in color, viscosity, acidity, saponification value, iodine value, unsaponifiable matter, sulphuric acid test of the U. S. Pharmacopoeia, Carr and Price test, in a few samples, in the Vitamin A value, generally as determined spectrographically but in a few samples biologically. The Vitamin A content was easily diminished by oxidation without affecting any of the other constants, but if any of these became seriously altered, the Vitamin A was also found to be completely destroyed.

Colorimetric Reaction

Among the first to use the colorimetric method of assaying the Vitamin A content of Cod Liver Oil were Rosenheim and Drummond (186). They used arsenic trichloride, 1 c.c. to one drop of oil. They found that the oil dissolved rapidly and gave a blue solution which in a few seconds changed to purple and gradually faded. Complete agreement was found to occur between the color intensity and growth-promoting activity as tested biologically.

Drummond, Coward and Hardy (65) demonstrated the sensitivity of color reactions with trichloroacetic acid or dimethyl sulphate, and found that they seemed to be of about the same order as the animal feeding test. They stated, however that the reaction with arsenic trichloride was decidedly more delicate.

Fearon (81) found that phosphorus pentoxide formed a deep violet color on addition to oils containing Vitamin A. Using a 12% solution of trichloroacetic acid in dry light petroleum as a condensing agent, pyrogallol and other polyphenols intereacted with oils containing Vitamin A to give stable pigments which were suitable for colorimetry.

Continuing this work were Willimott, Moore and Wokes (256). They noted that concentrated sulphuric acid and phosphorous pentoxide were less sensitive tests for Vitamin A than were arsenic trichloride or antimony trichloride. They

concluded, "In view of the transient nature of the colors obtained with both these reagents, it is suggested that readings be taken not more than thirty seconds after mixing.

Using the antimony trichloride test Wilson (261) observed that the fatty extract from the human liver gave the same color reaction as Vitamin A found in Cod Liver Oil.

In a study of effect of heat and oxidation on Cod Liver Oil, Wokes and Willimott (263) used four color tests--concentrated sulphuric acid and phosphorus pentoxide being qualitative only and arsenic and antimony trichlorides being quantitative as well as qualitative. They obtained results in agreement with those of workers using animals.

Drummond and Morton (66) measured the color reactions with a Lovibond tintometer and found their results were in agreement with those obtained by Rosenheim and Drummond (186) and also those obtained by the absorption bands. They found that colorimetric analysis was in agreement with the spectroscopic methods and recommended either for giving a rapid, reliable quantitative measurement of the Vitamin A content of Cod Liver Oils.

Wokes (264) conducted a spectroscopic study of the colors produced by the "Vitamin Reagents" (arsenic and antimony trichlorides) on a series of Cod Liver Oils and concentrates whose Vitamin A content had been ascertained by feeding tests. In each case he found two absorption bands which appeared to

be characteristic of the chromogen. Arsenic trichloride gave bands at about 587 and 475 μ and antimony trichloride gave bands at about 614 and 530 μ . He observed that the chromogen on standing in contact with either reagent gradually passed from the stage giving the initial band (at about 587 or 615 μ) to the stage giving the second band (at about 475 or 530 μ). This change was accompanied by a gradual loss in blue color and gain in red color, which can be measured by means of the tintometer. He also stressed the time effect in reading the results as (256) had done previously.

According to Smith and Hazley (221) the unsaponifiable fraction of Cod Liver Oil gives with antimony trichloride in chloroform a blue color proportional to its concentration. The line representing the dilution effect for the total unsaponifiable fraction is tangential at the origin to the dilution curve for the corresponding Cod Liver Oil. They describe a method for carrying out the color test on the unsaponifiable fraction extracted with chloroform.

Emmerie, Eekelen, and Wolff (76) found that by treating a Vitamin A preparation from Cod Liver Oil with drops of furan, methylfuran, pyrrol, indol or skatol prior to use of the antimony trichloride reagent, a purple color in place of the usual blue was given. The 610 μ absorption band was found to be suppressed, but the 572 μ band was unaltered. They conclude that the variation in different specimens of liver

oil may perhaps be due to variations in their content of indol-like substances.

Gillam and Morton (87) observed from their work that liver oils contained two chromogens which with antimony trichloride gave colored substances with absorption maxima at 606μ and 572μ respectively. In concentrates these maxima were displaced to 620μ and 583μ . A comparison of ultra-violet absorption spectra with a spectroscopic data on the color test disclosed, "(1) that the parallelism between the intensity of the 606μ band and the intensity of the 328μ band breaks down so seriously in extreme cases or to render it improbable that the 606μ chromogen is Vitamin A; (11) that the 572μ chromogen and the substance responsible for the 328μ band are probably identical; (111) that the blue color for rich oil and concentrates is often much deeper than would be expected on the basis of correlation between blue color and ultra-violet absorption. "Hence they concluded that the matching of blue colors with Lovibond glasses, though it may act as a rough guide to Vitamin A potency was theoretically unsound.

The ideal conditions for accurate colorimetric determinations are stated and reviewed by Heilbron, Gillam and Morton (109). They observed that in a considerable number of oils characterised in the color test by predominance of the 572μ band over the 606μ band, a large increase in the

intensity of the latter band could be obtained by treating the oil beforehand with ozonised oxygen, hydrogen peroxide or benzoyl peroxide. Oils which initially showed an excess of the 572 μ μ chromogen over the 606 μ μ chromogen underwent a slow spontaneous ageing which resulted in a marked increase in the intensity of the 606 μ μ band. Increases in the 606 μ μ absorption were not at the expense of the 572 μ μ chromogen and were not accompanied by similar increases in the latter, and the absorption at 328 m μ remained practically constant throughout.

Rosenthal and Erdelyi (187) (188) found that a modification of the antimony trichloride color reaction for Vitamin A by the addition of pyrocatechol distinguished between Vitamin A and the known carotenoids. The violet-red color resembling that of a dilute solution of potassium permanganate was more stable than the blue of the Carr-Price reaction.

A short time later these same two investigators (189) noted that a 5% guaiacol solution produced with Vitamin A the same red violet color as 5% catechol. The stability of the color rendered the guaiacol test suitable for quantitative purposes.

Continuing this work Rosenthal and Weltner (190) found that the purplish red color of the antimony trichloride-catechol test for Vitamin A, when examined spectroscopically

within the first ten minutes, showed maxima at 545 and 475 μ , on longer standing two other maxima appeared. When catechol was replaced by guaiacol the purple color remained unchanged for hours, with maxima at 545 and 478 μ .

Anderson and Levine (5 a) observed that on heating the reaction mixture to 60° C. the blue color given by Vitamin A with the antimony trichloride reagent was changed to red, whereas the color given by carotene remained blue. They concluded that the use of pyrocatechol, as recommended by (187), was not necessary to bring about the change of color in the case of Vitamin A.

According to Przydzicka (177) if the mixture is heated in the presence of guaiacol for two minutes on a water bath at 60° C. a rose or red color is produced which is stable and may be compared with a standard solution of suelan three.

Holmes and Bromund (119) found that the orthodox method of matching against potassium dichromate was satisfactory for estimating carotene when dissolved in petroleum ether. Solvents of higher refractive index, such as benzene and chloroform, however, caused a change in the color towards the red end of the spectrum, and accurate matching against a dichromate solution was impossible. Accurate results could be obtained by using solutions of crystalline bixin as a standard.

Absorption Spectrum

The absorption spectra of Vitamin A was observed first by Schultz and Morse (199) and Schultz and Zeigler (200). Shortly after these investigators were Heibron, Kamm and Morton (108).

In 1928 Morton and Heilbron (165) claimed that Vitamin A was characterised by an absorption band with a maximum at 328μ . They also suggested that one of the decomposition products of Vitamin A had an absorption band near 275 to 285μ .

Morton, Heilbron and Thomson (166) found that a crude Cod Liver Oil of high potency gave additional selective absorption between 565 and 585μ in the blue solution.

Dann (45) showed that Vitamin A was more rapidly destroyed than carotene by radiation of wave length 2650 \AA . He definitely denied the possibility that Vitamin A was the end product of the photochemical reaction of carotene with this radiation.

Chevallier and Chabre (32) applied a spectrophotometric method, which permitted a very accurate measurement of the ultra-violet absorption given by a substance to the measurement of intensity of absorption at 3280 \AA of different samples of Cod Liver Oil. The examination of a great number of oils of different origins showed, that, besides the presence of Vitamin A, the free acidity of the oil and in certain cases its pigment (if very concentrated) must be taken into account

in consideration of the absorption in the neighborhood of $3280 \overset{\circ}{\text{A}}$. They concluded that when the free acid content of the oil was low, the results of the physical and biological tests agreed fairly well, the differences not exceeding the usual experimental errors inherent in biological methods.

Bills (17) demonstrated that the spectrograph gave two adjacent spectrograms, and an optical wedge in front of the collimator provides means of revealing directly the intensity of the banded absorption due to Vitamin A.

Macwalter (150) found that the aeration of Cod Liver Oil for a short time reduced its absorption at 328μ but aeration for a long time increased its absorption greatly. He devised a method which uses a curve relating absorption at 328μ to duration of aeration, whereby an accurate measure of the absorption due to Vitamin A may be obtained. When this method was impracticable, the absorption of 328μ of the unsaponifiable fraction of an oil was a truer measure of Vitamin A than the absorption of the oil itself.

In Netevarp's (170) experiments the Hilyer Vitameter A was equipped with a simple photographic device which made it possible to determine E. 328μ with an accuracy approaching that attained by conventional spectrophotometric methods. The values thus obtained for pure and crude Cod Liver Oils, halibut liver oils, concentrates of herring oil, etc., agreed

well with the blue values obtained by the antimony trichloride method, when the latter were calculated according to a special formula. With Cod Liver Oils the absorption of the unsaponifiable matter was 85 to 90% of that of the original oil, the reduction being attributed chiefly to losses in the preparation of the concentrate. He also noted that the value for $E_{1\text{ cm}}^{1\%}$ at 328 μ was reduced from 0.52 to 0.027 in a Cod Liver Oil exposed for six months on a roof in a white glass bottle.

Shrum and How (210) found that the results of biological estimations of Vitamin A in a Cod Liver Oil and in a concentrate, agreed well with the value found by multiplying the extinction coefficients ($E_{1\text{ cm}}^{1\%}$ at 328 μ) by 1600. In two samples of pilchard oil, however, the physical method gave values more than three times the biological values for the oils and 2.5 times those for the unsaponifiable fractions. Removal of the pigment by absorption on diatomaceous larch reduced the absorption only slightly. To measure the absorption due to substances other than Vitamin A, the latter was destroyed by oxidation, and the absorption of the treated material measured. After subtraction of this value from the total for the untreated material, the remainder, due presumably to Vitamin A, yielded a value only 30% greater than the biological value. They believed that the high values obtained for pilchard oil were caused by other substances absorbing in the

region of $328 \mu \mu$. They concluded that the spectroscopic method can be applied to pilchard oil only when the Vitamin A is removed completely from the oil without otherwise modifying or affecting it.

Sample	Vitamin A Content	
	Biological	Physical
Standard Solution Cod	12,500	11,700
Liver Oil	650	620
Pilchard Oil No. 1	120	380
Pilchard Oil No. 2	175	580

The findings of Shrum and How.

According to McFarlane and Rudolph (148), at the University of Alberta, there is need for further investigation of pilchard oil to bring into closer agreement the results of the biological assay and physical measurements of this oil for Vitamin A.

Milne (156) found that spectrophotometric measurements of the Vitamin A content of pilchard oil did not equal the biological value of the oil. It was also found that the carotenoid pigment of this oil was too small to account for the high biological value.

Solvents for Carotene

Dann (43) reported that Vitamin A was rapidly oxidized by air when dissolved in some solvents, and slowly or not at all in others. He found it to be particularly stable in ethyl alcohol, alcoholic potash and ethyl acetate. In ethyl alcohol it was stable toward hydrogen peroxide. The solvent (or impurities associated with the vitamin or solvent) appears to play a leading part in determining the stability of Vitamin A.

McDonald (146) found that carotene dissolved in ethyl butyrate, laurate, or palmitate, or in peanut oil, and stored with access of air, was destroyed to the extent of 80% in four weeks, even at 5°C., while with the same conditions, only 8% was destroyed in Cod Liver, Wesson or Maize oil. Higher temperatures accelerated, and storage in vacuo retarded destruction.

Baumann and Steenbock (8) examined the stability of carotene in a number of solvents, quantitative estimation being made spectrophotometrically by observing the intensity of absorption at 485 and 460 m μ . Refined cottonseed oil (Wesson oil brand) was most satisfactory; cocoanut oil, among others, was less good. The effect of various conditions of storage was studied. It was calculated that when the solutions were kept in the conditions exacted by their use in a feeding test, 95% of carotene was lost after a month when dissolved in olive or

cocoanut oil, but only 26% when cottonseed oil was used. The addition of hydroquinone increased the stability of carotene in certain solvents (ethyl laurate, ethyl sebosate) but not in others.

An elaborate study of the stability of carotene in ethyl esters of fatty acids and in liver and vegetable oils was conducted by McDonald (147). A 0.05% solution of mixed alfa and beta carotene in ethyl butyrate, laurate, and palmitate, in peanut oil, cod liver oil, maize oil and Wesson oil was stored in partly filled stoppered bottles at 37°, 24°, and 5° C and in evacuated sealed tubes at 37°C. and the carotene content observed spectrographically (462 m^μ line) from time to time. The carotene was conserved best in the Wesson and maize oils and worst in the esters.

Dyer and Key (73) found the biological value of the carotene, used as international standard for Vitamin A, was greater in solution of cocoanut or arachis oil than in hardened cottonseed oil or ethyl laurate. Three samples of cocoanut oil, with and without the addition of quinol, gave approximately the same result. Cod liver oil gave a higher biological value for Vitamin A, when diluted with cocoanut oil, than with hardened cottonseed or olive oil.

Vitamin A and Antioxidants

Jones and Christiansen (127) found that hydroquinone in a concentration of 0.03% retarded the absorption of oxygen and the deterioration of the "blue value" of two samples of refined halibut liver oil. It also retarded without wholly preventing the deterioration of the Vitamin A value of one sample as estimated biologically. Maleic acid did not act as an antioxidant in halibut liver oil.

Lecithin and hydroquinone as antioxidants for Vitamin A was studied by Holmes, Corbet and Hartzler (120). The effect of lecithin and of hydroquinone in delaying the oxidation of Vitamin A, as measured by the SbCl_5 reaction, in halibut liver oil and cod liver oil was studied both at room temperature and in an atmosphere of air, and with the aid of specially designed apparatus, at 96°C ., in an atmosphere of oxygen. The antioxidant action of both substances was confirmed under all conditions and at all concentrations. The action of lecithin in supplementing the action of hydroquinone was much greater than would have been expected from its action alone, and the best results were obtained with a combination of both substances. In some instances, and notably with hydroquinone in cod liver oil, there appears to be an optimum concentration for the antioxidant, and less satisfactory results are obtained if this concentration is exceeded. At 96°C . the presence of water slightly retarded the rate of oxidation.

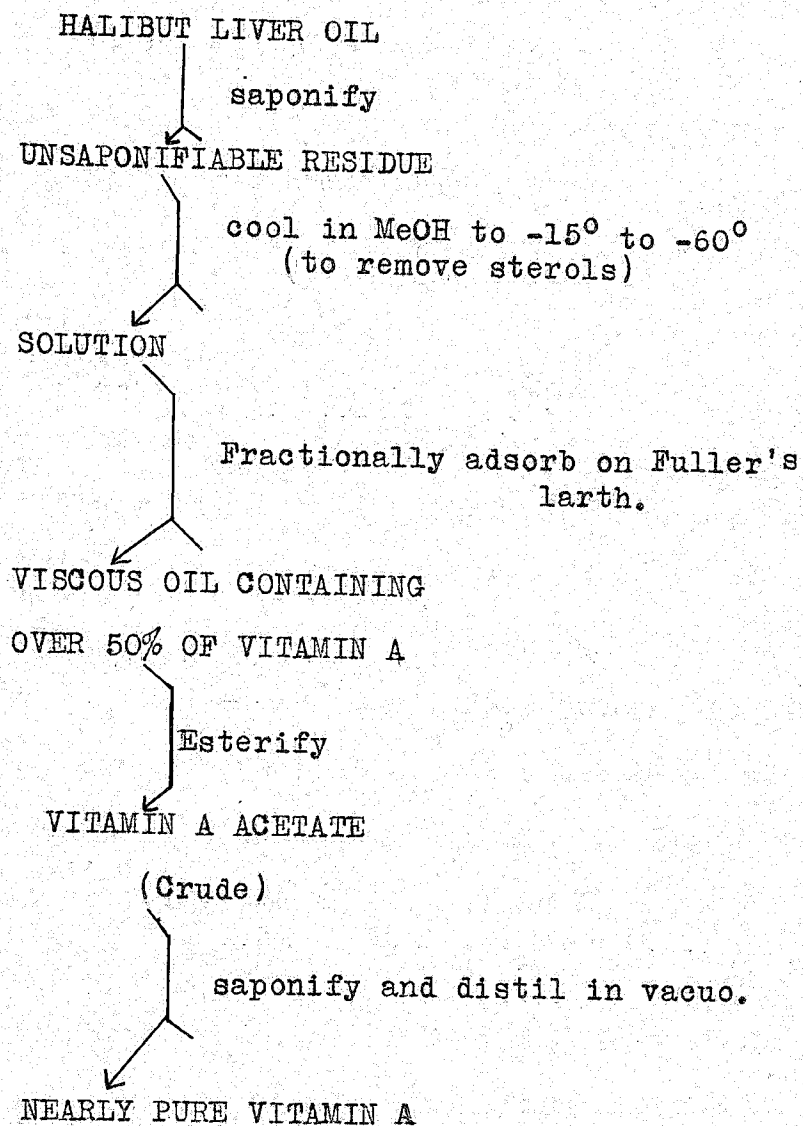
Concentration of Vitamin A

Willimit and Wokes (258), working with spinach, prepared an extract that was two hundred times as concentrated as fresh leaves. This extract, they found, was potent source of Vitamin A, in that 25 mg. daily gave complete freedom from xerophthalmia and induced satisfactory growth.

Moore (162) prepared concentrates from the liver oils of rats and pigs which had received diets containing large amounts of carotene as red palm oil and compared them with concentrates derived from turbot and sol-liver oils. Although the initial blue values of the oils varied widely, little difference could be detected in the activities of the final concentrates, which approached an average value of 2400 B.U. per mg. (Pharmacopoeia color value 45,000) in the Sbcl₁ test, corresponding to a minimal dose of about 0.001 mg. in rat growth experiments. He believed that the concentrates must have been consisted substantially of actual Vitamin A, or alternatively that the vitamin must have been associated with other substances of similar solubility properties in remarkably constant proportion.

The preparation of a potent Vitamin A concentrate was described in 1935 by Holmes et al. (118). They prepared concentrates of halibut liver oil having blue values up to 140,000, and claimed them to be 40% more potent than any other previously prepared samples.

Karrer et al (129) suggest the following process for concentrating Vitamin A.



(10,000 times as active as original material)

The Vitamin A concentrate obtained from the above process represents about one part in two thousand of the original oil. The dose needed to cure a rat is as little as 1/1000 of a milligram (= 1/30,000,000 of an ounce).

The Measurement of Vitamin A

(International Units)

The need for a unit measurement of Vitamin A has been recognized by investigators throughout the world. To avoid confusion the so called "International Unit" has been evolved after several years of intensive investigation. Until recently the Sherman and Munsell unit (206) has been extensively used in the Vitamin A determinations of foods.

Eekelen, Emmerie and Wolff (73 a) in 1934 converted the various "units" used to express the results of Vitamin A estimations by the antimony trichloride method, into International units. Their findings were as follows: 1 Cod Liver Oil unit (Rosenheim and Webster) equals 208 international units, 1 blue value (Drummond and Hilditch) equals 20.8 international units, 1 lovibond unit (Wolff) equals 4.2 international units, 1 blue unit (Moore) equals 0.39 international units. They also showed that by similar calculations an oil having $\frac{1}{1}$ percent $\frac{1}{\text{cm}}$. 328 u u equals 1 is assumed to contain 5 Cod Liver Oil units, 50 blue values, 250 lovibond units (Wolff), 2750 blue units (Moore) and 625 gamma Vitamin A or 1042 international units per gram.

The first International Conference for the Standardisation of Vitamins was held in 1931 (183) in London England. A provisional unit of Vitamin A was established in terms of the

biological activity of a sample of carotene. The second Conference was held in 1934 at which a solution of pure beta carotene was adopted as the permanent standard in terms of which units of Vitamin A were in future to be expressed (154).

The Pharmacopoeia of the United States has evolved a standard procedure for the biological testing of Vitamins A and D. Their standards are Reference Cod Liver Oils of known standard potency. See Appendix No. 1 for the procedure.

The above two standards are for the use of rats. Up to the present there is no standard procedure for Vitamin A determinations with chicks. Many investigators have used chicks as a test animal but all express their results differently and therefore it is very difficult to compare the results of one investigator with another. A standard procedure using chicks as the test animal is needed in order that the findings of different investigators may be comparable.

The definition of a unit of Vitamin A as outlined by the "Permanent Standards Commission of the Health Organization of the League of Nations" is as follows:

(a) International Standard

The Conference recommends that pure B--carotene be adopted as the International Standard for Vitamin A. The Standard Preparation shall conform to the requirements stated in Note 1 in regard to its chemical and physical constants.

(b) Definition of Unit

The Conference recommends that the unit for Vitamin A provisionally adopted at the 1931 Conference shall be maintained. It has been established that one such unit is contained in 0.6 microgram (0.6 gamma) of pure B--carotene.

The International Unit for Vitamin A recommended for adoption shall be defined as the Vitamin A activity of 0.6 microgram (0.6 gamma) of the International Standard Preparation.

Daily doses of two to four International Units of Vitamin A, when administered to young rats suitably prepared on a Vitamin A deficient diet, have been found adequate to restore growth; somewhat larger doses are required for the cure of xerophthalmia.

(c) Mode of Preparation

It is recommended that the Health Organization of the League of Nations shall be requested to obtain a sample of B--carotene as defined by the Conference (see Note 1), and that the National Institute for Medical Research, London, acting for this purpose as central laboratory on behalf of the Health Organization of the League of Nations, shall undertake the care, storage and distribution of the International Vitamin A Standard so obtained.

(d) Mode of Distribution

The Conference recommends that the International Standard

Preparation shall be issued in the form of a standard solution in oil, the strength of the solution being such that 1 gramme contains 500 International Units, or 300 micrograms (300 gamma) of B--carotene.

(e) Adoption of Subsidiary Standard of Reference

The Conference recommends that a sample of Cod Liver Oil, the potency of which has been accurately determined in terms of the International Standard Preparation of B--carotene, shall be provided as a Subsidiary Standard of Reference.

In view of the fact that the Reference Cod Liver Oil of the United States Pharmacopoeia, which has been accurately assayed in terms of the provisional International Standard adopted in 1931, has been in effective use in the United States of America for some time, the Conference recommends that the Board of Trustees of the United States Pharmacopoeia be approached and invited to place a quantity of their Reference Cod Liver Oil at the disposal of the Health Organization of the League of Nations, with a view to its adoption for international use as a Subsidiary Standard for Vitamin A.

In the event of the Reference Cod Liver Oil of the United States Pharmacopoeia not being available for international adoption, the Conference recommends that another sample of Cod Liver Oil be selected, its potency in terms of the International Standard Preparation of B--carotene accurately determined by biological comparison and independently by

spectrophotometric measurements, and that this selected sample be then adopted as a Subsidiary International Standard for Vitamin A.

VITAMIN A IN POULTRYSymptoms and Lesions Due to Lack of Vitamin A

Beach (12) in 1924, was the first investigator to describe avitaminosis A in poultry. He noted that the characteristic symptoms of this disease were confined to the head and involving the nasal passages, the mouth, pharynx, esophagus and the eyes. According to his early work avitaminosis A was associated with the following symptopathology:

"A discharge from the nostrils, of a watery or viscid fluid is nearly always present. Later this may collect in the infraorbital sinuses, become transformed into a caseous mass and cause a swelling of the face.

"The lesions in the mouth, pharynx, esophagus and crop consist of collections of white caseous material in the mucous glands.

"The lesions in the eye consist of an ophthalmia which produces puffiness of the eyelids, reddening of the conjunctiva, a profuse watery secretion which soon becomes viscid and may glue the eyelids together.

"The kidneys are usually pale and marked by a network of fine white lines which are urate-filled tubules. Occasionally there is a deposit of urates on the heart, pericardium, liver, omentum and intestines. In some cases the uraters are greatly distended with urates.

"The possibility that it is Vitamin D rather than

Vitamin A in the cod liver oil that prevents the development of this disease would seem to be controverted by the following:

1. "The similarity of the ophthalmia to that occurring in rats fed a ration deficient in Vitamin A and the total lack of any symptom of rickets suggestive of Vitamin D deficiency.

2. "Exposure of the fowls to an abundance of direct sunlight did not prevent the development of this disease, although it did prevent rickets.

3. "It would seem, therefore, that this disease which the writer has previously designated as a nutritional disease resembling roup should now be designated a nutritional disease caused by Vitamin A deficiency although the name nutritional roup might be more suitable for use by the poultrymen".

In 1930 Seifried (203) described the essential histopathological changes in the respiratory tract of chickens caused by avitaminosis A. He said, "There is first an atrophy and degeneration of the lining mucous membrane epithelium as well as of the epithelium of the mucous membrane glands. This process is followed or accompanied by a replacement or substitution of the degenerating original epithelium of these parts by a squamous stratified keratinizing epithelium. This newly formed epithelium develops from the primitive columnar epithelium and divides and grows very rapidly. The process appears to be one of substitution rather than a metaplasia, and resembles the normal keratinization of the skin or even more

closely the incomplete keratinization of the mucous membranes. All parts of the respiratory tract are about equally involved in the process, and the olfactory region as well, so that the sense of smell may be lost. The lesions, which first take place on the surface epithelium and then in the glands, show only minor differences ".

"The protective mechanism inherent in the mucous membranes of the entire respiratory tract is seriously damaged or even entirely destroyed by the degeneration of the ciliated cells at the surface and the lack of secretion with bactericidal properties. Secondary infections are frequently found, and nasal discharge and various kinds of inflammatory processes are common, including purulent ones, especially in the upper respiratory tract, communicating sinuses, eyes and trachea. The development of the characteristic histological process is not dependent upon the presence of these infections, since it also takes place in the absence of infection. The specific histological lesions make it possible to differentiate between A avitaminosis and some infectious diseases of the respiratory tract".

Continuing this work further Seifried (204) said "When fowls are placed on a diet lacking in Vitamin A lesions appear in the upper alimentary tract which are confined largely to the mucous glands and their ducts. Histologically it is shown that the original epithelium becomes replaced by a stratified

squamous keratinizing epithelium and that secondary infections are relatively common. The ducts of the glands may be blocked leading to distention with secretions and neurotic materials. These lesions macroscopically resemble very closely certain stages of fowl pox and the two conditions can be separated only by histological examination. These lesions produced by a lack of Vitamin A may enable bacteria and other viruses to enter the body".

Ackert, McIlvaine and Crawford (2) found that the resistance of growing chickens to the intestinal roundworm, *Ascaridia Lineata*, was lowered when the fowls, four to seven weeks of age, were kept on a diet deficient in Vitamin A. The larger number of the roundworms remaining in the chickens on the Vitamin A deficient diet were attributed in part to evidences of weakened peristalsis, thus making it less difficult for the young worms to withstand the rigors of peristalsis.

According to investigations conducted at the University of Idaho, (251) the symptoms and lesions of chicks fed a Vitamin A free ration are as follows:

"A characteristic wobbly gait, sore eyes, swelling under the throat, excessive mucous in the mouth, ruffled feathers, general inactivity and inability to maintain normal equilibrium, and extreme paleness of skin, beak, and shanks. The internal lesions observed consisted of extreme paleness of the kidneys, followed by a characteristic white network of urate deposit in

the kidney tissue, enlargement of the ureters with an accumulation of urates, and enlargement of the gall bladder and proventriculus. A white deposit often appeared over the surface of the internal organs and walls of the body cavity. A gelatinous substance was frequently found around the heart or over the breast muscles. In those chicks which survived to or beyond six weeks of age, typical white abscesses (pustules) developed on the mucous membrane of the throat, and cankerous growths appeared in the mouth. In some instances the small intestines exhibited extreme inflammation, with definite hemorrhagic spots. As previously stated, these lesions did not appear uniformly in all the chicks. The urate deposits in the kidneys and the enlargement of the ureters, gall bladder, and proventriculus, were the lesions which appeared more consistently. The above symptoms occur in chicks at about four weeks of age and become more severe before the chicks finally die".

Hinshaw and Lloyd (114) report that poults fed a Vitamin A deficient ration from the time of hatching developed symptoms of avitaminosis A in twenty-five days. Chicks kept as penmates to the poults showed similar symptoms in twenty-seven days. The disease was much more acute among the poults than among the chicks. Lesions in the poults were confined to mucous membranes of the head, the upper digestive tract, the respiratory tract and the Bursa of Fabricus. Deposits of urates in the kidneys and the ureters seldom occurred in the

poults. They also found that turkeys required a ration containing eight percent of dehydrated alfalfa leaf meal (containing approximately 130 gamma of carotene per gram) for normal growth to thirty weeks of age. Chicks kept as penmates to the turkeys made normal gains and showed no evidence of avitaminosis A on a level of four percent alfalfa meal. From their investigation they prepared tables showing the distribution of lesions in the turkeys and the chickens.

Seifried (205) in later work showed that chicks developed the first macroscopic epithelial changes after thirty-three days on a diet deficient in Vitamin A.

Miller and Bearse (155 a) noted that the first symptom observed in chicks in a Vitamin A deficient diet was a staggering gait. This condition became progressively worse until death occurred. Staggering gait, unthrifty appearance, and soiled fluff were the only external symptoms of deficiency in chicks dying early in the trial. Those dying later frequently showed a watery eye condition which was often accompanied by pus. They also found that the post mortem examination of chicks on a Vitamin A deficient diet revealed enlarged gall bladders, enlarged gray kidneys (due to accumulation of urates), ureters distended with urates, enlarged proventriculi and gray hearts. Gullet lesions were observed in only a few birds.

Requirements of Chicks For Growth

Sugiura and Benedict (240) in 1923 studied synthetic diets for the nutrition of pigeons. In their work they used two different rations and obtained normal growth and reproduction. No. 1 diet consisted of casein 22, cane sugar 10, starch 27, agar 2, salt mixture 3, butter fat 30 and yeast 6%. No. 2 diet had the starch increased to 37% and the butter fat replaced by lard 20%. They concluded from their work, "Pigeons on a diet of sufficient caloric value, even though the diet lacks fat and fat soluble vitamins, may maintain excellent condition, and may produce fertile eggs and rear healthy squabs. Hence fat-soluble vitamin is not essential in any stage of avian nutrition".

A few months later Emmett and Peacock (78) showed that young chicks require the fat soluble Vitamin A. They noted that in the absence of Vitamin A, the onset of the symptoms of ophthalmia appeared and unless the diet was properly reinforced, or an oral treatment rich in the Vitamin was given, death eventually ensued. They claimed that this eye condition resulting from the lack of Vitamin A was the same as the poultry nutritional rump described by Beach (12.). The presence of urates in the ureters, kidneys and at times on the surface of the heart, liver and spleen were observed and believed related to the deficiency of the fat soluble Vitamin A. They concluded that, "Young mature pigeons require very little if

any, fat soluble A. Vitamin A does however play a role in the nutrition of some species of avian".

The following year, Hart, Steenbock, Lephovsky and Haplin, (102) agreed with Emmett and Peacock (78) and disagreed with Suyiura and Benedict (240).

Hauge, Canick and Prange (105) found that when they used 25% of yellow corn in their basal ration, the fat soluble A requirements of growing chicks were met for the first ten weeks of their lives. On the other hand 50% of yellow corn met the requirements for the development of pullets, up to the laying age. Chicks fed on rations deficient in fat soluble A usually reflected such a deficiency in their growth responses at about four weeks of age.

Russell and Weber (192) investigated the role played by plant pigments in the nutrition of chickens. Four groups of chicks were placed on a Vitamin A deficient diet. After four weeks on this diet alone, daily supplements of 0.03 mg. of carotene, xanthophyll and chlorophyll were fed to the chicks in each of three of the four groups respectively, the fourth serving as controls. The group receiving the carotene thrived, but the chicks in the control group and in the two groups receiving the other pigments failed and died in two weeks. The uric acid in the blood of the Vitamin A deficient chicks was much increased but starving chicks yielded similar values.

The conversion of carotene into Vitamin A was definitely proven by Capper, McKibben and Prentice (31). They also observed that the beaks and shanks of chickens, which had become colorless through the absence of carotenoids from the diet, did not become more yellow when carotene was added to the ration. They showed that the Vitamin A content of hen liver oils was very high and that the Vitamin A requirements of fowl were large.

Elvenjem and New (75) used chicks instead of rats in their study of avitaminosis A. They evolved a basal diet consisting of : 58 parts ground white corn, 25 parts wheat middlings, 12 parts crude casein, 1 part common salt, 1 part precipitated calcium carbonate, 1 part precipitated calcium phosphate, 2 parts dried yeast.

They observed that the chicks grew normally to three weeks when they began to exhibit general incoordination, became drowsy and crouched on their haunches. The feathers were very ruffled and there was some soreness around the eyes, but there was no typical ophthalmia. The beaks and shanks became colorless and most of the birds were dead by the end of the fifth week. They also found that the uric acid content of the blood of normal chicks was approximately 5 mg. per 100 c.c. of whole blood, while that of Vitamin A deficient chicks went as high as 44 mg. per 100 c.c. of blood. The amount of uric acid in the blood was independent of the protein intake.

Vitamin A deficiency did not disturb the uric acid metabolism but injured the structure of the kidney sufficiently to prevent normal elimination of uric acid. The amount of uric acid in the blood was dependent upon the degree of kidney damage. The degree of incoordination, however, was independent of the uric acid content of the blood.

Kline, Schultze and Hart (132) found that Xanthophyll, m.p. 174⁰ prepared from spinach did not serve as a source of Vitamin A for the chick. There were, however no toxic effects, even when it was fed at levels of 0.25 mg. daily per chick. Carotene, m.p. 172.5⁰ prepared from spinach, when fed in adequate amounts served as a source of Vitamin A for chicks. When the chicks reached the age of seven to eight weeks, 0.03 mg. of carotene daily were not sufficient when it was the sole source of Vitamin A. Chicks that had been depleted of Vitamin A required more than 0.05 mg. of carotene daily in order to grow to maturity.

In determining the minimum amount of yellow corn necessary in a growing ration Smith (224) used a basal ration which contained: 35 pounds ground corn meal, 5 pounds oat meal, 20 pounds wheat middlings, 7 pounds meat scrap, 3 pounds fish meal, 5 pounds dried skim milk, 1½ pounds oyster shell, ½ pound salt.

When white corn was used in the basal ration one hundred percent mortality resulted by the end of the eighth

week. Normal growth and viability resulted when twenty-five percent and forty-five and one half percent of the total ration was made up of yellow corn. He concluded that the minimum amount of yellow corn required in a growing ration lay between twelve percent and twenty-five percent when other sources of Vitamin A were lacking.

The Idaho Agricultural Experiment Station (251) used the following basal ration for their work on avitaminosis A: 43 pounds ground wheat, 15 pounds ground oats, 20 pounds bran, 10 pounds dried milk, 7 pounds meat and bone meal, 4 pounds oyster shell, 1 pound salt. Vitamin D supplied by irradiation.

They observed that the first indication of Vitamin A deficiency occurred at about three weeks. A high death rate resulted after four weeks up to one hundred percent by eight weeks.

Studying avitaminosis A in turkeys Hinshaw and Lloyd (114) fed the following ration: 25 pounds white corn, 25 pounds barley, 25 pounds ground wheat, 10 pounds fish scrap, 10 pounds dried milk, 3 pounds bone meal, 2 pounds ground limestone and $\frac{1}{2}$ pound salt. They kept chicks as penmates to the poults and their observations are discussed later under pathology.

Gutteridge (99) found that the addition of pilchard oil or Cod Liver Oil to a ration otherwise deficient in Vitamin A, increased growth and prevented the development of deficiency symptoms in chicks. He also observed that neither pilchard oil

nor Cod Liver Oil, when fed with a Vitamin A deficient ration at levels of one percent and two percent of the total feed consumed brought about as rapid growth as was attained by feeding a well balanced ration. In conclusion he claimed that pilchard oil and Cod Liver Oil were of equal value in so far as Vitamin A content is concerned, and that pilchard oil was slightly more efficient in this respect.

In a study of the carotene and Vitamin A requirements for white leghorn chicks Frohring and Wyeno (86) used the following ration: $52\frac{1}{2}$ pounds ground white corn, 10 pounds wheat bran, 15 pounds wheat middlings, 10 pounds meat scrap, 10 pounds skimmed milk powder, 2 pounds calcium carbonate, $\frac{1}{2}$ pound sodium chloride. 100 A.D.M.A. units of Vitamin D per chick per day.

They found that the minimum requirements of Vitamin A for a chick at the age of about eight weeks was approximately 65 A.D.M.A. units per day. Practically all chicks depleted in Vitamin A showed marked ataxia three to fourteen days before complete depletion and death, even though given an adequate amount of Vitamin D. There was a wide variation in the number of days chicks lived on the Vitamin A free diet, no doubt due to variation in storage from the egg from which the chicks hatched, which in turn was dependent on the storage or ration of the parent fowl. The addition of carotene or Vitamin A to the carotene, and Vitamin A free ration delayed the appearance

of deficiency symptoms and prolonged life in a rather definite relation to the amount of carotene or Vitamin A added to the diet.

Sherwood and Fraps (214) fed rations containing 50, 100, 150 and 300 Sherman-Munsell (206) units per 100 grams of feed to chicks hatched from eggs produced by hens receiving rations containing 310, 440 and 560 units of Vitamin A per 100 grams of feed. During the first few weeks of the experiment the mortality of the chicks from the hens on the lowest Vitamin A level was so high that the different levels of Vitamin A in the chick feeds studied did not show the results on chick mortality that they did on the chicks from the hens on the higher levels. The mortality was lower in the case of the chicks from the hens receiving 440 and 560 units of Vitamin A per 100 grams of feed as the amount of Vitamin A in the ration increased. No advantage was shown in the weight of the chicks for the 300 units over the 150 units per 100 gms., in the chick rations.

In another study with chicks hatched from hens receiving an adequate supply of Vitamin A these same two investigators found that the mortality in twelve weeks was as follows: insignificant amount of Vitamin A, 100% mortality; 42 units per 100 gms. 41%; 84 units per 100 gms. 15%; and 126 units per 100 gms. 12%. The percentage of healthy chicks remaining at the close of the experiment was 0, 24, 72, 83 for the respected lots.

Record, Bethe and Wilder (181) used a ration consisting of: 58 pounds white corn, 25 pounds wheat middlings, 12 pounds domestic casein, $1\frac{1}{2}$ pounds dried yeast, $\frac{1}{2}$ pound irradiated yeast (200 D), 1 pound steamed bone meal, 1 pound ground lime stone, 1 pound salt.

They found in prophylactic trials that from fifty to one hundred gamma of carotene per 100 gms. of feed were required to produce normal growth and prevent symptoms of Vitamin A deficiency during the first eight weeks of a chicks' life. In curative trials chicks were fed the basal ration for twenty-six days, at which time a large percentage of the birds showed Vitamin A symptoms. They then divided the chicks into groups and fed different amounts of carotene or Cod Liver Oil and found that it required approximately fifty gamma of carotene or 60 to 100 international units daily of Cod Liver Oil to produce normal chicks for seven or nine weeks of supplemental feeding.

In 1935, Schroeder, Higgins and Wilson (202) reported that chicks up to nine weeks of age required 6000 international units of Vitamin A per pound of feed (about 1320 units per 100 gm) to prevent clinical and pathological symptoms of hypovitaminosis A. They also reported that 1200 units of Vitamin A per pound of feed appeared to be adequate to promote fair growth; but that there was a tendency for body weights to increase as the Vitamin A level was increased. The following

year these same investigators (262) used the same basal ration and found 1200 international units of Vitamin A per pound of feed were adequate to promote satisfactory growth. They suggested that their previous high requirements were due to the severe mixed infection that occurred amongst the experimental birds. They further noted that the storage of Vitamin A was cumulative and bore a marked relationship to the amount of the factor in the diet. Unit for unit, carotene and Vitamin A obtained from a fish oil concentrate were found to be utilized by the chick with equal efficiency.

In a study of the Vitamin A storage of growing chicks, Holmes, Tripp and Campbell (116) used the following ration: 32 pounds corn-meal attrition, 15 pounds wheat bran, 15 pounds wheat flour middlings, 12 pounds ground oat groats, 8 pounds dry skim milk, 5 pounds alfalfa leaf meal, 5 pounds meat scraps, 5 pounds fish meal, $\frac{1}{2}$ pound di calcium phosphate, 1 pound oyster shell meal, 1 pound salt.

When 0.5% sardine oil was added to the above basal ration and fed to young chicks the livers of these chicks contained significantly more Vitamin A than when 0.25% was added. The amount stored was approximately four times more for the higher level. The Vitamin A reserve in the liver increased in the period from eight to twelve weeks of age. Older birds showed wide variations both in egg producing power and in the Vitamin A content of their livers.

Working at Cornell, Ringrose and Norris (185) used the following ration: 55 pounds white corn-meal, 25 pounds wheat flour middlings, 10 pounds commercial casein, 5 pounds dried yeast, $2\frac{1}{2}$ pounds pulverized limestone, 1 pound steamed bone meal, 1 pound cottonseed oil, $\frac{1}{2}$ pound iodized salt. Vitamin D was supplied by irradiation.

They established the growth response curve for the chick to Vitamin A feeding, a biological assay expressing results in terms of Vitamin A per gram of material thus became possible. The assay was conducted by feeding the test material at such a percentage of the ration as to induce a growth response which was slightly, but definitely, subnormal. The growth results obtained at eight weeks of age were then applied to the growth response curve and the units of Vitamin A per 100 gms. of feed determined. By dividing the number of units per 100 gms. of feed by the percent of the test material in the ration, the potency of the test material in units per gm, was obtained. They found that the minimum Vitamin A requirement of the chick during the first eight weeks of life was about 150 U.S.P.X. Revised 1934 units per 100 gms. of feed.

Holmes, Tripp and Campbell (117) obtained embryos and young chicks from different sources and determined the Vitamin A stores in their livers and unabsorbed egg yolks by means of the antimony trichloride reaction. From the eighteenth day of incubation to the fifth day after hatching the Vitamin A

content of the liver rose and that of the unabsorbed yolk fell, the combined total from both sources falling slowly.

At the University of British Columbia Biely and Chalmers (16) fed chicks the diet of Elvehjem and Neu (75) to which irradiated yeast was added. Once a week a dose of the U.S. Pharmacopoeia Reference Cod Liver Oil was fed directly into the crop of each bird in an amount to supply 0, 25, 50, 75 and 100 international units of Vitamin A daily to the various groups. The group receiving no oil showed symptoms of Vitamin A deficiency after three weeks; these became marked at five weeks and after eight weeks all the birds were dead. Of the remainder those receiving 25 international units daily, began to lag in growth and to show some symptoms after four weeks. The remainder grew well and showed no symptoms. They concluded that 75 international units daily were sufficient to ensure normal growth and protect chicks up to eight weeks against any symptoms of Vitamin A deficiency, 100 units appeared to be above the immediate normal requirements of the chicks up to this age. When yellow corn was substituted for the white corn (59%) in the ration with the omission of Cod Liver Oil, they found approximately the same performance as the dose of 75 international units daily had given.

Record, Bethke and Wilder (182) used the basal diet of Elvehjem and Neu (75) in which irradiated yeast supplied 4,000 international units of Vitamin D per 100 grams of feed. They

conducted both prophylactic type and curative type of feeding. The prophylactic trials showed that, under the experimental conditions employed, it required a minimum of approximately 50 to 100 micrograms of carotene or 80 to 160 international units of Vitamin A from Cod Liver Oil per 100 gms. of ration for normal growth and the prevention of external and internal symptoms of Vitamin A deficiency in chicks to about eight weeks of age. The curative experiments showed that about 100 micrograms of carotene or 120 to 200 international units of Vitamin A from Cod Liver Oil were required every other day to cure and prevent symptoms of Vitamin A deficiency and restore good growth in chicks depleted of their Vitamin A reserves to ten to twelve weeks of age. Both types of experiments showed that the chick utilized carotene as a source of Vitamin A. The response of the chicks to carotene or Vitamin A was approximately similar when equivalent rat units were fed; indicating that the chick and rat utilize carotene as a source of Vitamin A in the same order. They presented data to show that the Vitamin A requirements of chicks increased with age. In conclusion they pointed out that these experiments showed no significant storage of Vitamin A in the livers of the chicks until several times the minimum level, as determined by growth and external and internal symptoms was fed.

At the Western Washington Experiment Station Bearse and Miller (13) used a basal ration consisting of: $44\frac{1}{2}$ pounds

ground white corn, 15 pounds ground wheat, 15 pounds ground oats, 10 pounds millrun, 7 pounds meat scrap, 7 pounds powdered skim milk, 1 pound oyster shell flour, $\frac{1}{2}$ pound salt. Vitamin D was supplied by irradiation.

They supplemented this ration with varying levels of dehydrated alfalfa for a twenty-four week growing period. These levels furnished approximately 87.5, 175, 350, 700 and 1400 Sherman-Munsell (206) Vitamin A units per 100 gms. of ration. The results based on avitaminosis A mortality, growth and liver storage of Vitamin A showed that 175 Sherman-Munsell Vitamin A units per 100 gm. of ration met the Vitamin A requirements of the chicks. Feeding efficiency was greater in the lots receiving the highest levels of Vitamin A during the first eight weeks of the trial. This difference was not so great for the entire twenty-four week period. There was a close correlation between mature body weight and total feed consumption.

In summarizing the findings of many investigators Dr. Parkhurst (174 a) stated: "When feeds are mixed previous to the feeding period, the minimum Vitamin A requirements of chickens to eight weeks of age would appear to be in the vicinity of 150 U.S.P. Vitamin A units per 100 grams of feed (680 units per pound) and the practical requirement for satisfactory growth and liver storage about 300 units per 100 grams

of feed (1362 units per pound. For chickens to thirty weeks of age, the requirement may be double. Turkeys require at least double the amount of Vitamin A in their ration as do chickens".

Requirements of Hens For Egg Production

Bethke, Kennard and Sassaman (15) reported in 1927 that the fat soluble vitamin content of hen's yolk was greatly influenced by the amount of these substances present in the ration and by environment of the laying hen. The yolks of eggs laid by hens which had access to a blue-grass range were approximately five times as potent in Vitamin A, and ten times as active antirachitically as the yolks of eggs laid by hens which received the same basal diet but were confined indoors. When two parts of Cod Liver Oil were added to the mash there was a five fold increase in the antirachitic and fat soluble A vitamin content of the egg yolks.

Holmes, Doolittle and Moore (115) found that the addition of fat soluble vitamins to the poultry rations definitely stimulated egg production. The average weight of eggs, produced by the oil fed birds was slightly greater than that of the eggs produced by the control birds. In this experiment they observed that the percentage of eggs containing blood clots decreased consistently with the increase of Cod Liver Oil in the experimental ration. The number of eggs discarded during incubation either from being infertile or on account of containing a weak germ was less for the oil fed group. The number of chicks obtained and the viability of these chicks was greater in the birds fed the supplement. Contrary to

previously observed conditions, the body weight of the high producing birds did not decrease but their weight at the end of the experiment exceeded that of the controls. As the experiment progressed the fat soluble vitamin potency of the eggs from the experimental birds increased while those from the controls decreased. No detectable flavor was imparted to either the eggs or the flesh of the birds fed the Cod Liver Oil. They definitely concluded that supplementary fat soluble vitamin feeding increased the reproductive performance of domestic fowl.

From the work of Sherwood and Fraps (211) in 1932, it was found that yellow corn alone was not sufficiently high to supply enough Vitamin A to pullets for egg production. They estimated that pullets required for maintenance alone, about 105 units of Vitamin A potency per day, or 33 units per pound per day, (this is eight times the estimate of 4 units per day per pound for maintenance of growing rats). Ordinary alfalfa was found not to supply enough Vitamin A to produce eggs of high potency in this vitamin, even if fed at 8% of the mash. They calculated that one unit of Vitamin A in the egg required 6.3 units in the feed at 270 units, 5.7 units at 336 units, and 4.0 units at 444 units in the feed.

The following year Sherwood and Fraps (212) published further data on the subject. In this experiment three rations were used:

No. 1

20 pounds yellow corn
 20 " wheat shorts
 20 " wheat bran
 20 " ground oats
 20 " meat and bone scrap

No. 2

50 pounds yellow corn
 50 " white corn

No. 3

All White Corn

These rations were fed to groups of pullets that had been normally fed. At the close of the experiment the yellow corn group (No. 1) averaged 25% more in weight than the white corn group (No. 3) and the mixed corn group (No. 2) weighed 17% more than No. 3. These rations had a definite effect on the number of eggs laid after the experiment had been in progress two months. During the last four months of the experiment the yellow corn group laid approximately 65% more eggs than did the white corn group and the mixed corn group laid 35% more eggs than did the white corn group. The Vitamin A content of the eggs showed definitely, as the experiment advanced, that none of the rations contained enough Vitamin A for high egg production. The Vitamin A content of the eggs from all of the groups became lower as the experiment progressed. They concluded that it required approximately 1,365 units of Vitamin A (Sherman-Munsell units) per day for a laying pullet for maintenance and production of eggs at the rate of twenty eggs per month.

Russell (193) also observed that the Vitamin A content of eggs from hens receiving yellow corn was greater than those from hens receiving white corn. He found that 47 to 48% of the Vitamin A fed was contained in the eggs.

In 1934 Sherwood and Fraps (213) stated, "The percentage of vitamin recovered in the eggs is greatest at the lowest level and least at the highest level of feeding. This apparent percentage recovery is not correct because the Vitamin A stored by the hen is not taken into account and some of the Vitamin A or carotene fed is used for maintenance while some of it is probably not digested". They found that on the average 1 unit of Vitamin A in the egg required 4.7 units in the feed. The units of Vitamin A required in the feed for 1 unit in the eggs decreased as the average number of units fed increases.

Bisbey, Appleby, Weis and Cover (20) conducted an investigation to correlate the Vitamin A activity of egg yolks with the amounts of carotinoid pigments they contained. They observed that there was a distinct gradation of the color in the egg yolks from the hens on the different rations and that the color of the yolks on a given ration was uniform. They concluded that while there seemed to be some relationship between color and growth, the Vitamin A activity of egg yolks could not be explained on the basis of the carotinoid pigments they contained. They also noted that the Vitamin A

activity of the egg yolks was directly dependent upon the rations of the hens.

Mohler (157) claimed that the optimum level of Cod Liver Oil feeding in the diet of chickens was between 1 and 2%. If this level was exceeded, impairment in egg production and hatchability was likely to occur.

The work of DeVaney, Litus and Nestler (51) showed that extra Vitamin D, as 0.5% viosterol (160 D), in the diet of chickens receiving graded amounts of Cod Liver Oil had no effect on the transfer of Vitamin A to the egg. Eggs from pullets receiving 8% of Cod Liver Oil were several times richer in Vitamin A than those from pullets receiving lower doses.

Russell and Taylor (194) determined the Vitamin A content of the egg yolk and the diet by the method of Sherman and Munsell with a reference oil as a standard. The results were expressed in U.S.P. 1934 (international) units by reference to a curve of response. They found that with varying egg production and intake of Vitamin A, the output of Vitamin A in the eggs, expressed as a percentage of intake, varied from 11 to 32%.

In 1935 Sherwood and Fraps (215) estimated that hens laying per year one hundred and fifty eggs, high in Vitamin A potency required approximately 600 Sherman-Munsell biological units of Vitamin A per day, or 7.5 units per gram of feed.

Koenig, Kramer and Payne (133) found that the eggs of pullets laid in the winter about the fourth month of egg production, and tested by the method of Sherman and Munsell, contained about 25 Sherman-Munsell units of Vitamin A per gram, whether the birds were of high or low productivity. Autumn eggs produced near the end of the first year contained 20 and 33 Sherman-Munsell units per gram, when laid by birds of high and low productivity, respectively. They conclude that egg production made pronounced nutritive demands upon the hen and that the recognized demand for Vitamin A was more pronounced the greater the number of eggs produced and the longer the laying period.

Working at the Western Washington Experiment Station, Bearse and Miller (14) found that eggs from hens receiving different quantities of Vitamin A in their rations contained different quantities of Vitamin A in their egg yolks in proportion to the amount of Vitamin in the ration. Chicks hatched from such eggs lived and grew on Vitamin A deficient ration in proportion to the amount of Vitamin A in the breeding hen ration. They concluded that 500 Sherman-Munsell units of Vitamin A per 100 gm. of feed in the breeding hen ration supplied sufficient Vitamin A for maximum hatchability.

EXPERIMENTALExperiment No. 1.

Preliminary experiments conducted at The University of British Columbia by Wood (266) indicated that Pilchard Oil, as produced in British Columbia, contained an appreciable amount of Vitamin A. In fact, Wood's experiments showed that one-half on one percent of a sample of commercial Pilchard Oil when fed in combination with a basal ration free from Vitamin A, promoted normal growth and development of chicks to eight weeks of age.

The purpose of the present experiment was to determine the Vitamin A potency of a sample of commercial Pilchard Oil in terms of International Units, using the chick as the test animal. The results of this test were compared with those of Biely and Chalmers (16), who determined the Vitamin A requirements of growing chicks up to eight weeks of age by feeding them graded doses of Reference Cod Liver Oil (251a).

The method followed by Biely and Chalmers was to feed 0, 25, 50, 75, and 100 units of the Reference Cod Liver Oil once a week with a pipette per orem. The chicks in the experiment described herein, were fed their Vitamin A supplement in the mash, but were brooded under similar conditions. Consequently, the data may be considered as comparable.

Experimental Methods:

Day-old single comb White Leghorn cockerels were

placed in battery brooders under the same conditions as described by Biely and Chalmers (16). These cockerels had been separated from the pullets by the Japanese method of "chick sexing". The basal ration fed to the chicks consisted of 59 pounds ground white corn, 25 pounds wheat middlings, 12 pounds crude casein, 1 pound calcium carbonate, 1 pound calcium phosphate, 1 pound salt, and 1 pound irradiated yeast (70 D). The chemical composition of this ration was as follows: Protein 20.84%, Calcium 1.32%, Phosphorus .54%.

This basal ration is similar to that used by Elvehjem and New (75) in their vitamin determinations, except that irradiated yeast (70 D) served as the source of Vitamin D in the present experiment in place of ultra-violet light as used by the above investigators. It differs from the basal ration used by Hart, Kline, and Keenan (101a) for the production of rickets in chicks, in that the yellow corn is replaced by white corn, and the yeast is changed to irradiated yeast.

The basal ration was supplemented with Pilchard Oil as follows:

Lot I	1/8%	Pilchard Oil	No. 1
Lot II	1/4%	Pilchard Oil	No. 1
Lot III	1/2%	Pilchard Oil	No. 1
Lot IV	1/4%	Pilchard Oil	No. 2
Lot V	1/2%	Cod Liver Oil	
Lot VI	Control		

Wesson Oil was used to dilute the 1/8% and 1/4% lots of oil up to 1/2% by weight while the controls received 1/2%

Wesson Oil only.

Results:

The average weekly weights of the various lots of chicks are given in Table No. 1, and the growth curves are shown in Figure 1.

It will be readily seen from Table No. 1 that the control chicks began to lag in growth between the second and third week. From then on the difference in the weight of the control chicks, and those which received Vitamin A as contained in Pilchard Oil (1/4% or 1/2%) or Cod Liver Oil (1/2%), became progressively greater as the experiment neared completion. This is strikingly shown in Figure No. 1. Furthermore, the control chicks showed distinct symptoms of avitaminosis A at two weeks of age, and characteristic lesions at the time of death which usually followed three to four days after the first appearance of symptoms. In every instance, the control chicks showed considerable loss of weight before death. The control chicks were all dead by the end of the eighth week.

From Table No. 1, it will be seen that the groups of chicks which received various amounts of Pilchard Oil or Cod Liver Oil, grew at a fairly uniform rate during the first four weeks of the experiment. After the fourth week, the group which received 1/2% Pilchard Oil No. 1, and the group which received 1/2% Cod Liver Oil, grew faster than the groups which received either 1/8% or 1/4% Pilchard Oil No. 1. At eight weeks of age, the average weight of the chicks which were fed 1/2% Pilchard Oil No. 1 exceeded the average weight of the chicks

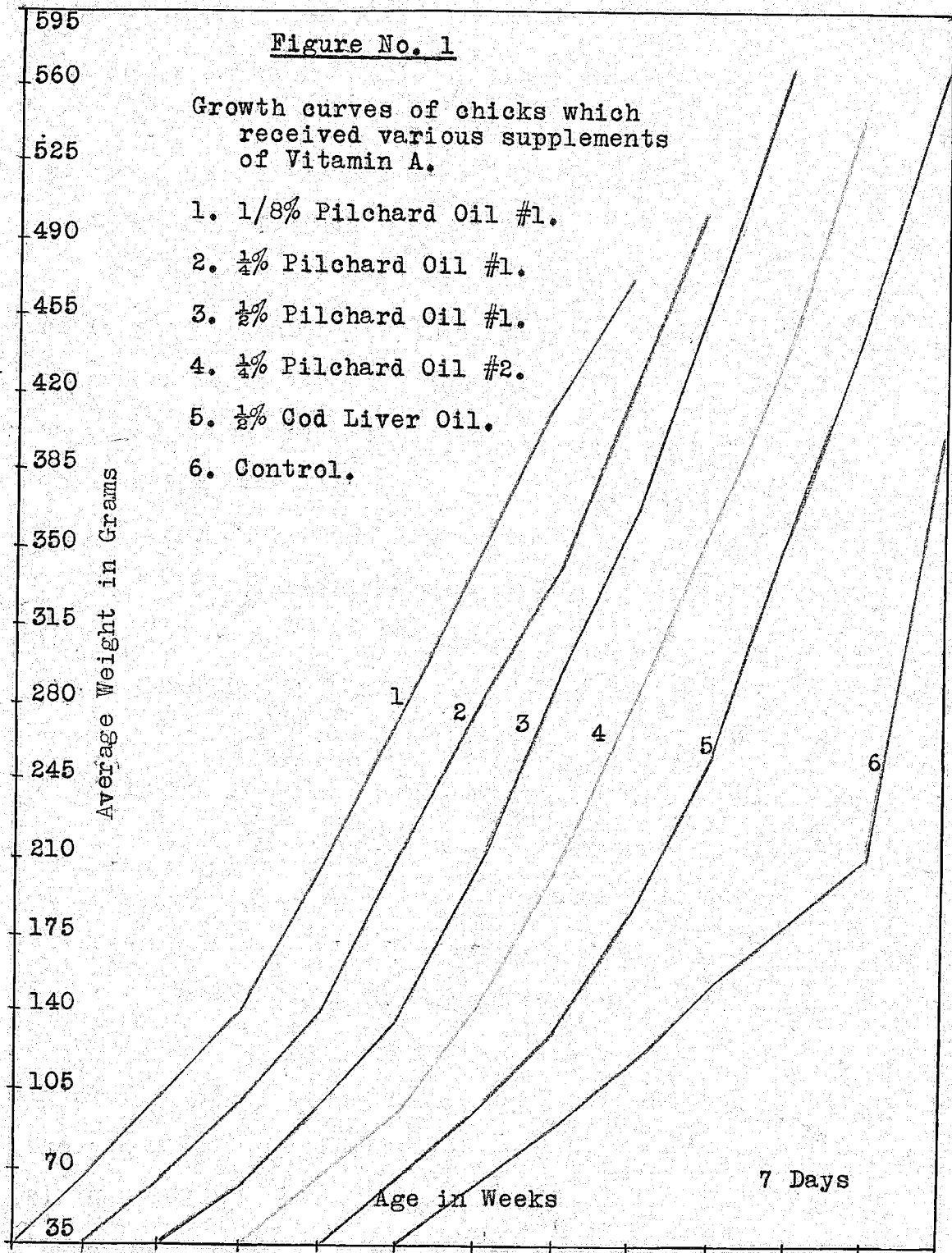
TABLE NO 1 AVERAGE WEEKLY WEIGHTS OF CHICKS.

Average weight in grams.									
Supplement.	Day-old	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks
1/8 % Pilchard Oil # 1.	36.58	64.58	99.17	140.05	208.23	276.12	345.88	416.87	478.40
1/4 % Pilchard Oil # 1.	37.10	64.15	96.26	137.89	206.42	276.84	335.58	424.73	508.11
1/2 % Pilchard Oil # 1.	38.35	62.17	95.82	134.35	203.88	290.00	366.35	462.87	569.00
1/4 % Pilchard Oil # 2.	38.47	63.47	94.35	141.82	204.94	285.88	358.47	442.58	549.62
1/2 % Cod Liver Oil	36.27	61.50	95.44	129.55	184.33	251.22	351.06	448.59	558.82
Control.	34.80	58.10	87.10	115.90	151.30	176.8	209.3	387.0	all dead
								2 chicks only	

Figure No. 1

Growth curves of chicks which
received various supplements
of Vitamin A.

1. $\frac{1}{8}\%$ Pilchard Oil #1.
2. $\frac{1}{4}\%$ Pilchard Oil #1.
3. $\frac{1}{2}\%$ Pilchard Oil #1.
4. $\frac{1}{4}\%$ Pilchard Oil #2.
5. $\frac{1}{2}\%$ Cod Liver Oil.
6. Control.



which were fed 1/2% Cod Liver Oil. It should be noted that the average weight of the chicks which received 1/4% of Pilchard Oil No. 2 were only 9 grams less than the average weight of the chicks which received 1/2% Cod Liver Oil and also, only 20 grams below the average weight of the chicks which were fed 1/2% Pilchard Oil No. 1.

With the exception of a few chicks which received 1/8% of Pilchard Oil No. 1, none of the chicks showed any evidence of avitaminosis A. As judged externally, the chicks appeared to be quite normal and healthy in every respect. Post-mortem examination of a few chicks in each lot did not reveal any indications of Vitamin A deficiency in the internal organs.

The statistical analyses of the data are given in Tables No. 2 and 3. From these results, it will be seen that the difference between the average weight of the chicks which received the basal ration supplemented with either 1/2% Pilchard Oil No. 1 or 1/2% Cod Liver Oil and the chicks which received 1/8% of Pilchard Oil No. 1, was statistically significant. Furthermore, it is important to note that the difference between the average weight of the chicks which were fed 1/4% Pilchard Oil No. 2 and the chicks which were fed 1/8% Pilchard Oil No. 1, was statistically significant. As previously stated, it is of considerable importance to note that the average weight of the chicks which received 1/4% of Pilchard Oil No. 2 compared very favorably with the average weight of the chicks which received either 1/2% Pilchard Oil No. 1, or 1/2% Cod Liver Oil. The results of the statistical analysis

TABLE NO. 2.

STATISTICAL ANALYSIS OF WEIGHTS AT EIGHT WEEKS OF AGE.

Supplement	Mean Weight	Standard Deviation	Coefficient of Variability
1/8 % Pilchard Oil	478.40 \pm 17.00	97.56 \pm 12.00	20.39 \pm 2.51
1/4 % Pilchard Oil	508.11 \pm 11.97	73.14 \pm 8.46	14.39 \pm 1.70
1/2 % Pilchard Oil	569.00 \pm 21.40	126.90 \pm 15.12	22.30 \pm 2.76
1/2 % Cod Liver Oil	558.82 \pm 16.03	97.89 \pm 11.33	17.52 \pm 2.09
1/4 % Pilchard Oil	549.63 \pm 16.77	99.45 \pm 11.85	18.09 \pm 2.22

TABLE NO. 3.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison	Difference of Means	Difference		Significance
		Probable Error		
1/8 % Pilchard Oil & 1/4 % Pilchard Oil	29.71 ± 20.79	1.43	Not Significant	
1/8 % Pilchard Oil & 1/2 % Pilchard Oil	90.60 ± 27.33	3.32	Significant	
1/8 % Pilchard Oil & 1/2 % Cod Liver Oil	80.42 ± 23.36	3.44	Significant	
1/8 % Pilchard Oil & 1/4 % Pilchard Oil # 2	71.23 ± 23.87	2.98	Almost Significant	
1/4 % Pilchard Oil & 1/2 % Pilchard Oil	60.89 ± 24.52	2.48	Not Significant	
1/4 % Pilchard Oil & 1/2 % Cod Liver Oil	50.71 ± 20.00	2.54	Not Significant	
1/4 % Pilchard Oil & 1/4 % Pilchard Oil # 2	41.52 ± 20.59	2.02	Not Significant	
1/2 % Pilchard Oil & 1/2 % Cod Liver Oil	10.18 ± 26.73	0.38	Not Significant	
1/2 % Pilchard Oil & 1/4 % Pilchard Oil # 2	19.37 ± 27.18	0.71	Not Significant	
1/2 % Cod Liver Oil & 1/4 % Pilchard Oil # 2	9.19 ± 23.19	0.40	Not Significant	

156.

(Table No. 3) show that the difference in the weights of the chicks which were fed 1/2% Pilchard Oil No. 1 or 1/4% Pilchard Oil No. 2, or 1/2% Cod Liver Oil were definitely of no statistical significance.

Discussion:

At the end of eight weeks, the average weight of the chicks that received 1/2% Pilchard Oil No. 1 or 1/4% Pilchard Oil No. 2 or 1/2% Cod Liver Oil compared very favorably with those reported by Buckner et. al. (24a), Ringrose and Norris (185) and Record et. al. (182).

According to Record et. al. (182), at the end of eight weeks the average weights of chicks which were fed various amounts of Vitamin A supplement per 100 grams of feed were as follows:

<u>Supplement</u>	<u>Aver. Weight</u> <u>Gm.</u>
40 units as C.L.O.	504 9.4
80 " " C.L.O.	532 8.6
160 " " C.L.O.	564 13.8
240 " " C.L.O.	581 13.0
320 " " C.L.O.	542 9.9
1% poultry C.L.O. (700 units)	564 7.7

Records et. al. data show that the chicks which received 160 units of Vitamin A per 100 grams of basal ration grew as well as the chicks which received 240 and 320 units respectively. Furthermore, the average weight of the chicks which received 160 units was equal to those which received 1% Cod Liver Oil (700 units). Since the chicks which were fed 40 or

80 units of Vitamin A showed symptoms of avitaminosis A, they concluded that 160 units of Vitamin A per 100 grams of feed may be considered as the minimum requirements of chicks up to eight weeks of age.

The average weight of the chicks which were fed 1/2% Pilchard Oil No. 1, or 1/4% Pilchard Oil No. 2, or 1/2% Cod Liver Oil (Table No. 1) were almost identical with the average weights of the chicks of the above mentioned investigators. It may be concluded, therefore, that the chicks which were fed 1/4% Pilchard Oil No. 2 or 1/2% Pilchard Oil No. 1 received at least 160 units of Vitamin A per 100 grams of feed from their respective quantities of Pilchard Oil. Since 1/4% Pilchard Oil No. 2 produced a rate of growth equally as good as 1/2% Pilchard Oil No. 1, it would appear that the Vitamin A potency of Pilchard Oil No. 2 was greater than that of Pilchard Oil No. 1.

Since 160 units of Vitamin A per 100 grams of feed is considered by several investigators to be the minimum requirement of chicks up to eight weeks of age, it would appear that 1/4% of a fish oil containing a minimum of 600 units of Vitamin A per gram of oil (minimum requirements of U.S. Pharmacopeia) would meet the minimum requirements of growing chicks to eight weeks of age. The results of this experiment show that 1/4% of Pilchard Oil No. 2, and 1/2% of Pilchard Oil No. 1 amply supplied the above requirements. It may be concluded, therefore, that the Vitamin A content of the two samples of Pilchard Oil used in this investigation ranged between 300 and 600 units

of Vitamin A per gram of oil.

Possibly another way of estimating the Vitamin A potency of the Pilchard Oil used in this experiment is through estimating the total feed consumption and the amount of Pilchard Oil contained therein. Unfortunately no record was kept of the amount of feed consumed by each group of chicks. However, the data reported by several investigators (126a), (127a), and (128a) show that normally developing White Leghorn chicks will consume 3.6 pounds or 1,634.4 grams feed up to eight weeks of age. This amount of feed would contain 4.086 grams of Pilchard Oil when 1/4% is added or 8.172 grams when 1/2% is added. Since the basal ration was practically free from Vitamin A, the chicks must have of necessity derived their Vitamin A requirements from the above amounts of oil in the 1/4% and 1/2% groups. Since, as has been shown before, a ration must contain 160 units of Vitamin A per 100 grams of feed to promote normal growth of single comb White Leghorn chicks to eight weeks of age, the total number of Vitamin A units supplied in the ration to this age would have to be approximately 2615.04 units. In view of the normal rate of growth attained by the chicks which received either 1/4% Pilchard Oil No. 2 (4.086 gm. oil) or 1/2% Pilchard Oil No. 1 (8.172 gm. oil) it may be concluded that the above number of units were supplied in the respective amounts of oil. If 4.086 grams supplied 2615.04 units of Vitamin A, then 1 gram of Pilchard Oil No. 1 would contain 640 units of Vitamin A. Similarly the 1/2% Pilchard Oil No. 1 would contain at least

320 units of Vitamin A per gram of oil.

Biely and Chalmers (16) have shown that the minimum requirements of growing chicks up to eight weeks of age are 50 units of Vitamin A per day. On this basis the chicks in the present experiment must have received during the entire period (50x56) 2,800 units of Vitamin A. In accordance with the method of calculation shown above, the sample of Pilchard Oil No. 1 would have contained 343 units of Vitamin A, while the sample No. 2 would have contained 685 units of Vitamin A.

Whether the Vitamin A potency of Pilchard Oil is estimated on the basis of Record et.al. (182) minimum requirement per 100 grams of feed or Biely and Chalmers (16) minimum requirements of 50 units per day, the results are in close agreement, i.e. the Vitamin A content of Pilchard Oil No. 1 is over 300 units and Pilchard Oil No. 2 over 600 units.

Both methods of calculation show that Pilchard Oil No. 2 would meet the minimum requirements for Vitamin A as specified by the United States Pharmacopoeia for Cod Liver Oil.

Experiment 2.

The basal ration used in the tests in Experiment 1 was similar to that employed by Elvehjem and New (75) in their studies of Vitamin A. It consisted of 59 pounds of white corn, 25 pounds of wheat middlings, 12 pounds of casein, 1 pound calcium carbonate, 1 pound calcium phosphate, 1 pound salt, and 1 pound irradiated yeast. Because of the fact that white corn is very expensive and not obtainable in Canada, it was deemed necessary to develop a ration consisting of commonly available Vitamin A-free grains. For this purpose, a ration similar to that used by Frohring and Wyeno (86) was compounded and a preliminary test conducted. Unfortunately a large percentage of the experimental chicks developed slipped tendons. Consequently, the ration had to be discarded.

At the time the present experiment was in progress the cause of slipped tendons was not known. It was generally believed then that an improper balance of calcium and phosphorous was mainly responsible for the occurrence of slipped tendons. However, several investigators have since shown that 10 to 20% of ground oats, added to a ration which ordinarily induced slipped tendons, tends to prevent or diminish their occurrence. For this reason, it was decided to include 16% oats in the rations used in this experiment and modify the calcium-phosphorous ratio by using various amounts of milk and meat. The latter also provided the animal protein in the ration, instead of casein which was used in the ration of the first experiment. Casein, unfortunately, contains a variable, but

appreciable, amount of Vitamin A, and therefore , is not quite suitable for use in a basal ration for Vitamin A experiments. To obtain further information on the residual amount of Vitamin A in casein, it was decided to compare a basal ration containing casein with other rations containing varying amounts of milk and meat. Thus the rations used in the present experiment varied in their calcium and phosphorus content, and also in the source of animal protein. The percentage of Calcium and Phosphorus in the various rations was as follows:

<u>Ration</u>	<u>Calcium %</u>	<u>Phosphorus %</u>
No. I	1.426	0.628
No. II	1.579	0.699
No. III	1.865	0.853
No. IV	0.978	0.608

All rations contained oats for the prevention of slipped tendons, while none contained white corn.

Table No. 4 shows the various rations that were used in this experiment. It will be seen that rations I, II, and III contained $17\frac{1}{2}$ pounds of animal protein by weight. Ration No. I contained $12\frac{1}{2}\%$ powdered skim milk, and 5% meat scrap. Ration NO.II: contained 10% skim milk, and $7\frac{1}{2}\%$ meat scrap. Ration No. III contained $7\frac{1}{2}\%$ milk, and 10% meat scrap. Ration No. IV contained 12% commercial casein only.

Experimental Methods:

Day-old single comb White Leghorn cockerels were obtained from a local hatchery and divided into 16 groups of 25 chicks in each. At 24 hours of age, the chicks were placed

TABLE NO. 4 RATIOMS

No. 1	No. 2	No. 3	No. 4
Barley 20 lbs.	Barley 20 lbs.	Barley 20 lbs.	Barley 20 lbs.
Wheat 28½lbs.	Wheat 28½lbs.	Wheat 28½lbs.	Wheat 24 lbs.
Oats 10 "	Oats 10 "	Oats 10 "	Oats 20 "
Middlings 10 "	Middlings 10 "	Middlings 10 "	Middlings 10 "
Bran 10 "	Bran 10 "	Bran 10 "	Bran 10 "
Milk 12½ "	Milk 10 "	Milk 7½ "	Casein 12 "
Meat Scrap 7½ "	Meat Scrap 7½ "	Meat Scrap 10 "	Calcium Carbonate 1 "
Oyster Shell 2 "	Oyster Shell 2 "	Oyster Shell 2 "	Calcium Phosphate 1 "
Salt 1 "	Salt 1 "	Salt 1 "	Salt 1 "
Irradiated Yeast 1 "	Irradiated Yeast 1 "	Irradiated Yeast 1 "	Irradiated Yeast 1 "
100 lbs.	100 lbs.	100 lbs.	100 lbs.

in four-tier battery brooders, each tier being 24 x 60 inches and divided into two equal sized compartments. One compartment in each tier contained a heating unit of three carbon filament lamps.

The four basal rations used are shown in Table No. 4. Each basal ration was divided into four lots and supplemented with various amounts of Vitamin A. The amount of supplement added to Lot 1 was 1/4% of Pilchard Oil; Lot 2, 1/2% Pilchard Oil; Lot 3, 1% Pilchard Oil, and Lot 4 served as the control. Wesson Oil was used to dilute the 1/4% Pilchard Oil up to 1/2% by weight in order to assure thorough mixing of the oil in the mash. One half percent Wesson Oil was added to the control rations. The respective amounts of oil were mixed into the mash and run through a fine-meshed sieve.

Mash and water were constantly available to the chicks. A time clock controlled the number of hours of artificial light that the chicks received. It was set for a twelve-hour day, from 6:00 A.M. to 6:00 P.M.

At the end of the experiment, several chicks were examined to determine the percentage of ash in the tibia fibula. The average ash analysis proved to be over 45%, thus indicating normal bone calcification.

Results:

The average weekly weights of the chicks in the various groups and rations are given in Tables No. 5 and No. 6, and the growth curves, in Figures 2 to 9, inclusive. The mortality of the control groups is shown in Table No. 7.

TABLE NO. 5 AVERAGE WEEKLY WEIGHTS OF MALE CHICKS.

Average weight in grams.									
<u>Ration Plus Supplement.</u>									
	<u>Day-old</u>	<u>1 week</u>	<u>2 weeks</u>	<u>3 weeks</u>	<u>4 weeks</u>	<u>5 weeks</u>	<u>6 weeks</u>	<u>7 weeks</u>	<u>8 weeks</u>
<u>Ration No. 1.</u>									
1/4 % Pilchard Oil		57.56	95.12	155.94	236.12	355.43	445.12	563.06	667.62
1/2 % Pilchard Oil		68.16	107.52	177.60	265.84	376.88	510.24	643.44	774.80
1 % Pilchard Oil		68.78	104.57	172.64	254.50	358.85	476.71	613.93	736.64
Control.		67.58	100.20	147.08	190.76	251.27	275.30	284.00	279.43
125.									
<u>Ration No. 2.</u>									
1/4 % Pilchard Oil	40.00	64.50	103.83	162.78	241.71	337.71	472.64	589.42	705.64
1/2 % Pilchard Oil	40.36	66.27	112.07	178.90	262.62	373.24	502.64	637.50	766.42
1 % Pilchard Oil	40.36	65.93	109.72	181.35	263.14	377.28	507.14	649.21	785.00
Control.	40.00	60.76	102.92	138.40	166.10	203.20	250.00	254.28	261.00

TABLE NO. 6 AVERAGE WEEKLY WEIGHTS OF MALE CHICKS.

Average weight in grams.								
<u>Ration Plus Supplement.</u>								
<u>Ration No. 3.</u>	<u>Day-old</u>	<u>1 week</u>	<u>2 weeks</u>	<u>3 weeks</u>	<u>4 weeks</u>	<u>5 weeks</u>	<u>6 weeks</u>	<u>7 weeks</u> <u>8 weeks</u>
1/4 % Pilchard Oil		73.41	118.60	178.96	255.55	346.81	450.22	559.03 658.66
1/2 % Pilchard Oil		67.86	114.07	181.72	256.51	373.24	493.93	620.48 738.62
1 % Pilchard Oil		69.53	120.27	197.53	283.66	386.40	480.20	631.00 764.66
Control.		72.61	115.07	155.70	185.20	235.43	293.33	280.00 336.00
126.								
<u>Ration No. 4.</u>								
1/4 % Pilchard Oil	40.60	75.92	121.21	189.42	275.07	377.28	493.00	594.92 718.07
1/2 % Pilchard Oil	39.00	76.64	123.64	200.42	287.85	402.78	537.71	632.80 759.44
1 % Pilchard Oil	40.00	75.36	113.20	190.41	283.57	403.70	543.11	670.18 794.00
Control.	40.00	71.04	111.56	144.16	194.44	253.25	278.25	304.71 324.00

Figure No. 2

Growth curves of chicks which
received Ration #1, plus
various supplements of
Vitamin A.

1. $\frac{1}{4}\%$ Pilchard Oil.
2. $\frac{1}{2}\%$ Pilchard Oil.
3. 1% Pilchard Oil.
4. Control.

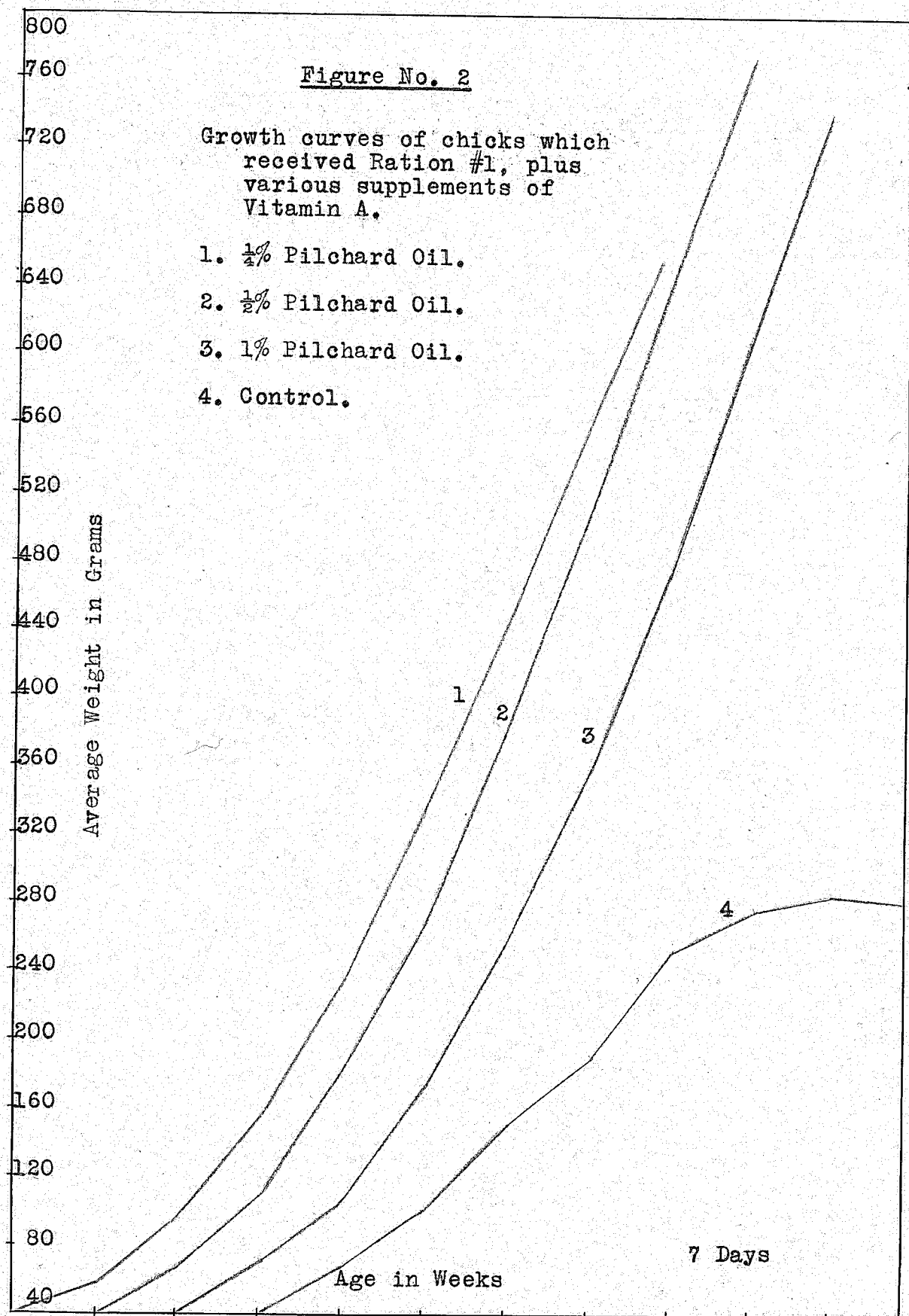


Figure No. 3.

Growth Curves of Chicks which
received Ration No. 2, plus
various Supplements of
Vitamin A.

- 1. $\frac{1}{4}\%$ Pilchard Oil.
- 2. $\frac{1}{2}\%$ Pilchard Oil.
- 3. 1% Pilchard Oil.
- 4. Control.

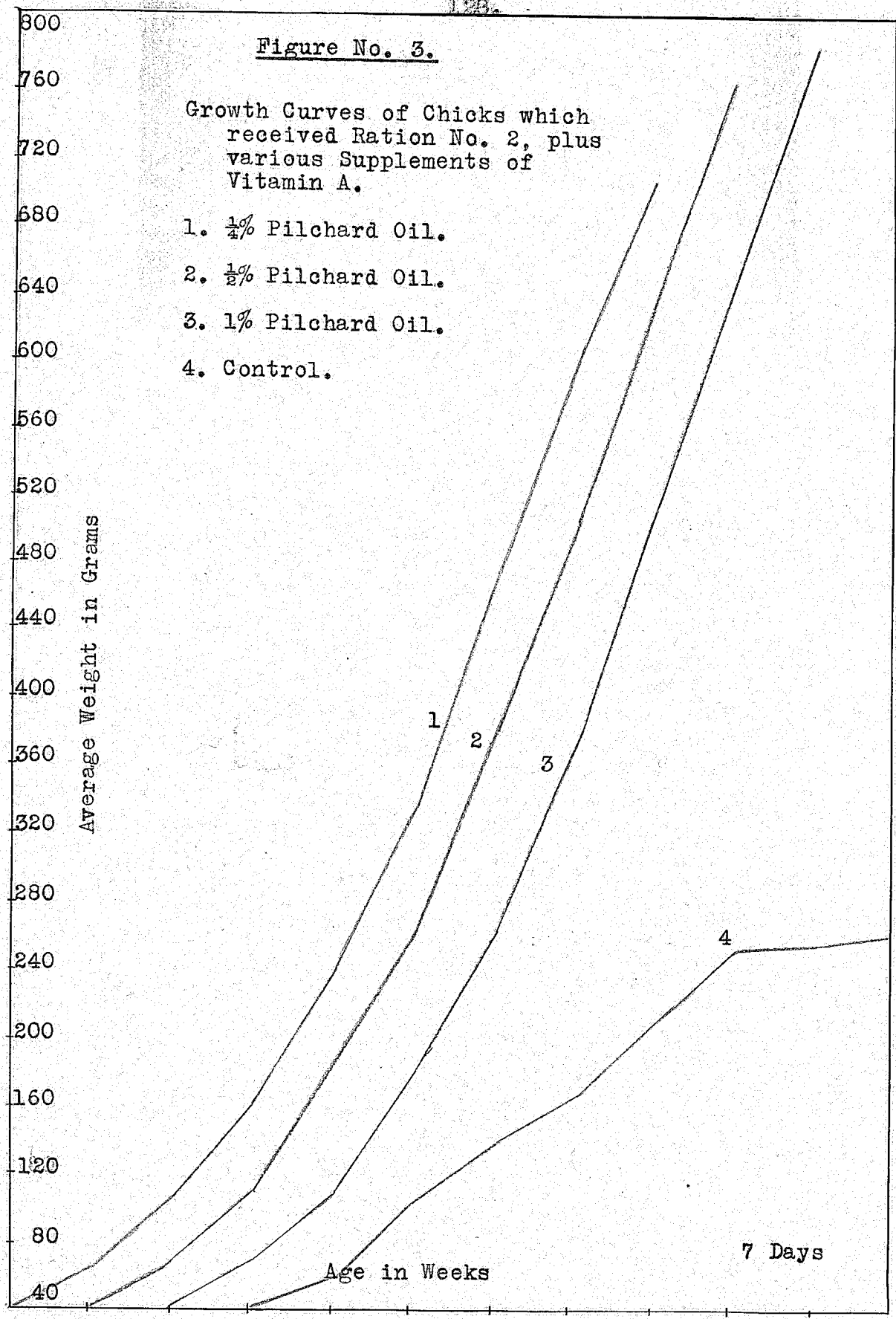


Figure No. 4

Growth Curves of Chicks which
received Ration No. 3, plus
various Supplements of
Vitamin A.

1. $\frac{1}{4}$ % Pilchard Oil.
2. $\frac{1}{2}$ % Pilchard Oil.
3. 1% Pilchard Oil.
4. Control.

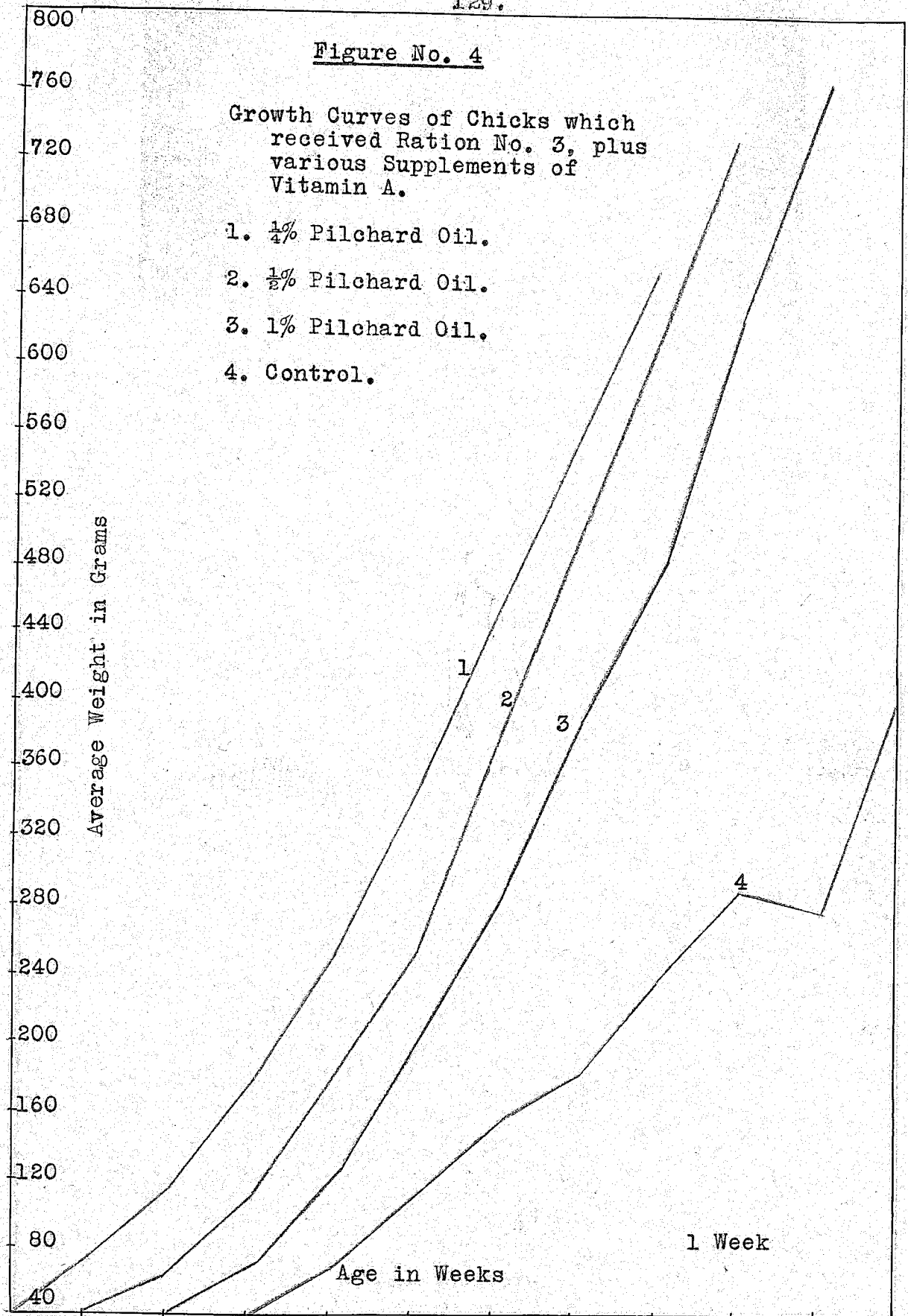


Figure No. 5

Growth Curves of Chicks which
received Ration No. 4, plus
various Supplements of Vitamin
A.

1. $\frac{1}{4}\%$ Pilchard Oil.
2. $\frac{1}{2}\%$ Pilchard Oil.
3. 1% Pilchard Oil.
4. Control.

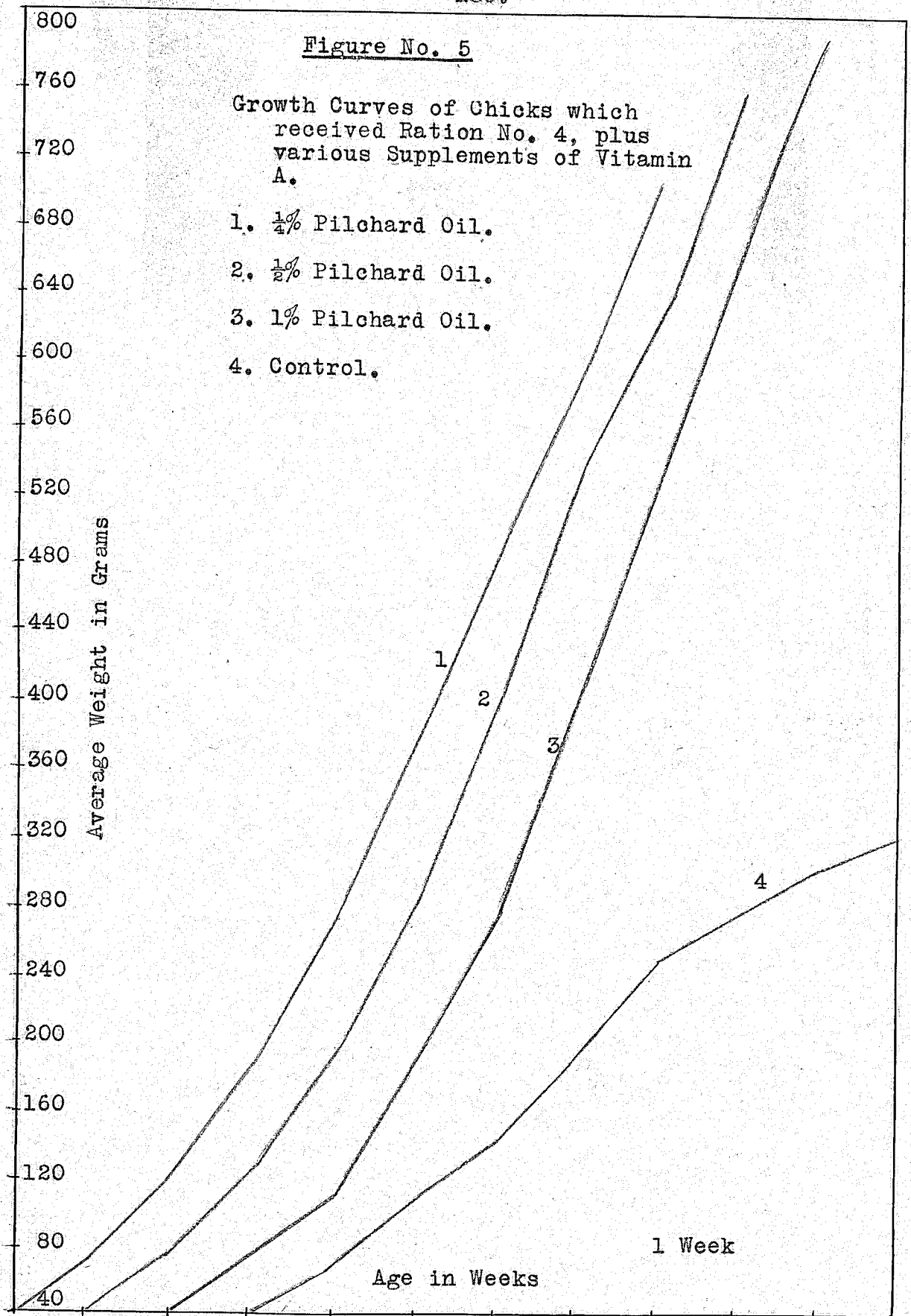


Figure No. 6.

Growth Curves of Chicks which
received $\frac{1}{4}\%$ Pilchard Oil.

1. Ration No. 1.
2. Ration No. 2.
3. Ration No. 3.
4. Ration No. 4.

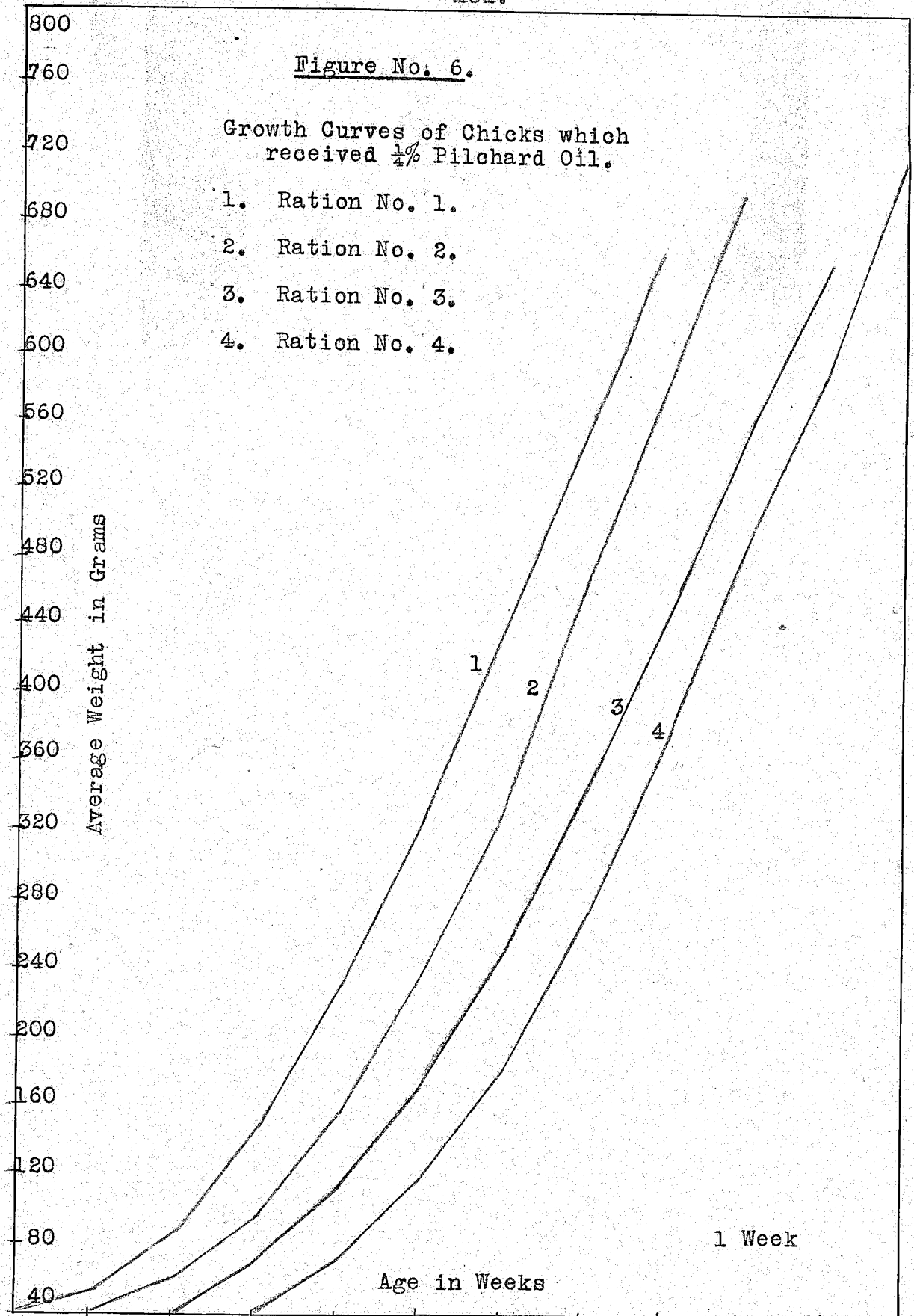


Figure No. 7

Growth Curves of Chicks which
received $\frac{1}{2}\%$ Pilchard Oil.

1. Ration No. 1.
2. Ration No. 2.
3. Ration No. 3.
4. Ration No. 4.

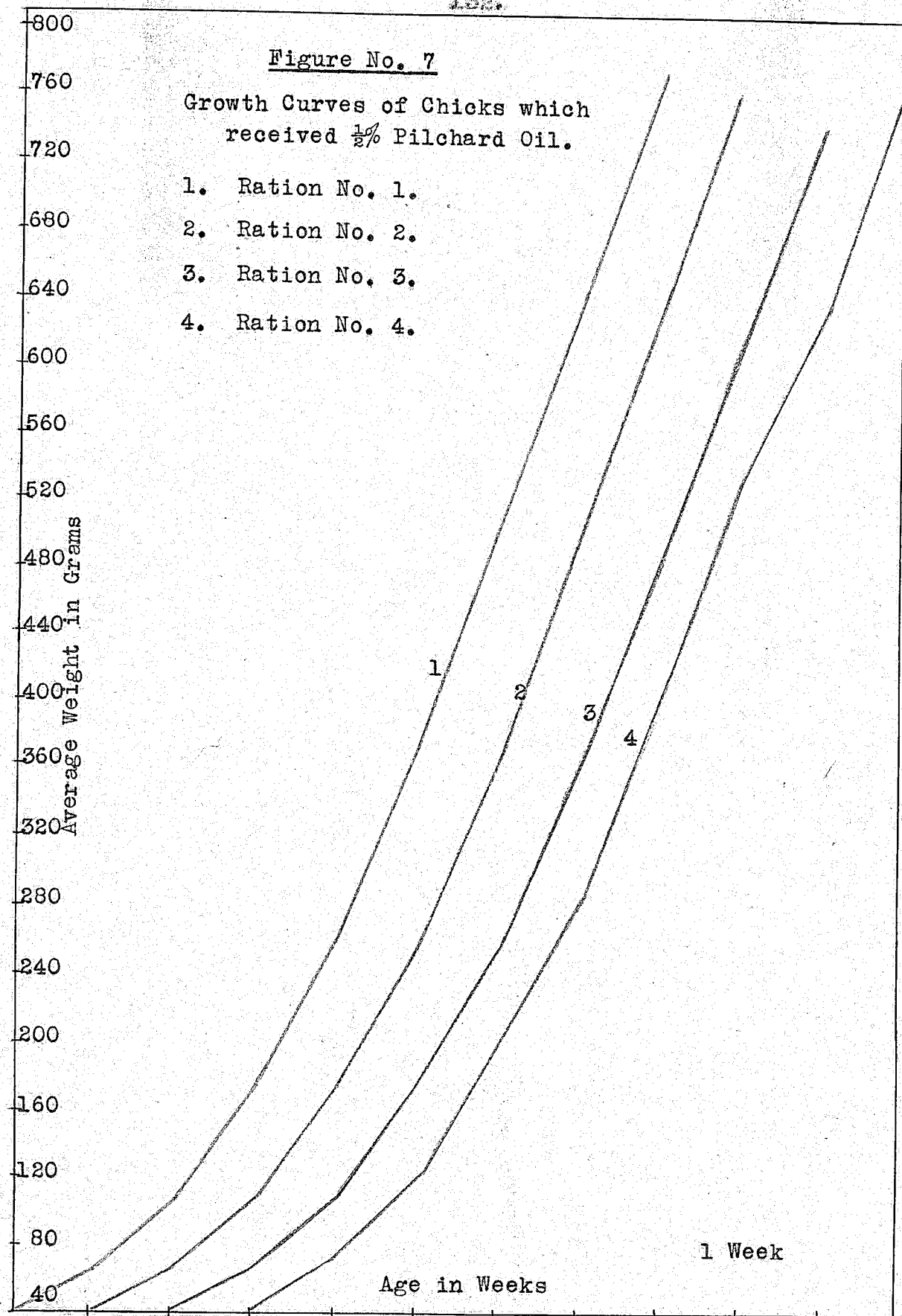
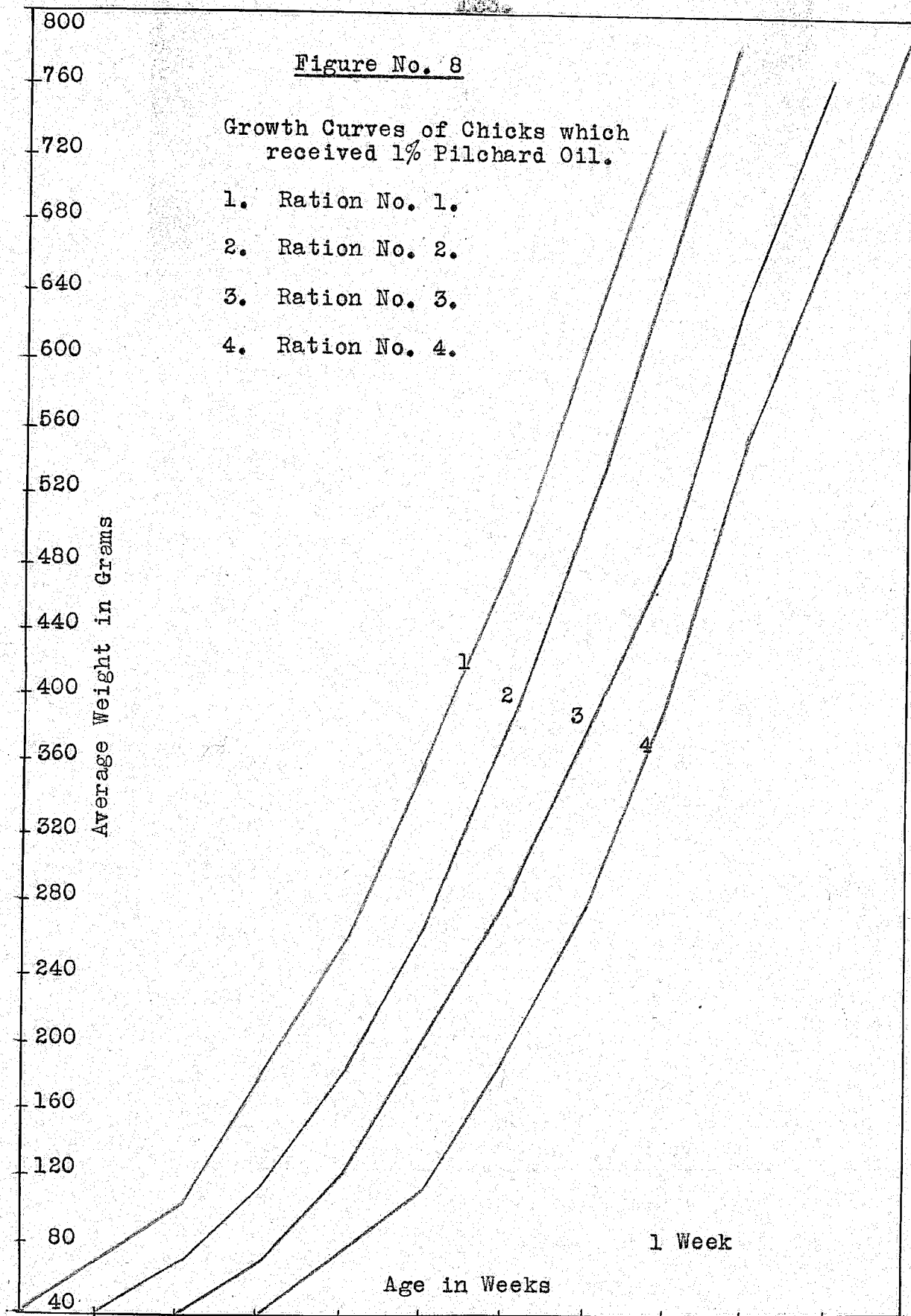


Figure No. 8

Growth Curves of Chicks which
received 1% Pilchard Oil.

1. Ration No. 1.
2. Ration No. 2.
3. Ration No. 3.
4. Ration No. 4.



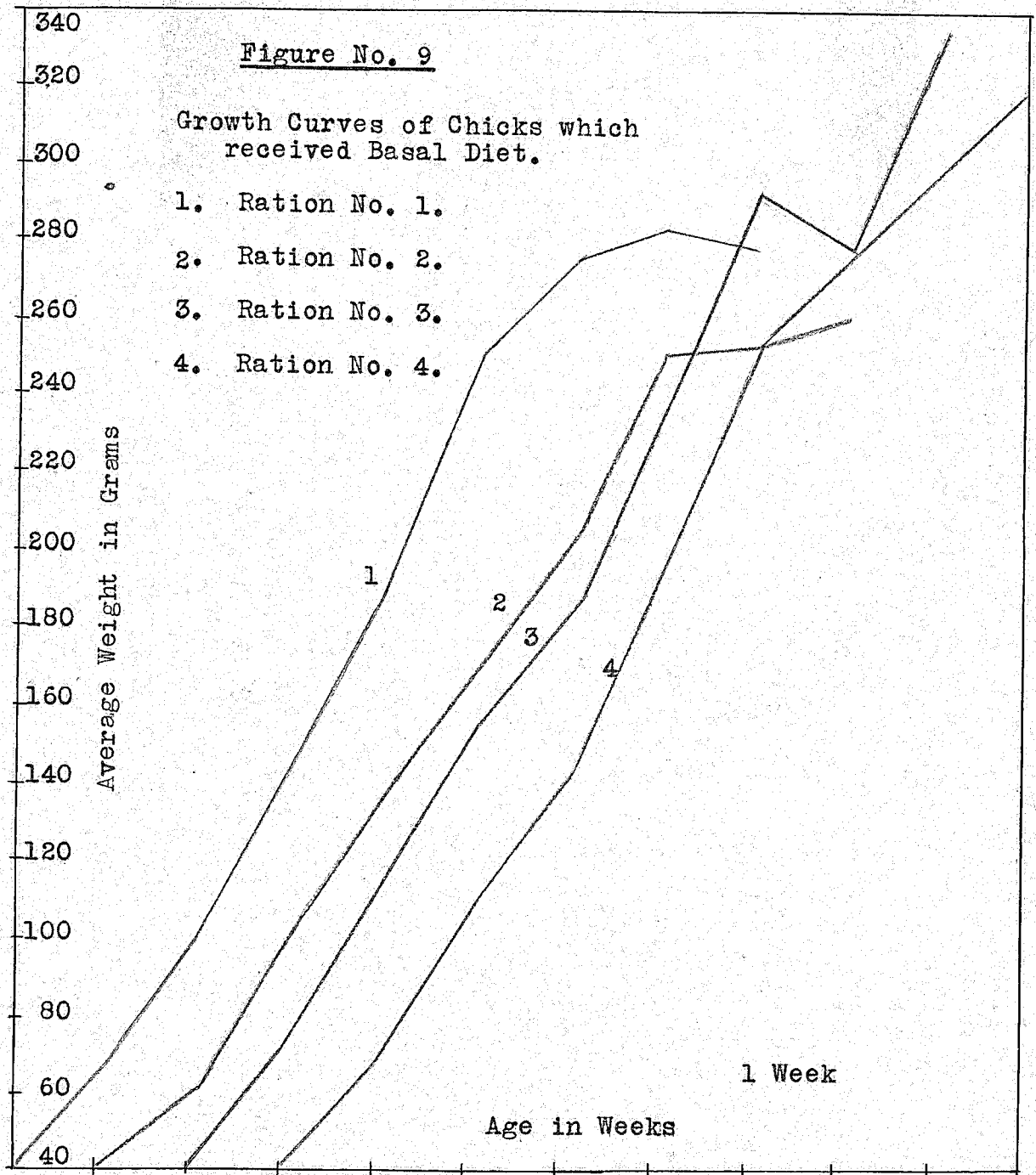


TABLE NO. 7.

MORTALITY OF CONTROL CHICKS.

Control Ration	No. Chicks at Start	No. Chicks at End	No. Chicks Dying	Condition of Kidneys	Condition of Ureters	Remarks
# 1.	29	7	22	8 with +	7 with +	1. showed unabsorbed yolk.
			5	" + +	2 " + +	5. off legs before death.
			2	" + + + +	2 " + + + +	1. showed spot at point of ventricle of heart.
						1. showed heratinized epithelium of the oesophagus.
# 2.	29	2	27	7 with +	7 with +	1. heart showed
			3	" + +	3 " + +	
			3	" + + +	1 " + + +	
			1	" + + + +	1 " + + + +	
# 3.	26	1	25	9 with +	11 with +	4. showed unabsorbed yolks.
			4	" + +	4 " + +	2. off legs before death.
			2	" + + +	2 " + + +	
			3	" + + + +	1 " + + + +	
# 4.	25	6	19	6 with +	6 with +	2. showed unabsorbed yolks.
			2	" + + +	2 " + + +	
			3	" + + + +	1 " + + + +	
			1	" + + + +	1 " + + + +	

The growth rates of the control chicks are in accord with those of Elvehjem and Neu (75), and Biely and Chalmers(16)

By the end of the third week avitaminosis A symptoms were observed amongst the control chicks fed Rations No. I, No. II, and No. III. The chicks fed Ration No. IV did not show symptoms until the fourth week. By the fifth week, all control chicks showed definite symptoms and were unsteady on their feet, and crouched on their haunches. In advanced stages, the chicks were found lying on one side with their heads fallen forward. The feathers were very ruffled, and the beaks and shanks were without any pigmentation. In spite of being in a weakened condition, the birds made considerable effort to eat and drink, but they all lost weight, however, before dying.

In all rations used, a condition was noted in which feed adhered to the beaks and palate of the control chicks, and the chicks which received 1/4% of oil. It was necessary to remove this dry, hard accumulation at least two or three times a week. At four weeks of age, some of the chicks showed a lateral displacement of the mandibles, while in others, the upper and lower mandibles became permanently curved. The cause of this condition was, no doubt due to an impairment of the mucous secreting glands of the mouth, induced by a lack of Vitamin A in the ration.

No typical ophthalmic lesions were observed, yet several chicks sho^wed slight soreness around the eyes. These observations are in agreement with Elvehjem and Neu (75).

All chicks that died during the course of the experiment were autopsied and the results or findings recorded (Table 7). In a number of the control chicks the renal tubules were greatly distended, and the kidneys filled with accumulations of urates. Several control chicks, however, exhibited ataxia, without any visible accumulation of urates in the urinary tract. The chicks still living on the control diets at the end of the experiment (8 weeks) were killed and post-mortemed. The two chicks living on Ration No. II, and one on Ration No. III, showed urates in the kidneys and ureters, and their general appearance and condition indicated that they would have died, probably during the ensuing week. Those on Rations No. I and No. IV showed slight accumulations of urates in the kidneys, and no doubt, they would have died in one or two weeks.

The fact that several of the control chicks lived to the end of the eighth week would indicate that the basal rations used in these experiments were not absolutely free from Vitamin A. Such results are in agreement with the findings of other investigators (169). Of the four rations used, Ration No. I (containing 12½% milk and 5% meat scrap) and Ration No. IV (containing 12% commercial casein) apparently had appreciable amounts of Vitamin A. There were seven and six chicks, respectively, living on these control diets at the end of the experiment. On the other hand, it would appear that Rations No. II and No. III (10% and 7½% of milk respectively) contained the least amount of Vitamin A, since only two chicks on Ration

No. II, and one chick on Ration No. III survived till the end of the eighth week. The survival of only one or two chicks on these latter two diets is most likely due to the individual reserves of Vitamin A in the chick as received from the mother hen.

The mortality in the groups which received the various supplements of Vitamin A during the experiment was less than $2\frac{1}{2}\%$, demonstrating that the chicks were brooded under satisfactory conditions.

Up to four weeks of age, the chicks in all groups, except the controls, grew at an almost uniform rate. After this, with one exception, the rate of growth of the different groups varied directly, according to the percentage of Pilchard Oil fed. For some unaccountable reason, the group receiving the $\frac{1}{2}\%$ Pilchard Oil in Ration No. I grew faster than the 1% group. This variation was noticeable at the end of the second week of the experiment. As the same sample of Pilchard Oil was used to supplement all rations, the variation in this ration cannot be attributed to the Vitamin A potency of the oil used. (See Tables No. 5 and No. 6, and Figures 2 to 5 inclusive).

The growth curves of the chicks which were fed the various basal rations, but the same amount of Vitamin A supplement, are shown in Figures 6, 7, 8, and 9.

The growth curves of the chicks which received $\frac{1}{4}\%$ Pilchard Oil in Rations No. I, No. II, No. III, and No. IV are shown in Figure 6. It will be seen that Ration No. IV (12% commercial casein) produced the fastest growth while

Ration No. II (10% milk) was next in order. The rate of growth on Rations No. I ($12\frac{1}{2}\%$ milk) and No. III ($7\frac{1}{2}\%$ milk) was practically the same, although considerably behind that produced by the other two rations.

In the case of the chicks which received $\frac{1}{2}\%$ of Pilchard Oil in the respective rations (Figure 7) it will be noted that Rations No. I, No. II and No. IV gave practically the same growth while Ration No. III gave the poorest growth.

In the case of the chicks which received 1% of Pilchard Oil (Figure 8) it will be seen that Ration No. IV produced the greatest growth while the other rations were in the following order; Ration No. II; Ration No. III; and Ration No. I producing the poorest growth.

The growth curves of the various control groups are found in Figure 9. A study of these curves reveals that at the end of the eight-week experimental period, the chicks on Ration No. I (7 chicks living) were rapidly losing weight while those on Ration No. II (2 chicks living), showed a marked decrease in weight, weighing considerably less than the chicks fed Rations No. I; No. III; or No. IV. The individual weekly weights showed that the control chick, that lived till the end of the experiment on basal Ration No. III, grew at a normal rate. The rapid rise in the growth curve between the seventh and the eighth week of the control chicks which received basal Ration No. III is due to the survival of this exceptionally large chick. The chicks fed Ration No. IV (6 chicks living) showed a gradual

rise in the growth curve. This rise in the growth curve may be taken as conclusive evidence that the commercial casein used in this experiment contained appreciable amounts of Vitamin A.

The mean body weights of the different lots were subjected to statistical interpretation in Tables No. 8 to No. 14, inclusive. For the results to be considered significant, the difference of the two means must be equal to or greater than three times its probable error.

In all rations except No. IV, there was a significant difference between the average weight of the $\frac{1}{2}\%$ and $\frac{1}{4}\%$ groups and the $\frac{1}{2}\%$ and 1% groups. Ration No. IV, however, showed only a significant difference between the average weight of the $\frac{1}{4}\%$ and 1% groups. The fact that in Ration No. IV the difference between the average weight of the $\frac{1}{2}\%$ and the $\frac{1}{4}\%$ groups was not significant, indicated further that Vitamin A was present in the commercial casein used in this experiment. Thus, the superior growth found in the $\frac{1}{4}\%$ group of Ration No. IV may be accounted for by the assumption that the Vitamin A present in the casein supplemented that supplied by the Pilchard Oil.

There was no significant difference between the average weights of the $\frac{1}{2}\%$ and 1% groups in any of the rations. This evidence demonstrated that $\frac{1}{2}\%$ of the Pilchard Oil used in this experiment supplied sufficient Vitamin A to promote normal growth of chicks up to eight weeks of age. This further substantiated the findings of Wood (266), that $\frac{1}{2}\%$ of a good grade of Pilchard Oil meets the Vitamin A requirements of growing chicks to eight weeks of age. Furthermore, these results show

TABLE NO. 8.

STATISTICAL ANALYSIS OF WEIGHTS' AT EIGHT WEEKS OF AGE.

Ration Plus Supplement	Mean Weight	Standard Deviation	Coefficient of Variability
<u>Ration no. 1.</u>			
1/4 % Pilchard Oil	667.62 ± 11.90	99.70 ± 8.41	14.94 ± 1.28
1/2 % Pilchard Oil	774.80 ± 13.22	97.99 ± 9.35	12.64 ± 1.23
1 % Pilchard Oil	736.64 ± 12.75	99.97 ± 9.01	13.57 ± 1.25
Control.	279.43 ± 13.08	51.30 ± 9.25	18.36 ± 3.41
<u>Ration No. 2.</u>			
1/4 % Pilchard Oil	705.64 ± 12.01	94.20 ± 8.49	11.35 ± 1.23
1/2 % Pilchard Oil	766.42 ± 11.01	86.45 ± 7.79	11.20 ± 1.02
1 % Pilchard Oil	785.00 ± 11.37	89.20 ± 8.04	11.36 ± 1.03
Control.	261.00		

TABLE NO. 9

STATISTICAL ANALYSIS OF WEIGHTS AT EIGHT WEEKS OF AGE.

Ration Plus Supplement	Mean Weight	Standard Deviation	Coefficient of Variability
<u>Ration No. 3.</u>			
1/4 % Pilchard Oil	658.66 ± 11.90	91.70 ± 8.43	13.93 ± 1.30
1/2 % Pilchard Oil	738.62 ± 12.20	97.40 ± 8.62	12.96 ± 1.17
1 % Pilchard Oil	764.66 ± 8.38	68.07 ± 5.92	8.90 ± 0.78
Control.	336.00		
<u>Ration No. 4.</u>			
1/4 % Pilchard Oil	718.07 ± 12.47	96.10 ± 8.82	13.37 ± 1.25
1/2 % Pilchard Oil	759.44 ± 11.84	87.80 ± 8.38	11.56 ± 1.11
1 % Pilchard Oil	794.00 ± 13.75	95.60 ± 9.73	12.04 ± 1.24
Control.	324.00 ± 23.30	84.60 ± 16.49	26.11 ± 5.45

TABLE NO. 10.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison.	Difference of Means.	$\frac{\text{Difference}}{\text{Probable Error}}$	Significance.
<u>Ration No. 1.</u>			
1/4 % & 1/2 %	107.18 \pm 17.78	6.03	Significant
1/4 % & 1 %	69.02 \pm 17.44	3.96	Significant
1/2 % & 1 %	38.16 \pm 18.36	2.08	Not Significant
<u>Ration No. 2.</u>			
1/4 % & 1/2 %	60.78 \pm 16.28	3.73	Significant
1/4 % & 1 %	79.36 \pm 16.53	4.80	Significant
1/2 % & 1 %	18.58 \pm 15.82	1.17	Not Significant

TABLE NO. 11.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison.	Difference of Means.	Difference Probable Error	Significance.
<u>Ration No. 3.</u>			
1/4 % & 1/2 %	79.69 ± 17.03	4.69	Significant
1/4 % & 1 %	106.00 ± 14.54	7.29	Significant
1/2 % & 1 %	26.04 ± 14.80	1.76	Not Significant
<u>Ration No. 4.</u>			
1/4 % & 1/2 %	41.37 ± 17.19	2.41	Not Significant
1/4 % & 1 %	75.93 ± 18.56	4.09	Significant
1/2 % & 1 %	34.56 ± 18.14	1.90	Not Significant

TABLE NO. 12.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison	Difference of Means	Difference Probable Error	Significance
1/4 % # 1 & 1/4 % # 2.	38.02 \pm 13.63	2.79	Not Significant
1/4 % # 1 & 1/4 % # 3.	8.96 \pm 13.72	0.65	Not Significant
1/4 % # 1 & 1/4 % # 4.	50.45 \pm 17.23	2.93	Almost Significant
1/4 % # 2 & 1/4 % # 3.	46.98 \pm 13.63	3.45	Significant
1/4 % # 2 & 1/4 % # 4.	12.43 \pm 17.31	0.72	Not Significant
1/4 % # 3 & 1/4 % # 4.	59.41 \pm 17.23	3.45	Significant

TABLE NO. 13.
SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison	Difference of Means	Difference		Significance
		Probable Error		
1/2 % # 1 & 1/2 % # 2	8.38 ± 17.20	0.49	Not Significant	
1/2 % # 1 & 1/2 % # 3	36.18 ± 17.98	2.01	Not Significant	
1/2 % # 1 & 1/2 % # 4	15.36 ± 17.74	0.87	Not Significant	
1/2 % # 2 & 1/2 % # 3	27.80 ± 16.43	1.69	Not Significant	
1/2 % # 2 & 1/2 % # 4	6.98 ± 16.16	0.43	Not Significant	
1/2 % # 3 & 1/2 % # 4	20.82 ± 17.00	1.22	Not Significant	

TABLE NO. 14.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison	Difference of Means	Difference Probable Error	Significance
1 % # 1 & 1 % # 2	48.36 \pm 17.08	2.83	Almost Significant
1 % # 1 & 1 % # 3	28.02 \pm 15.25	1.84	Not Significant
1 % # 1 & 1 % # 4	57.36 \pm 18.75	3.06	Significant
1 % # 2 & 1 % # 3	20.34 \pm 14.12	1.44	Not Significant
1 % # 2 & 1 % # 4	9.00 \pm 17.84	0.50	Not Significant
1 % # 3 & 1 % # 4	29.34 \pm 16.10	1.82	Not Significant

that no immediate advantage was derived from feeding an excess of Vitamin A.

The groups which received the same percentage of Vitamin A supplement, but in different rations, were compared statistically in Tables No. 12 ($\frac{1}{4}\%$), No. 13 ($\frac{1}{2}\%$), and No. 14 (1%).

A study of Table No. 12 ($\frac{1}{4}\%$) revealed that the average weight of the chicks fed Ration No. IV (commercial casein) was significantly higher than the average weight of chicks fed Ration No. I ($12\frac{1}{2}\%$ milk). Similarly, the average weight of chicks fed Ration No. II (10% milk) was significantly higher than the average weight of chicks receiving Ration No. III ($7\frac{1}{2}\%$ milk), and the average weight of chicks fed Ration No. IV was significantly higher than that of those fed Ration No. III. These results demonstrated the superiority of Rations No. IV and No. II over the other rations. Since it has been shown above that Ration No. IV contained appreciable amounts of Vitamin A, this ration cannot be regarded as a suitable Vitamin A-free basal ration.

Table No. 13 ($\frac{1}{2}\%$) does not show any significant difference in the weights of the chicks which were fed the various rations. Any trace of Vitamin A that may have been present in any of the rations did not influence the rate of growth of these groups as it did in the case of the $\frac{1}{4}\%$ groups. Such evidence again points out the fact that when chicks up to eight weeks of age are given $\frac{1}{2}\%$ Pilchard Oil, their Vitamin A requirements for normal growth are met.

The comparisons of the 1% groups are shown in Table No. 14. It will be noted that the only significant difference was that between the average weight of the chicks fed Ration No. IV and the weight of those fed Ration No. I. The difference between Ration No. II and Ration No. I was nearly significant. As previously stated, there was some unaccountable reason for the slow growth of the group fed Ration No. I with 1% oil as compared with the group fed $\frac{1}{2}$ % oil. It is doubtful if any significance should be attached to this difference.

Discussion:

It will be seen from Table No. 7 that, at the end of the eighth week, the group fed Ration No. II had two control chicks living, and Ration No. III, only one, and that these three chicks showed external symptoms of avitaminosis A. Since several investigators (111) have demonstrated that chicks show marked variation in their individual stores of Vitamin A at time of hatching, it may be concluded that the variation in livability of the chicks receiving the various rations was due not entirely to varying traces of Vitamin A in the rations, but to variations in individual reserves of Vitamin A in the chicks themselves.

Since it is apparent that traces, if any, of Vitamin A in Rations No. II and No. III were the same, the rate of growth of the groups fed the Vitamin A supplement becomes the most important factor for consideration. The weights of the chicks in the various groups at the end of the experiment definitely show that Ration No. II is superior to Ration No.

III in growth promoting powers.

The combination of ten pounds of milk and seven and one-half pounds of meat scrap as used in Ration No. II can, therefore, be definitely accepted as a satisfactory protein supplement—practically free from Vitamin A—in place of casein for biological tests with chicks.

The growth obtained with Rations No. I and No. IV is decidedly greater than that with Ration No. II, but it has already been shown that these former rations contained an appreciable amount of Vitamin A. Thus in spite of the increased growth obtained, these rations cannot be recommended for use in Vitamin A determinations with chicks.

Besides the cost and time involved in obtaining Vitamin A-free casein by either alcoholic extraction or by heating in an oven, there is the possibility that some of the amino acids may be changed or destroyed in either or both of these processes. In the event of any such changes taking place, it can be readily seen how detrimental this would be to growing chicks. On the other hand, when a small quantity of milk, along with a good grade of meat scrap (Ration No. II), is substituted for treated casein, the cost per pound protein is lowered considerably, while at the same time animal protein of high biological value is provided.

In order to avoid the influence of the individual reserve of Vitamin A, it would be desirable to extend the length of the Vitamin A experiments from eight to ten weeks. This extended period would allow the control chicks more time in

which to exhaust their store of Vitamin A, thus assuring 100% mortality.

If the supply of Vitamin A fed to the breeding stock is not controlled, it would appear desirable to establish for chicks a depletion period similar to that used in the rat tests. While no definite time regarding the most satisfactory length of this depletion period can be suggested here, the idea would appear to be worthy of investigation. The depletion periods of chicks hatched from flocks receiving different amounts of Vitamin A would have to be ascertained in each case. Such a procedure, if adopted, would lead to the more uniform occurrence of avitaminosis A symptoms, death of control chicks, and more precise evaluation of the potency of Vitamin A carriers.

Frohning and Wyeno (86) suggested that in Vitamin A tests, chicks from only one breeding flock should be used, or if they are obtained from different breeding flocks, that they be distributed equally amongst all the groups. The latter suggestion entails the keeping of extensive records by the person or persons hatching the chicks, and would tend to increase the factor of variability due to the different viability of the chicks from the different flocks. The idea of using only one breeding flock as a source of chicks for Vitamin A determinations would appear to be more commendable. At present, the chicks used in biological assays do not vary only from laboratory to laboratory, but they also vary within a laboratory, according to the season of the year, strain of

birds, and other conditions. In Vitamin A assays the indiscriminate use of chicks from various flocks is undoubtedly responsible for the great variation noted in the rate of growth and final weight of individual chicks. It probably also accounts for some of the variability in the weight of chicks within a group at the end of the test period. To avoid such variability, it would be necessary to breed and maintain a strain of breeding stock genetically pure for rate of growth.

Since the rate of growth and the final average weight of chicks at the end of the experimental period are the main criteria used in evaluating the potency of Vitamin A supplements, it is essential that the chicks in the various groups should grow at a uniform level. To accomplish this, the following conditions would have to be assured:

- (a) The strain of birds should be genetically pure for rate of growth.
- (b) The Vitamin A intake of the breeding stock should be controlled.
- (c) The Vitamin A reserve of chicks should be depleted before the actual assay period is begun.
- (d) A Vitamin A-free ration that ensures rapid development of avitaminosis A symptoms should be used.
- (e) The Vitamin A-free ration in the presence of sufficient Vitamin A should promote rapid, normal growth.
- (f) The conditions of brooding and rearing should be uniform.

With all of the above conditions under complete experimental control, it should be possible within four to six weeks following the depletion period to evaluate with great

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precision the potency of Vitamin A carriers.

Experiment No. 3.

The object of this experiment was to compare the biological and chemical methods of assaying British Columbia Pilchard Oil for Vitamin A. For this purpose, three samples of Pilchard Oil produced at different stages of the fishing season were used. Thus, information was also secured on the effect of seasonal variation on the Vitamin A content of Pilchard Oil.

Oil No. 1 was produced early in the season and Oil No. 2, in the middle of the season, and Oil No. 3, late in the season. These oils were kept in large volumes in commercial storage tanks. A sample of each oil was taken from the respective tanks and brought to the laboratory for biological assay.

A portion of each oil was tested by the antimony trichloride test (Carr-Price), and the blue unit value recorded. The results of this test showed that Oil No. 1 contained four blue units, Oil No. 2, two blue units, and Oil No. 3, one blue unit.

Experimental Methods:

Day-old single comb White Leghorn cockerels were divided into seven groups of fifteen chicks each, and placed in battery brooders as in the previous experiments. They were fed a basal ration which consisted of the following ingredients:

Ground Wheat	38 $\frac{1}{2}$ pounds.
Ground Oats	10 "
Wheat Middlings	20 "

Wheat Bran	10 pounds
Skim Milk Powder	10 "
Meat Scrap	7½ "
Ground Oyster Shell	2 "
Salt	1 pound
Irradiated Yeast	<u>1</u> "
Total	100 pounds

The amount of Vitamin A supplement added to the ration was either 1/8% or 1/4% of each of the three samples of Pilchard Oil. Each oil was diluted up to 1/2% by weight with Wesson Oil, while the controls received 1/2% of Wesson Oil only. All details of procedure were similar to those described in Experiment II.

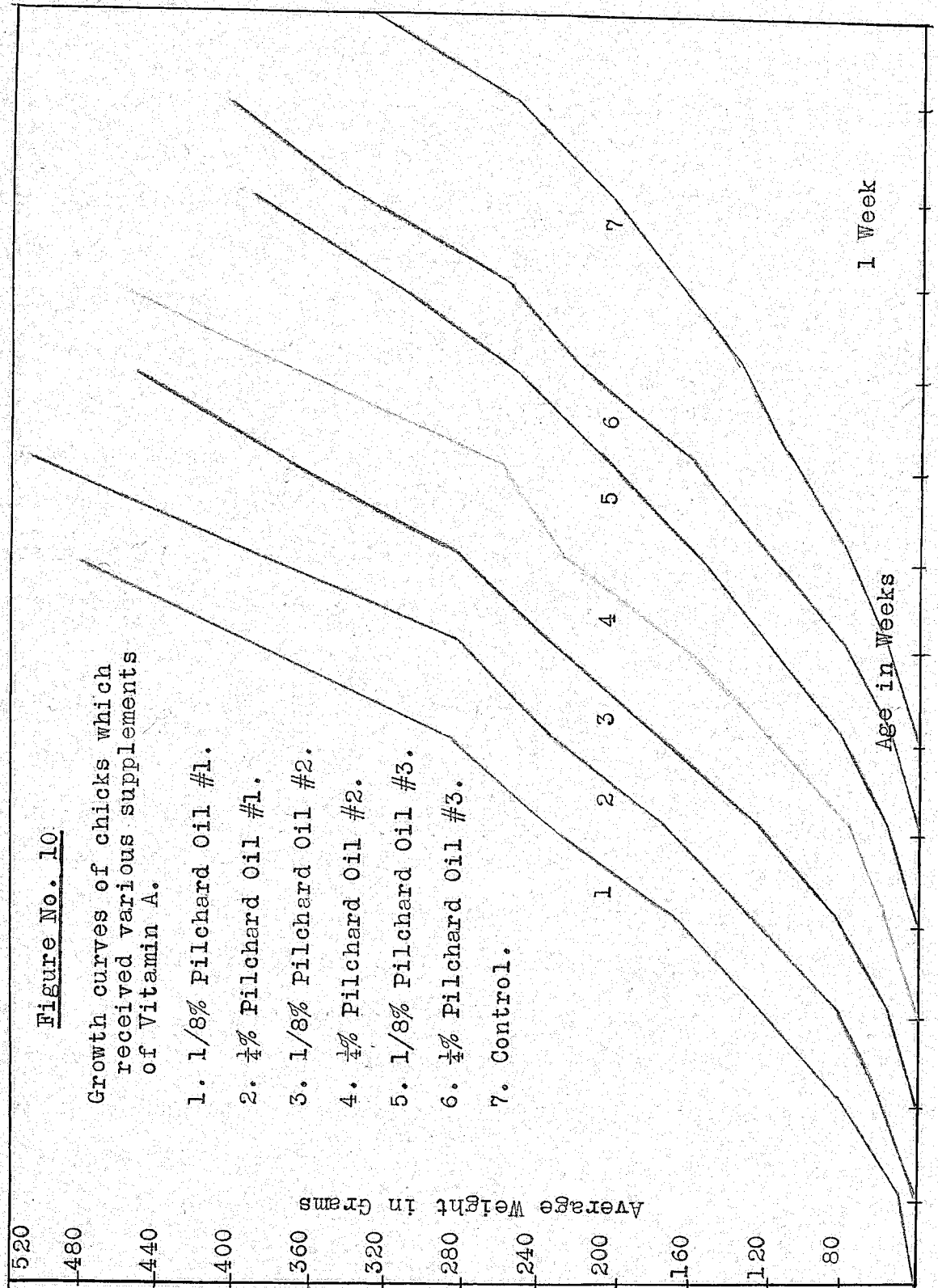
Results:

The average weekly weights of the various groups are given in Table No 15. Growth curves of these groups are shown in Figure No. 10.

A study of the growth curves shows that the average weight of the groups which received Oil No. 1 are heavier at the end of eight weeks than the groups which received Oil No. 2 or Oil No. 3 or the control. Similarly the chicks which were fed Oil No. 2 show higher average weights than the chicks which were fed Oil No. 3 or the basal ration alone. It should be noted, however, that the average weight of the chicks which received Oil No. 3 was only slightly higher than the average weight of the control chicks.

TABLE NO. 15 AVERAGE WEEKLY WEIGHTS OF CHICKS

Supplement.	Average weight in grams.							
	Day-old	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks 8 weeks
1/8 % Pilchard Oil # 1.		53.19	85.00	127.20	173.53	235.67	292.80	387.20 489.47
1/4 % Pilchard Oil # 1.		59.25	84.42	129.67	181.58	238.58	286.83	394.75 507.50
1/8 % Pilchard Oil # 2.		58.38	84.38	124.31	178.23	238.85	283.85	374.00 454.15
1/4 % Pilchard Oil # 2.		59.62	79.92	122.00	169.23	229.15	265.85	372.00 468.15
1/8 % Pilchard Oil # 3.		57.60	80.00	121.20	160.40	200.40	254.20	318.60 402.20
1/4 % Pilchard Oil # 3.		57.67	81.00	122.67	164.17	220.33	262.83	345.83 414.66
Control.		58.33	78.33	106.00	135.67	173.67	210.00	256.00 334.00



The control chicks showed the early symptoms of avitaminosis A at three weeks of age. On post-mortem examination, considerable accumulations of urates in the kidneys and ureters were observed. There were only three chicks living in this group at the end of the experiment. These three chicks were killed and examined internally. They showed enlarged kidneys containing large deposits of urates. Keratinization of the epithelial tissue of the respiratory tract was observed on several of the control chicks that died during the course of the experiment. The fact that three chicks survived to eight weeks of age on the control diet may be due to late hatching (autumn) and to the large stores of Vitamin A that they could, as a consequence, receive from the parent stock at that time.

With the exception of the chicks which were fed Oil No. 3 there were no external symptoms of Vitamin A deficiency in any other groups. The chicks which received 1/8% Oil No. 3 showed distinct symptoms of avitaminosis A at four weeks of age. At the termination of the test at eight weeks, 50% of these chicks had died. On post-mortem examination, accumulations of urates were found in the kidneys and ureters of these chicks. At the end of the experiment, several of the chicks that were still living in this group were selected for post-mortem examination. They too, showed typical avitaminosis A lesions of the kidneys. The symptoms of Vitamin A deficiency in the group fed 1/4% Oil No. 3 were not so pronounced as those found in the group which received 1/8% Oil No. 3.

The statistical analysis of the average mean weights

of the different lots of chicks are given in Table No. 16, and the significance of the difference between these weights in Tables No. 17 and No. 18. A study of these tables reveals, that at eight weeks of age the difference between the average weight of the chicks which were fed either 1/8% or 1/4% Pilchard Oil No. 3, and the control chicks, is not significant. This indicates that Pilchard Oil No. 3 does not supply sufficient Vitamin A at these levels to promote normal growth of chicks up to eight weeks of age. Furthermore, it will be seen that the difference between the average weight of the chicks which received 1/8% Pilchard Oil No. 1, and that of the chicks which received either 1/8% or 1/4% Pilchard Oil No. 3 is statistically significant. The difference is even more significant between the former and the control group. Similarly the difference between the average weight of the chicks which received the supplement of 1/4% Pilchard Oil No. 1 and any of these three latter groups, is significant. Comparison of the other groups did not reveal any significant difference.

Discussion:

It may be readily seen from the above results that the sample of Pilchard Oil No. 1 (early season) is decidedly superior in Vitamin A potency to the other two samples of oil. Similarly, the sample of Pilchard Oil No. 2 (middle season) is superior to the sample of Pilchard Oil No. 3 (late season). Because these oils had been stored immediately upon production, any deterioration that may have taken place during storage would be most pronounced in sample No. 1, and least in sample

TABLE NO. 16.
STATISTICAL ANALYSIS OF WEIGHTS AT EIGHT WEEKS OF AGE.

Supplement	Mean Weight	Standard Deviation	Coefficient of Variability
1/8 % Pilchard Oil # 1.	489.47 ± 12.46	71.48 ± 8.80	14.60 ± 1.83
1/4 % Pilchard Oil # 1.	507.50 ± 14.98	76.84 ± 10.58	15.14 ± 2.13
1/8 % Pilchard Oil # 2.	454.15 ± 14.55	77.88 ± 10.30	17.15 ± 2.31
1/4 % Pilchard Oil # 2.	468.15 ± 16.61	88.92 ± 11.76	18.99 ± 2.59
1/8 % Pilchard Oil # 3.	402.20 ± 17.79	83.36 ± 12.58	20.73 ± 3.25
1/4 % Pilchard Oil # 3.	414.66 ± 20.60	105.66 ± 14.54	25.48 ± 3.72
Control.	334.00 ± 36.55	93.74 ± 25.81	28.07 ± 8.35

TABLE NO. 17.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison		Difference of Means	Difference Probable Error	Significance
1/8 % Pilchard Oil # 1 & Control		155.47 ± 38.61	4.03	Significant
1/8 % Pilchard Oil # 2 & Control		120.15 ± 39.34	3.05	Significant
1/8 % Pilchard Oil # 3 & Control		68.20 ± 40.65	1.68	Not Significant
1/4 % Pilchard Oil # 1 & Control		173.50 ± 39.50	4.39	Significant
1/4 % Pilchard Oil # 2 & Control		134.15 ± 40.14	3.34	Significant
1/4 % Pilchard Oil # 3 & Control		80.66 ± 41.95	1.92	Not Significant
1/8 % P.O. # 1 & 1/8 % P.O. # 2		35.32 ± 19.15	1.84	Not Significant
1/8 % P.O. # 1 & 1/8 % P.O. # 3		87.27 ± 21.71	4.02	Significant
1/8 % P.O. # 1 & 1/4 % P.O. # 1		18.03 ± 19.47	.93	Not Significant
1/8 % P.O. # 1 & 1/4 % P.O. # 2		21.32 ± 20.76	1.03	Not Significant
1/8 % P.O. # 1 & 1/4 % P.O. # 3		74.81 ± 24.06	3.11	Significant

TABLE NO. 18.

SIGNIFICANCE OF DIFFERENCES BETWEEN MEAN WEIGHTS AT EIGHT WEEKS OF AGE.

Comparison	Difference of Means	Difference	
		Probable Error	Significance
L/4 % Pilchard Oil # 1 & 1/8 % Pilchard Oil # 2	53.35 ± 20.88	2.56	Not Significant
L/4 % Pilchard Oil # 1 & 1/8 % Pilchard Oil # 3	165.30 ± 23.24	4.53	Significant
L/4 % Pilchard Oil # 1 & 1/4 % Pilchard Oil # 2	39.35 ± 22.36	1.76	Not Significant
L/4 % Pilchard Oil # 1 & 1/4 % Pilchard Oil # 3	92.84 ± 25.46	3.65	Significant
1/8 % Pilchard Oil # 2 & 1/8 % Pilchard Oil # 3	51.95 ± 22.98	2.26	Not Significant
1/8 % Pilchard Oil # 2 & 1/4 % Pilchard Oil # 2	14.00 ± 22.07	.63	Not Significant
1/8 % Pilchard Oil # 2 & 1/4 % Pilchard Oil # 3	39.49 ± 25.22	1.57	Not Significant
1/4 % Pilchard Oil # 2 & 1/8 % Pilchard Oil # 3	65.95 ± 24.33	2.71	Not Significant
1/4 % Pilchard Oil # 2 & 1/4 % Pilchard Oil # 3	53.49 ± 26.46	2.02	Not Significant
1/8 % Pilchard Oil # 3 & 1/4 % Pilchard Oil # 3	12.46 ± 27.22	.46	Not Significant

No. 3. Therefore, it may be concluded that Pilchard Oil No. 1 (early season) must have contained more Vitamin A at the time of production than the other two oils. This evidence demonstrates that Pilchard Oil produced early in the season is more potent in Vitamin A than oils that are produced at later stages of the fishing season.

It is important to note that the results of the blue unit test were in agreement with the biological assay in that they showed the difference in the Vitamin A content of the three samples of Pilchard Oil. Consequently the blue unit test may be used as a preliminary test for estimating the Vitamin A potency of Pilchard Oil. This is of considerable commercial importance inasmuch as it would enable commercial refineries to select their oils on the basis of this test. Such a practice would permit the use of the oils with the highest Vitamin A content for medicinal purposes, while the oils of low Vitamin A potency could be used for paints and other manufactured products.

Bailey (6) at Prince Rupert has reported that the blue value of Pilchard Oil is only a rough estimation of the Vitamin A content of the oil. Under such circumstances, this test may be used only as a rough measure of the relative potency of various samples of Pilchard Oil. At the present stage of our knowledge, the actual Vitamin A activity of Pilchard Oil has still to be determined by the biological assay.

The foregoing experiment has definitely shown that the Vitamin A content of Pilchard Oil varies with the season of the

year, and that oil produced early in the season has the highest Vitamin A potency. It would be interesting to ascertain if the Vitamin D potency of Pilchard Oil would be parallel to the Vitamin A potency at various stages of the fishing season.

SUMMARY.Experiment No. 1

(1) 140 day-old single comb White Leghorn Cockerels were divided into seven different groups. The Vitamin A-free basal ration used was similar to that of Elvehjem and Neu(75). It was supplemented with $1/8\%$, $1/4\%$, $1/2\%$ and 1% Pilchard Oil #1, $1/4\%$ Pilchard Oil #2, and $1/2\%$ Cod Liver Oil. One lot of chicks was fed the basal ration only.

(2) When either $1/2\%$ Pilchard Oil #1 or $1/4\%$ Pilchard Oil #2 was added to the basal ration, the rate of growth was as good as when $1/2\%$ Cod Liver Oil was added to the ration.

(3) The rate of growth of the chicks in this experiment was compared with the rate of growth of chicks that had been fed graded amounts of Vitamin A as supplied by Reference Cod Liver Oil (U.S. Pharmacopoeia, 3000 units of Vitamin A per gram).

(4) It was found that Pilchard Oil #1 contained at least 300 International Units of Vitamin A per gram and Pilchard Oil #2 at least 600 International Units per gram.

(5) Pilchard Oil #2 would appear to meet the minimum requirements specified by the U.S. Pharmacopoeia for Cod Liver Oil (600 units of Vitamin A per gram).

Experiment No. 2.

(1) 400 day-old single comb White Leghorn Cockerels were divided into 4 groups and each group was subdivided into

4 lots. Each group was fed a different basal ration. Ration No. 1 contained $12\frac{1}{2}\%$ powdered skim milk and 5% meat scrap; Ration No. II 10% powdered skim milk and $7\frac{1}{2}\%$ meat scrap; Ration No. III $7\frac{1}{2}\%$ powdered skim milk and 10% meat scrap; and Ration No. IV $12\frac{1}{2}\%$ commercial casein.

(2) The rations were supplemented with 0%, $\frac{1}{4}\%$, $\frac{1}{2}\%$ and 1% of Pilchard Oil.

(3) It was shown that Rations No. I and No. IV contained appreciable traces of Vitamin A.

(4) Since basal Ration No. II (10% powdered skim milk and $7\frac{1}{2}\%$ meat scrap) contained the least traces of Vitamin A of all the rations used in this experiment it was found to be the most suitable for Vitamin A studies.

(5) $\frac{1}{2}\%$ Pilchard Oil was found to supply adequately the Vitamin A requirements of growing chicks up to 8 weeks of age.

(6) At 8 weeks of age the average weights of the different lots of chicks (in grams) were as follows:

<u>Ration</u>	<u>Control</u>	<u>$1/4\%$ P.O.</u>	<u>$1/2\%$ P.O.</u>	<u>1% P.O.</u>
#1	279 \pm 13	667 \pm 11	774 \pm 13	736 \pm 12
#2	261	705 \pm 12	766 \pm 11	785 \pm 11
#3	336	658 \pm 11	738 \pm 12	764 \pm 8
#4	324 \pm 23	718 \pm 12	759 \pm 11	794 \pm 13

(7) Up to 8 weeks of age, the mortality in all the groups except the controls was less than $2\frac{1}{2}\%$.

(8) Several recommendations are suggested for im-

proving the biological Assay of Vitamin A carriers, using the chick as the test animal.

Experiment No. 3.

(1) 105 day-old single comb White Leghorn chicks were divided into 7 groups. The basal ration used in this experiment was similar to that of Ration No. II in Experiment No. 2.

(2) Three samples of Pilchard Oil that had been produced at different stages of the fishing season were used as Vitamin A supplements. These oils were added to the basal ration in amounts of 0%, 1/8% and 1/4%.

(3) It was found that the Pilchard Oil produced in the early stages of the fishing season was a more potent source of Vitamin A than the oils produced later in the season.

(4) These three samples of Pilchard Oil were tested by the antimony tri-chloride test (Carr-Price reaction).

(5) The results of the antimony tri-chloride test paralleled the results obtained with the Biological Assay.

(6) It is recommended that oils that are produced in the early stages of the fishing season should be used for medicinal purposes, because of their higher Vitamin A content.

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U.S.P. X
Interim Revision Announcement No. 2

**A 1934 REVISION OF THE TEXT AND ASSAYS
FOR COD LIVER OIL**

**OF THE
PHARMACOPŒIA OF THE UNITED STATES
TENTH DECENNIAL REVISION**

This announcement by interim revision of new standards and assays for medicinal Cod Liver Oil is in recognition of the notable advancement in scientific knowledge concerning vitamins, and for the purpose of officially establishing in this country the new International Units for Vitamins A and D.

The Pharmacopœia has accepted these International Vitamin A and Vitamin D Units as the basis for its new standards, and the new "*U.S.P. X 1934 Vitamin A Unit*" and "*U.S.P. X 1934 Vitamin D Unit*" are identical with the corresponding International Units.

It is hoped that this action by the Pharmacopœia will largely overcome the present confusion in label and literature statements concerning the Vitamin A and Vitamin D potency of many products. This confusion in the past has been due to the use of unofficial units of varying vitamin value.*

Following the established policy of pharmacopœial revision the Committee has had the advice and coöperation of many experts in the preparation of this new text. Two conferences, with more than thirty recognized authorities in vitamin work, present at each, laid the foundations for the assays. There has since been established what is known as the "*U.S.P. Vitamin Advisory Board*." This Board has completed the assay texts and supervised the preparation of an official "*Reference Cod Liver Oil*" of known Vitamin A and Vitamin D potency, expressed in International Units. This is now being distributed by the Pharmacopœia Board of Trustees as a basis of comparison in assays. The new assays require the use of this official "*Reference Cod Liver Oil*" as a basis of comparison.

This "*Reference Cod Liver Oil*" was independently assayed, in com-

* A statement of the approximate relationship of various unofficial vitamin units, now employed in the study and labeling of products containing Vitamin A and Vitamin D, has been issued by the U.S.P. Vitamin Advisory Board. Information concerning this can be obtained by addressing the Chairman of the U.S.P. Committee of Revision.

parison with the international standards, by fifteen different laboratories who reported their results to the Vitamin Board. The members of this Board compiled and evaluated the data submitted and determined from these figures the Vitamin A and Vitamin D potency of the "Reference Oil." The Board has also arranged for the periodic recheck of the potency of this "Reference Oil" so that it may serve as a satisfactory standard when used by manufacturers of products containing Vitamin A and/or Vitamin D. The "Reference Cod Liver Oil" is obtainable through the office of the Chairman of the Committee of Revision.

This Revision for the first time establishes an official vitamin standard for Cod Liver Oil. Heretofore, the Pharmacopoeial requirement for vitamin content and assay has been optional and reflected the situation when the U.S.P. X became official some years ago. At that time there was little or no quantitative information upon which to base the proper minimum Vitamin A potency for a medicinal Cod Liver Oil and the requirement that, when assayed Cod Liver Oil should contain at least 50 Vitamin A units per gram, was primarily to insure the exclusion of manipulated oils in which all vitamins had been destroyed. At that time "Vitamin D" was not even known by that designation but was referred to as "an antirachitic factor." The new official standards insure a Cod Liver Oil of excellent quality, corresponding in potency to what is now largely being sold in this country as "U.S.P. Oil."

In addition to the introduction of the vitamin standards and methods of assay, the following physical or chemical standards have been introduced or amended:

A standard for permissible color intensity for the official oil has been added.

The limit of free acid and the saponification number take into consideration the occasional use of carbon dioxide as a preservative for the Oil.

The directions for the determination of unsaponifiable matter have been so modified as to eliminate possible sources of error.

A chill-test has been introduced to insure the absence of excessive amounts of stearin.

The new official vitamin standards and assays and the chemical and physical standards as prepared by the Sub-Committee on Organic Chemicals of the Committee of Revision, have been officially adopted by the General Committee of Revision and the Board of Trustees. By action of the Board of Trustees the new standards for Cod Liver Oil will become official on January 1, 1935.

E. FULLERTON COOK

43rd St. and Woodland Ave..

Philadelphia, Pa.

*Chairman of the Committee of Revision
of the U.S. Pharmacopoeia.*

May 1, 1934

"SALT MIXTURES"

For Diets in Biological Assays

In reviewing the salt mixtures during the revision of the Pharmacopoeia it was found that the formulas published in the U.S.P. Interim Revision Announcement No. 2 were not strictly in conformity with the formulas as originally published. The formulas now have been calculated to represent the same content of salts and acids as in the original, but in terms of official products. This will enable those using these formulas to secure more easily the ingredients and prepare the mixtures.

The formulas as they will appear in the U.S.P. XI are as follows:

Salt Mixtures

For preparing the salt mixtures the available form of each chemical is taken to furnish the stipulated equivalent of each chemical.

Salt Mixture No. 1

Calcium Carbonate (U.S.P.).....	134.8 Gm.
Magnesium Carbonate (U.S.P., 40 per cent MgO).....	28.9 Gm.
Sodium Carbonate, anhydrous (U.S.P. Reagent).....	34.2 Gm.
Potassium Carbonate (U.S.P., dried at 180°C).....	141.3 Gm.
Phosphoric Acid (U.S.P., 86.5 per cent).....	119.3 Gm.
Hydrochloric Acid (U.S.P., 36 per cent).....	148.3 Gm.
Sulfuric Acid (U.S.P., 96 per cent).....	9.6 Gm.
Citric Acid (U.S.P.).....	111.1 Gm.
Ferric Citrate (U.S.P. Reagent, 17.5 per cent Fe).....	7.44 Gm.
Potassium Iodide (U.S.P.).....	0.020 Gm.
Manganese Sulfate (U.S.P. Reagent, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$).....	0.117 Gm.
Sodium Fluoride (U.S.P. Reagent).....	0.062 Gm.
Potassium Alum (U.S.P.).....	0.044 Gm.

Dissolve the citric acid in a sufficient quantity of hot distilled water and add the solution to the mixed carbonates. Then add the potassium iodide, manganese sulfate, sodium fluoride and potassium alum, previously dissolved in distilled water. Then add the ferric citrate dissolved in the hydrochloric acid. Dilute the sulfuric acid with distilled water; add the phosphoric acid and add this acid mixture to the mixture previously prepared and stir until effervescence ceases. Evaporate the final mixture to dryness, in a current of air at from 90° to 100°C. and reduce the resulting product to a fine powder.

Salt Mixture No. 2

Sodium Chloride (U.S.P.).....	1.73 Gm.
Magnesium Sulfate (U.S.P.).....	5.45 Gm.
Sodium Biphosphate (U.S.P.).....	3.47 Gm.
Potassium Phosphate (K_2HPO_4).....	9.54 Gm.
Calcium Biphosphate [$CaH_4(PO_4)_2 \cdot H_2O$].....	5.40 Gm.
Ferric Citrate (U.S.P. Reagent, 17.5 per cent Fe).....	1.18 Gm.
Calcium Lactate (U.S.P.).....	13 Gm.
Mix the finely powdered salts uniformly.	

Respectfully submitted,

E. FULLERTON COOK,
Chairman

OLEUM MORRHUÆ

Cod Liver Oil

Ol. Morrh.

The partially destearinated fixed oil obtained from fresh livers of *Gadus Morrhua* Linné and other species of the Family *Gadidæ*. Cod Liver Oil may be flavored by the addition of not more than 1 per cent of any one or any mixture of flavoring substances recognized in this Pharmacopœia. Cod Liver Oil contains in each Gm. at least 600 U.S.P. Units of Vitamin A and at least 85 U.S.P. Units of Vitamin D.*

The Vitamin A potency and Vitamin D potency of Cod Liver Oil when designated shall be expressed in "United States Pharmacopœia Units" per gram of oil and may be referred to as "U.S.P. Units" per gram of oil. To indicate the adoption of the new standards the statement "U.S.P. X—Revised 1934" may be used.

Description and physical properties—A thin oily liquid, having a peculiar, slightly fishy, but not a rancid odor, and a fishy taste.

Cod Liver Oil is slightly soluble in alcohol, but is freely soluble in ether, chloroform, carbon disulphide, and in ethyl acetate.

Tests for identity and purity—Specific gravity 0.918 to 0.927 at 25° C.

A solution of 1 drop of Cod Liver Oil in 1 cc. of chloroform, when shaken with 1 drop of sulphuric acid, acquires a violet-red tint, gradually changing to reddish-brown.

When viewed transversely in a tall, cylindrical, standard oil-sample bottle of colorless glass of about 120 cc. capacity, the color of Cod Liver Oil shall not be more intense than that of a mixture of 11 cc. of colorimetric cobalt, T.S.,† 76 cc. of colorimetric ferric T.S.†, and 33 cc. of distilled water, in a similar bottle of the same internal diameter.

* One "United States Pharmacopœia Unit of Vitamin A" is equal, in growth promoting and antiophthalmic activities for the rat, to one International Unit of Vitamin A as defined and adopted by the Conference of Vitamin Standards of the Permanent Commission on Biological Standardisation of the League of Nations in June of 1931; one "United States Pharmacopœia Unit of Vitamin D" is equal, in antirachitic potency for the rat, to one International Unit of Vitamin D as defined and adopted by the Conference of Vitamin Standards of the Permanent Commission on Biological Standardisation of the League of Nations in June of 1931.

† Reagents for Color Determination:

Colorimetric Cobalt T.S.—Containing 59.496 Gm. of cobaltous chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) in 1000 cc. of solution. Dissolve about 65 Gm. of cobaltous chloride in enough of a fluid made by mixing 25 cc. of hydrochloric acid with 975 cc. of distilled water to make a volume of 1000 cc. Measure 5 cc. of this solution into a 250 cc. flask, add 15 cc. of a solution of sodium hydroxide (1 in 4) and 5 cc. of solution of hydrogen dioxide. Boil the mixture gently for ten minutes, cool, add 2 Gm. of potassium iodide and 20 cc. of sulphuric acid (1 in 4). When the precipitate has dissolved titrate the liberated iodine with tenth-normal sodium thiosulphate, using starch T.S. as the indicator. One cc. of tenth-normal sodium thiosulphate corresponds to 0.023799 Gm. of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Adjust the volume of the cobaltous chloride solution so that 1 cc. contains 0.059496 Gm. of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Preserve the solution in a bottle with a well-fitting glass stopper.

Colorimetric Ferric T.S.—Containing 45.053 Gm. of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in 1000 cc. of solution. Dissolve about 55 Gm. of ferric chloride in enough of a fluid made by mixing 25 cc. of hydrochloric acid with 975 cc. of distilled water to make a volume of 1000 cc. Assay the solution as directed under *Liquor Ferri Chloridi*, U.S.P. X, using 10 cc. of the solution, accurately measured. Adjust the volume of the solution so that 1 cc. contains 0.045053 Gm. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Preserve the solution in a bottle of amber glass having a well-fitting glass stopper.

Dissolve 2 Gm. of Cod Liver Oil, accurately weighed, in 30 cc. of a mixture of equal volumes of alcohol and ether, the mixture having been previously neutralized with tenth-normal sodium hydroxide, using 5 drops of phenolphthalein T.S. as the indicator, and boil the oil solution gently under a reflux condenser for ten minutes. Cool and titrate the mixture with tenth-normal sodium hydroxide to the production of a pink color which persists after shaking for thirty seconds. Not more than 1 cc. of tenth-normal sodium hydroxide is required (*free acid*).

Weigh 5 Gm. of Cod Liver Oil into a 250 cc. flask, add a solution of 2 Gm. of potassium hydroxide in 40 cc. of alcohol and heat under a reflux condenser for two hours, keeping the alcohol gently boiling. Evaporate the alcohol on a water bath, dissolve the residue in 50 cc. of hot distilled water, and transfer the solution to a separatory funnel, rinsing the flask with two 25-cc. portions of distilled water which are added to the solution in the separator. Cool the mixture to from 15° to 20° C., and extract with two successive portions of 50 cc. each of ether, adding a few drops of alcohol. Combine the ether extracts in another separator, and wash the ether solution, first with 20 cc. of tenth-normal potassium hydroxide, then with 20 cc. of fifth-normal potassium hydroxide, and finally with successive 15-cc. portions of distilled water until the washings are not reddened by the addition of 2 drops of phenolphthalein T.S. Transfer the ether solution to a tared beaker, rinse the separator with 10 cc. of ether, and add the rinsings to the beaker. Evaporate the ether just to dryness on a water bath and dry the residue for thirty minutes at 100° C. Cool the beaker in the desiccator for thirty minutes and weigh. The residue shall not exceed 1.30 per cent of the weight of Oil taken for the assay (*unsaponifiable matter*).

Fill a tall, cylindrical, standard oil-sample bottle of about 120 cc. capacity with Cod Liver Oil, at a temperature between 23° and 28° C., stopper, and immerse the bottle in a mixture of ice and distilled water for five hours. The oil remains fluid and does not deposit stearin (*undestearinated cod liver oil*).

Saponification value: not less than 180 and not more than 192. When carbon dioxide has been used as a preservative, the oil must be exposed in a shallow dish in a vacuum desiccator for twenty-four hours before weighing the sample for determination of the saponification value.

Iodine value: not less than 145 and not more than 180.

Preserve in a cool place, in well-closed containers which have been thoroughly dried before filling. Cod Liver Oil may be bottled or packaged in a vacuum or in the presence of an inert gas.

Assay—Proceed as directed under *Assays for Vitamins A and D in Cod Liver Oil*, pages 4 to 11.

Preparation—Emulsum Olei Morrhuae.

AVERAGE DOSE—(Administer three times daily)—Infants: Metric, 4 cc.—Apothecaries, 1 fluidrachm. Adults: Metric, 8 cc.—Apothecaries, 2 fluidrachms.

Assays for Vitamins A and D in Cod Liver Oil

(To replace the Vitamin Assay on page 489 in the U.S.P. X)

Definitions—As used herein, unless the context otherwise indicates, the term *assayer* means the individual immediately responsible for the interpretation of the assay; the term *assay group* means a group of rats to which the assay oil shall be administered during the assay period; the term *assay oil* means a Cod Liver Oil under examination for its vitamin potency; the term *assay period for the Vitamin A assay* means the interval in the life of a rat between the last day of the depletion period and the twenty-ninth day thereafter or between the last day of the depletion period and the death of the rat; the term *assay period for the Vitamin D assay* means the interval in the life of a rat between the last day of the depletion period and the eleventh day thereafter; the term *assemble* means the procedure by which rats are selected and assigned to groups for the purpose of feeding, care, and observation; the

term *control group* means a group of rats receiving no Cod Liver Oil during the assay period; the term *daily*, for the Vitamin A assay, means six days of each week of the assay period; the term *daily*, for the Vitamin D assay, means each of the first eight days of the assay period; the term *declining weight* means a condition of a rat when the body weight of the rat on any given day is equal to or less than the body weight of the rat on the seventh day prior to the given day; the term *depletion period* means the interval in the life of a rat between the last day of the preliminary period and the first day of the assay period; the term *dose* means the quantity of the Reference Oil or of the assay oil to be fed daily to a rat during the assay period; the term *fed* means made readily available to the rat or administered to the rat by mouth; the term *ground gluten* means the clean, sound product made from wheat flour by the almost complete removal of starch, and contains not more than 10 per cent of moisture, and, calculated on the water-free basis, not less than 14.2 per cent of nitrogen, not less than 15 per cent of nitrogen-free extract (using the protein factor 5.7), and not more than 5.5 per cent of starch (as determined by the diastase method); the term *group for the Vitamin A assays* means six or more rats maintained on the same required dietary regimen during the assay period; the term *group for the Vitamin D assay* means seven or more rats maintained on the same required dietary regimen during the assay period; the term *ophthalmia* means a pathological state of the eye and/or the conjunctiva and/or the tissues anatomically related to the eye, readily discernible macroscopically and usually associated with Vitamin A deficiency; the term *preliminary period* means the interval in the life of a rat between the seventh day after birth and the first day of the depletion period; the term *rachitogenic diet* means a uniform mixture of the food materials, and in the proportions named, in either of the following formulas:

Rachitogenic Diet No. 1

Whole Yellow Maize, ground.....	33 per cent
Whole Wheat, ground.....	33 per cent
Ground Gluten.....	15 per cent
Gelatin.....	15 per cent
Calcium Carbonate (CaCO_3).....	3 per cent
Sodium Chloride (NaCl).....	1 per cent

Rachitogenic Diet No. 2

Whole Yellow Maize, ground.....	76 per cent
Ground Gluten.....	20 per cent
Calcium Carbonate (CaCO_3).....	3 per cent
Sodium Chloride (NaCl).....	1 per cent

The term *Vitamin A test diet* means a food material consisting of the following proportions of the named ingredients of the quality specified:

Vitamin A Test Diet

Casein.....	18 per cent
Salt Mixture (see page 6).....	4 per cent
Yeast, dried.....	8 per cent
Starch.....	65 per cent
Vegetable Oil.....	5 per cent
Vitamin D, a sufficient amount	

Not less than 3 U.S.P. Units of Vitamin D shall be provided in each gram of diet and this vitamin shall be carried by the yeast or the vegetable oil. The ingredients of the Vitamin A test diet shall be free from Vitamin A or shall have been treated so as to reduce the Vitamin A content to such a degree that when the Vitamin A test diet is fed to the control group two-thirds or more of the rats shall manifest, prior

to the eleventh day of the assay period, symptoms of Vitamin A deficiency characterized by both declining weight and ophthalmia. The dried yeast shall carry the Vitamin B complex in such concentration that a daily dose of 0.15 Gm. shall permit an average gain in weight of at least 3 Gm. per week in rats during an interval of four weeks between the twenty-fifth and sixtieth days of age, when the rats are provided ad libitum with a ration which is adequate for optimal growth, except that the ration shall be devoid of the Vitamin B complex.

Salt Mixture No. 1

Calcium Carbonate (CaCO_3).....	134.8 Gm.
Magnesium Carbonate (MgCO_3).....	24.2 Gm.
Sodium Carbonate (Na_2CO_3).....	34.2 Gm.
Potassium Carbonate (K_2CO_3).....	141.3 Gm.
Phosphoric Acid (100 per cent).....	119.3 Gm.
Hydrochloric Acid (100 per cent).....	166.9 Gm.
Sulphuric Acid (100 per cent).....	9.8 Gm.
Citric Acid (H_2O).....	111.1 Gm.
Ferric Citrate ($1\frac{1}{2}\text{H}_2\text{O}$).....	5.7 Gm.
Potassium Iodide (KI).....	0.020 Gm.
Manganese Sulphate (MnSO_4).....	0.079 Gm.
Sodium Fluoride (NaF).....	0.248 Gm.
Potash Alum [$\text{K}_2\text{Al}_2(\text{SO}_4)_4$].....	0.0245 Gm.

The available form of each chemical substance is taken in sufficient quantity to furnish the stipulated equivalent quantity of each chemical. The mixed carbonates and ferric citrate are added to the mixed acids. The specified quantities of KI, MnSO_4 , NaF, and $\text{K}_2\text{Al}_2(\text{SO}_4)_4$ are added as solutions of known concentrations and the resulting mixture is evaporated to dryness in a current of air at from 90° to 100° C. and ground to a fine powder.

Salt Mixture No. 2

Sodium Chloride (NaCl).....	0.173 Gm.
Anhydrous Magnesium Sulphate (MgSO_4).....	0.266 Gm.
Sodium Phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$).....	0.347 Gm.
Potassium Phosphate (K_2HPO_4).....	0.954 Gm.
Calcium Acid Phosphate [$\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$].....	0.540 Gm.
Ferric Citrate ($1\frac{1}{2}\text{H}_2\text{O}$).....	0.118 Gm.
Calcium Lactate.....	1.300 Gm.

Uniformly mix the finely powdered salts.

The assay of Cod Liver Oil for Vitamin A and Vitamin D potency shall be by comparison with the U.S.P. "Reference Cod Liver Oil," by assay procedures conforming in all respects to the following specifications:

Method of Assay for Vitamin A

The Vitamin A assay, comprising the recording of observations of groups of rats throughout specified periods of their lives, while being maintained on specified dietary regimens, and the interpretation of such data, is as follows:

Preliminary period—Throughout the preliminary period each rat shall be raised under the immediate supervision of or according to directions specified by the assayer. Throughout the preliminary period the rats shall be maintained on a dietary regimen which shall provide for normal development in all respects, except that the supply of Vitamin A, or precursors of Vitamin A, shall be limited to such a degree that rats weighing between 40 and 50 Gm. and not exceeding twenty-eight days of age and

subsisting on a suitable Vitamin A deficient ration and water for an interval not exceeding forty-five days shall manifest symptoms characteristic of Vitamin A deficiency.

Depletion period—A rat shall be suitable for the depletion period when the age of the rat does not exceed twenty-eight days, and if the body weight of the rat shall exceed 39 Gm., and does not exceed 50 Gm., and if the animal manifests no evidence of injury, or disease, or anatomical abnormality which might hinder growth and development. Throughout the depletion period each rat shall be provided with the Vitamin A test diet and distilled water ad libitum, and during this period no other dietary supplement shall be available to the animal.

Assembling rats into groups for the assay period—Rats which are suitable for the assay period shall be assembled into groups. For each assay oil there shall be one or more assay groups. In the assay of one assay oil there shall be provided at least one control group and at least one reference group, but one control group and one reference group may be used for the concurrent assay of more than one assay oil. The interval of assembling rats into groups shall not exceed sixty days. On any one day during the interval of assembling rats into groups, the total number of rats that shall have been assigned to make up any one group shall not exceed by more than two the number of rats that shall have been assigned to make up any other group. When the assembling of all groups shall have been completed the total number of rats in each group shall be the same, and the number of rats of one sex in each group shall be the same. Not more than three rats from one litter shall be assigned to one group. When the assembling of all groups shall have been completed, the average weight of the rats in any one group on the day beginning the assay period shall not exceed by more than 10 Gm. the average weight of the rats in any other group on the day beginning the assay period.

Assay period—A rat shall be suitable for the assay period, provided that the depletion period shall have exceeded twenty-four days and shall not have exceeded forty-five days, and provided that a rat shall manifest evidence of Vitamin A deficiency characterized by declining weight and/or ophthalmia. Throughout the assay period each rat of the control, reference, and assay groups shall be kept in an individual cage and shall be provided with the Vitamin A test diet and distilled water, ad libitum. Throughout the assay period each rat in any assay group shall be fed daily a dose of the assay oil, and throughout the assay period each rat in any one reference group shall be fed daily a dose of the reference oil. The reference oil and/or the assay oil may be diluted before feeding with an edible vegetable oil free from Vitamin A. Diluted oil shall be stored in the dark at a temperature not exceeding 50° F. The period of storage shall not exceed seven days. Not more than 0.1 cc. of the diluted oil shall be fed as a daily dose. During the assay period all conditions of environment shall be maintained as uniformly as possible with respect to the assay, reference, and control groups.

Recording of data—On the day beginning the depletion period and at intervals of not more than seven days for the first twenty-one days of that period there shall be a record made of the body weight of each rat. From the twenty-first day of depletion period until the end of the assay period a record shall be made of the body weight and eye condition of each rat at intervals not exceeding five days. The eye condition shall be designated as normal, watery, sensitive to light, swollen, bloody exudate, purulent, opacity of cornea, or any combination of these terms. A record shall be made of the failure of a rat to consume the prescribed daily dose of reference or assay oil.

Vitamin A potency of the assay oil—In determining the Vitamin A potency of the assay oil the performance of the rats of the assay and reference groups shall be calculated for each group on the basis of the difference between the average weight of the

surviving rats and the average weight of the same rats on the day beginning the assay period. The data from the reference group shall be considered valid for establishing the Vitamin A potency of the assay oil only when two-thirds or more of the total number of animals comprising a reference group shall have made individually between the beginning day of the assay period and the twenty-eighth day thereafter an increase in body weight which shall equal or exceed 12 Gm. and shall not exceed 60 Gm., and the data from an assay or reference group shall be considered valid for establishing the Vitamin A potency of the assay oil only when two-thirds or more, but not less than six, of the rats of an assay or reference group have survived twenty-eight days of the assay period. The data from an assay group shall be considered valid for establishing that an assay oil conforms with the U.S. Pharmacopœia standard for Vitamin A in cod liver oil only when two-thirds or more but not less than six rats shall have made individually between the beginning day of the assay period and the twenty-eighth day thereafter an increase in body weight which shall equal or exceed 12 grams. The data from a rat shall be considered valid for establishing the average performance of a reference or assay group only on the condition that the rat has consumed the prescribed dose of oil for at least twenty-two days of the assay period. A Vitamin A assay shall not be considered valid unless two-thirds or more of the total number of animals comprising the control group shall, prior to the eleventh day of the assay period, manifest symptoms of Vitamin A deficiency characterized by both declining weight and ophthalmia. The Vitamin A potency of the assay oil is then calculated according to the following procedure:

Let "R" equal the daily dose in milligrams of the reference oil necessary to produce in a reference group an average gain in weight, "G," of not less than 12 Gm. and not more than 60 Gm.

Let "A" equal the daily dose in milligrams of the assay oil that will produce in an assay group an average gain in weight equal to or greater than "G."

If the product of $\left[\frac{R}{A} \right] \times$ [units per gram of Vitamin A contained in the reference oil] is equal to or greater than 600, then the assay oil contains 600 or more units of Vitamin A per gram of oil and complies with the U.S. Pharmacopœial requirement for Vitamin A potency.

Method of Assay for Vitamin D

The Vitamin D assay, comprising the recording of observations of groups of rats, throughout specified periods of their lives, while being maintained on specified dietary regimens, and the interpretation of such data, is as follows:

Preliminary period—Throughout the preliminary period each rat shall be raised under the immediate supervision of or according to directions specified by the assayer. Throughout the preliminary period the rats shall be maintained on a dietary regimen which shall provide for normal development in all respects, except that the supply of Vitamin D shall be limited to such a degree that rats, weighing between 40 and 60 Gm. at an age of twenty-one to twenty-eight days, and subsisting for an interval of three weeks on a suitable rachitogenic diet, shall manifest evidence of severe rickets.

Depletion period—A rat shall be suitable for the depletion period when the age of the rat does not exceed thirty days, and if the body weight of the rat shall exceed 44 Gm., and does not exceed 60 Gm., and if the animal manifests no evidence of injury, or disease, or anatomical abnormality which might hinder growth and development. Throughout the depletion period each rat shall be provided with the

rachitogenic diet and distilled water ad libitum, and during this period no other dietary supplement shall be available to the animal.

Assembling rats into groups for the assay period—Rats which are suitable for the assay period shall be assembled into groups. For each assay oil there shall be one or more assay groups. In the assay of one assay oil there shall be provided at least one reference group, but one reference group may be used for the concurrent assay of more than one assay oil. The interval of assembling rats into groups shall not exceed sixty days. On any one day during the interval of assembling rats into groups, the total number of rats that shall have been assigned to make up any one group shall not exceed by more than two the number of rats that shall have been assigned to make up any other group. When the assembling of all groups shall have been completed the total number of rats in each group shall be the same, and the number of rats of one sex in each group shall be the same. Not more than three rats from one litter shall be assigned to one group. When the assembling of all groups shall have been completed the average weight of the rats in any one group on the day beginning the assay period shall not exceed by more than 8 Gm. the average weight of the rats in any other group on the day beginning the assay period.

Assay period—A rat shall be suitable for the assay period, provided that the depletion period shall have exceeded eighteen days and shall not have exceeded twenty-five days, and provided that a rat shall manifest evidence of rickets characterized by a distinctive, wobbly, rachitic gait and by enlarged joints. The presence of rickets may also be established by examination of a leg bone of one member of a litter by the "line test" described below. Each rat shall be kept in an individual cage and shall be provided with the rachitogenic diet and distilled water, ad libitum. On any calendar day of the assay period the assay and reference groups shall receive a rachitogenic diet compounded from the same lots of ingredients. Throughout the first eight days of the assay period each rat in any one assay group shall be fed daily a dose of the assay oil, and throughout the first eight days of the assay period each rat in any one reference group shall be fed daily a dose of the reference oil, except that the following deviation from the daily feeding shall be permissible: that the daily dose may be doubled on the day preceding a one-day holiday falling within the first eight days of the assay period. During the remainder of the assay period (i. e., the ninth day and tenth day) neither the assay oil nor the reference oil shall be fed. On the eleventh day of the assay period each rat shall be killed and one or more leg bones examined for healing of the rachitic metaphysis according to the "line test" described below. The reference oil and/or the assay oil may be diluted before feeding with an edible vegetable oil free from Vitamins A and D. The diluted oil shall be stored in the dark at a temperature not exceeding 50° F. and the duration of this storage shall not exceed thirty days. Not more than 0.1 cc. of the diluted oil shall be fed as a daily dose. During the assay period all conditions of environment (particularly with reference to physiologically active radiations) shall be maintained as uniformly as possible with respect to the assay and reference groups.

Line test—The line test shall be made on the proximal end of a tibia or distal end of a radius or ulna. The end of the desired bone is removed from the animal and cleaned of adhering tissue. A longitudinal median section shall be made through the end of the bone with a clean, sharp blade to expose a plane surface through the junction of the epiphysis and diaphysis. In any one assay the same bone of all the animals must be used and sectioned through the same plane. Both sections of the bone shall be rinsed in distilled water and shall then immediately be immersed in a 2 per cent aqueous solution of silver nitrate for one minute. The sections shall then be rinsed in distilled water and the sectioned surfaces of the bone shall be exposed

in water to daylight or other source of actinic light until the calcified areas have developed a clearly defined stain without marked discoloration of the uncalcified areas. Records shall be made immediately of the extent and degree of calcification of the rachitic metaphysis of every section. It shall be permissible to use modifications of the described procedure for staining, provided that such modified procedures clearly differentiate between calcified and uncalcified areas.

Recording of data—On the day beginning the assay period and on the tenth day thereafter a record shall be made of the body weight of each rat. A record shall be made of the quantity of rachitogenic diet consumed per rat per diem during the assay period. Numerical values shall be assigned to the extent and degree of calcification of the rachitic metaphysis of the bones examined by the line test so that it will be possible to average the performance of each group.

Vitamin D potency of the assay oil—In determining the Vitamin D potency of the assay oil the average performance of groups with respect to healing of the rachitic metaphysis shall be considered, provided that the average performance of a reference group with respect to calcification of the rachitic metaphysis shall be determined by the data from rats which individually show an extent and degree of calcification in the rachitic metaphysis as determined by the line test equal to or greater than a condition described as a positive macroscopic evidence of calcification, but less than an extent and degree of calcification described as complete healing. The data from a reference group shall be considered valid for establishing the Vitamin D potency of the assay oil only when two-thirds or more, but not less than seven rats, show individually an extent and degree of calcification of the rachitic metaphysis equal to or greater than a condition described as positive macroscopic evidence of calcification, but less than an extent and degree of calcification described as complete healing. The data from an assay group shall be considered valid for establishing that an assay oil conforms with the U.S. Pharmacopœia standard for Vitamin D in cod liver oil only when two-thirds or more, but not less than seven rats, show individually an extent and degree of calcification of the rachitic metaphysis equal to or greater than a condition described as positive macroscopic evidence of calcification. The data from a rat shall be considered valid for establishing the average performance of a group only on the condition that the weight of the rat on the eleventh day of the assay period shall equal or exceed the weight of the rat on the beginning day of the assay period and that the rat has consumed 2 or more Gm. of the rachitogenic diet on each day of the assay period and 40 Gm. or more of the rachitogenic diet during the assay period and on the condition that the rat has consumed each prescribed dose of cod liver oil within twenty-four hours from the time it was fed. The Vitamin D potency of the assay oil is then calculated according to the following procedure.

Let "R" equal the daily dose in milligrams of the reference oil necessary to produce in a reference group an average extent and degree of calcification "C" not less than a condition described as a narrow continuous line of calcification but less than an extent and degree of calcification described as complete healing.

Let "A" equal the daily dose in milligrams of the assay oil that will produce in an assay group an average extent and degree of calcification equal to or greater than "C."

If the product of $\left[\frac{R}{A} \right] \times$ [units per gram of Vitamin D, contained in the reference oil] is equal to or greater than 85, then the assay oil contains in each gram 85 or more units of Vitamin D and complies with the U.S. Pharmacopœial requirement for Vitamin D potency.