

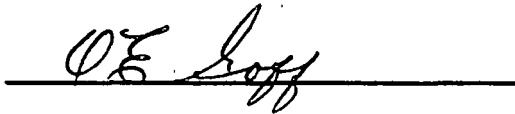
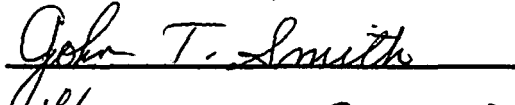

August 17, 1970

To the Graduate Council:


I am submitting herewith a dissertation written by Lloyd Henry Littlefield, entitled "The Site of Absorption of Xanthophylls and Factors Affecting Pigmentation of Chickens, Egg Yolks, and Products Made From Egg Yolks." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.


Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:


Vice Chancellor for
Graduate Studies and Research

THE SITE OF ABSORPTION OF XANTHOPHYLLS AND FACTORS
AFFECTING PIGMENTATION OF CHICKENS, EGG YOLKS,
AND PRODUCTS MADE FROM EGG YOLKS

A Dissertation
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Lloyd Henry Littlefield
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ABSTRACT

A total of four experiments were conducted to determine the location of the site of absorption of xanthophylls, to determine the relationship of egg yolk color produced by various feed xanthophylls to the color of mayonnaise, and to study the effect of the level of dietary cow manure, age, ambient temperature and feed consumption on xanthophyll pigmentation of hens and egg yolks.

Increases in the level of blood xanthophylls and visual pigmentation of xanthophyll depleted hens were used to measure the absorption of xanthophylls. Surgical removal of either the duodenum, jejunum, ileum or large intestine resulted in a slight but significant decrease in absorption of xanthophylls when compared to the sham operated chicks and ligation of the ceca resulted in a slight increase. Only the removal of that section of the jejunum-ileum affected by E. maxima resulted in chicks with no significant absorption when compared to chicks fed a diet free of xanthophylls. It is concluded that most absorption of xanthophylls in the chick takes place in this middle section of the jejunum-ileum.

Mayonnaise was made with yolks from hens fed diets containing no xanthophyll, 23 milligrams of xanthophyll per kilogram and the latter diet plus 66 milligrams of xanthophyll per kilogram from yellow, orange or red concentrate. The egg yolks appeared different, but no significant differences were found between the yolks from hens fed the diets with the added concentrates.

As measured by a triangle test, mayonnaise made from the egg

yolks from hens fed the xanthophyll free diet had the lightest color. Mayonnaise made from egg yolks from hens fed the "layer" plus yellow concentrate and "layer" plus orange concentrate were not significantly different.

The red concentrate had the greatest carry-over effect. The color of mayonnaise depends on the kind and amount of xanthophylls present.

Dried cow manure was added at the rate of 0, 2.5, 5, or 10 kilograms per 100 kilograms of diets containing 0 and 23 milligrams of xanthophylls per kilogram to determine the effect on pigmentation. There was a high positive linear correlation between the amount of cow manure added and the amount of xanthophyll in the blood. There was a high negative linear correlation between pigmenting efficiency and the amount of cow manure added to the diet. Cow manure was a good source of xanthophylls, but its xanthophylls were not efficiently utilized.

The effect of age, temperature and amount of feed consumed on yolk pigmentation was studied. Both young and old hens were housed in rooms designed to simulate winter and summer temperatures. Half of the hens of each age group at each temperature had access to feed ad libitum and the other half received feed limited to 90 percent of that consumed in the hot rooms by hens on the ad libitum regime.

The hens in the "cool" rooms produced egg yolks with the greatest pigmentation. A four months difference in age was not a major factor in causing light colored yolks. However, egg yolk color generally became lighter as the hens grew older. In one

trial limited feeding resulted in deeper pigmented yolks as measured by yolk xanthophyll levels. It is concluded that the reduction of the yolk xanthophyll levels during the summer is associated more with high temperature per se, and less with aging and a drop in feed consumption as generally assumed.

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I. INTRODUCTION

It has long been known that the yellow color of egg yolks, skin, adipose tissue and blood serum in the domestic fowl is due to a group of carotenoids called xanthophylls. The consumer preference for the degree of pigmentation in dressed poultry and eggs varies from country to country, and from area to area in these countries (Raskopf, et al., 1961; DeGrootte, 1970). The light-colored egg yolk is preferred in some areas while the dark-colored egg yolk is desired in other locations (Mountney, et al., 1962; Roy, 1968). Consumers will often pay more for eggs with yolks the color of their choice. Manufacturers of noodles, mayonnaise, cakes and other products made from eggs demand and will often pay a premium for eggs with deeply pigmented yolks (Sullivan and Holleman, 1962; Anonymous, 1963).

Table eggs, until the late 1930's, were primarily from barnyard flocks and had a deep yellow yolk color. The typical hen of that period ate grass, insects, yellow corn, a little mash and some morsels salvaged from the path of the cow. With confinement of layers it has become necessary for the poultry researcher to find ways to get maximum use of the xanthophyll sources remaining in the diet, to search for new sources of the pigments and to examine the factors involved in the absorption and utilization of these pigments. Little has been reported about the site of absorption of xanthophylls, and this basic information is needed before steps can be taken to aid the chicken in the maximum

utilization of these pigments.

The manufacturers of egg yolk-containing products normally pay less for eggs during the summer months partly because the eggs are less deeply pigmented in this season. The decrease in pigmentation is associated with a decrease in feed intake as a result of high temperatures, but it is not known if it is due to age, feed consumption or the effect of the high temperature on the metabolism of xanthophylls.

The objectives of the research herein reported were:

- (1) To determine the site or sites of absorption of xanthophylls in the chicken.
- (2) To determine the relationships between xanthophylls in the feed, the color of the egg yolks produced and the color of mayonnaise produced from the egg yolks.
- (3) To determine the effect of various levels of cow manure added to the laying diet on the pigmentation of egg yolks and on the level of xanthophylls in egg yolks and blood.
- (4) To determine the variations in pigmentation of egg yolks from Leghorn-type hens due to such factors as amount of feed consumed, environmental temperature and age of the hens.

II. REVIEW OF LITERATURE

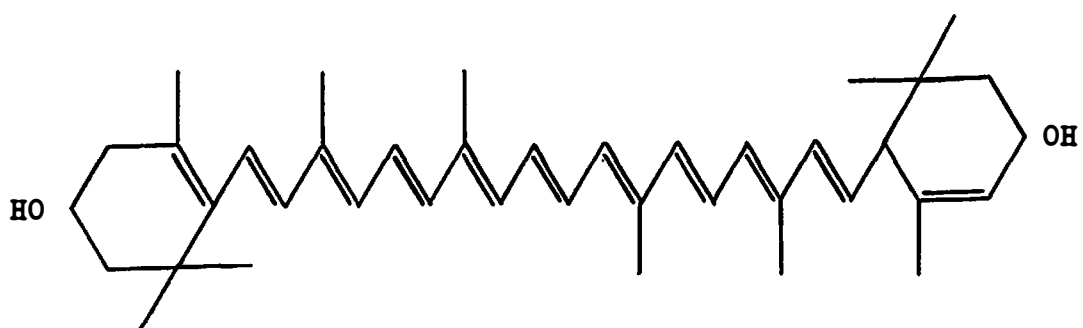
Discovery

The term xanthophyll was coined by Berzelius (1837) for the yellow, alcohol-soluble pigment in autumn leaves. This pigment was first shown to occur in green leaves by Frey in 1864 according to Goodwin (1954) and confirmed by Stokes (1864). It is now known that these researchers were dealing not with a single compound, but with a group of closely related chemicals.

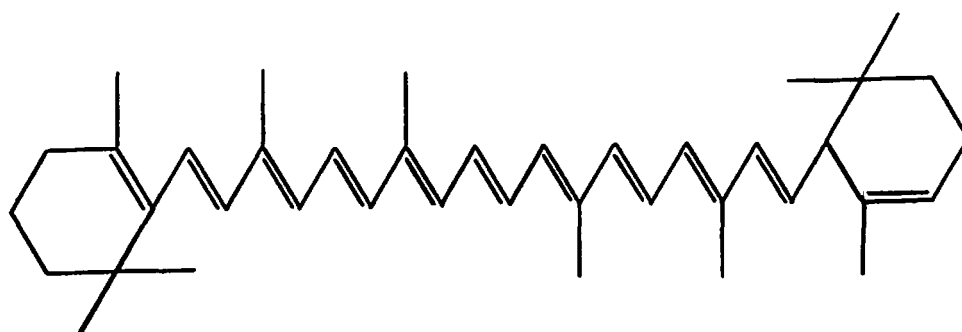
Nomenclature

The literature is not consistent in the use of the term "xanthophyll." The compound labeled "xanthophyll (lutein)" in Figure 1 was called "xanthophyll" by Karrer et al. (1929) and the "Union internationale de Chimie" (Anonymous, 1946), "lutein" by Kuhn et al. (1931), "beta-xanthophyll" by Goodwin (1954) and "xanthophyll B" by Letendre et al. (1968). The oxygen-containing carotenoids were called "phytoxanthins" by Karrer et al. (1929), "xanthophyll" by Kuhn et al. (1931) and "xanthophylls" by Goodwin (1954).

In this paper the term "lutein" or the term "xanthophyll (lutein)" is used for the yellow, leaf pigment $C_{40}H_{56}O_2$ (Figure 1). The terms "xanthophyll" or "xanthophylls" are used as the more general names to indicate all the oxygen-containing carotenoids as suggested by Wagner and Mitchell (1960) and as used in most scientific papers on poultry. The term "carotenoid" includes all



xanthophyll (lutein)



alpha-carotene

Figure 1. Structure of Xanthophyll (Lutein) and Alpha-Carotene.

yellow to red pigments of aliphatic or alicyclic structures composed of isoprene units, usually eight in number, linked so that the center two methyl groups of the molecule are in positions 1:6 and all other lateral methyl groups of the molecule are in position 1:5 (Figure 1, page 4). These carotenoids were described by Fruton and Simmonds (1958) as light yellow to purple pigments often found in the unsaponifiable lipids of plants, animals and microorganisms. Karrer and Jucker (1950) considered the naturally occurring carotenoids as derivatives of the red pigment lycopene which has the empirical formula $C_{40}H_{56}$.

Structure

The empirical formula ($C_{40}H_{52}O_2$), the molecular weight (565), the melting point ($172^\circ C.$), and other chemical and physical data were determined when Willstatter and Mieg (1907) isolated xanthophyll (lutein) from green plants in the crystalline state. Karrer et al. (1930a) and Strain (1938) showed the structure of lutein to contain two hydroxyl groups (Figure 1). The first evidence of the presence of double bonds was the reacting of lutein with $KMnO_4$ in the Bayer test for unsaturation by Karrer et al. (1930b). Karrer et al. (1930c) also determined that lutein differed from carotene only in the structure of the two carbon rings. Smith (1931) established that there were nine double bonds easily saturated and, therefore, that they were probably conjugated.

The structural difference in alpha-xanthophyll and beta-xanthophyll was discovered by Nilson and Karrer (1931). The same

year Karrer et al. (1931) found optical rotation of xanthophyll to be equal to 70° , and determined that the two hydroxyl groups were located on the rings. The location and number of methyl groups were determined by means of chromic acid and permanganate oxidation by Karrer et al. (1933).

According to Mackinney and Little (1962) the color of the xanthophylls comes from the conjugated double bonds found in all carotenoids in their central chains. These aliphatic central chains are made up of four dehydrogenated isoprene units and the two rings are alpha-ionone and beta-ionone rings (Figure 2).

Distribution

The largest source of xanthophylls is from green plants where they are found along with carotene and chlorophyll according to Kuhn and Winterstein (1930), but they are also found in red and yellow blossoms, in various fruits, green insects, fats, soil, peat, skin of birds, feathers, and in egg yolk. Mackinney (1935) reported that many plants contain xanthophylls almost exclusive of other carotenoids, however, many plants have various other pigments to give the total color (Dreosti et al., 1966). These carotenoids are primarily in the all-trans form (Fox, 1953). The xanthophylls of the leaves, unlike those in the non-photosynthetic tissues (flowers and fruits), are always unesterified (Goodwin, 1965). Xanthophylls have been isolated many times from egg yolks and identified as the same pigments found in leaves (Thudicum, 1869; Schunk, 1903; Willstatter and Escher, 1911; Palmer and Kempster, 1919a).

Kuhn et al. (1931) found that egg yolk pigments contained

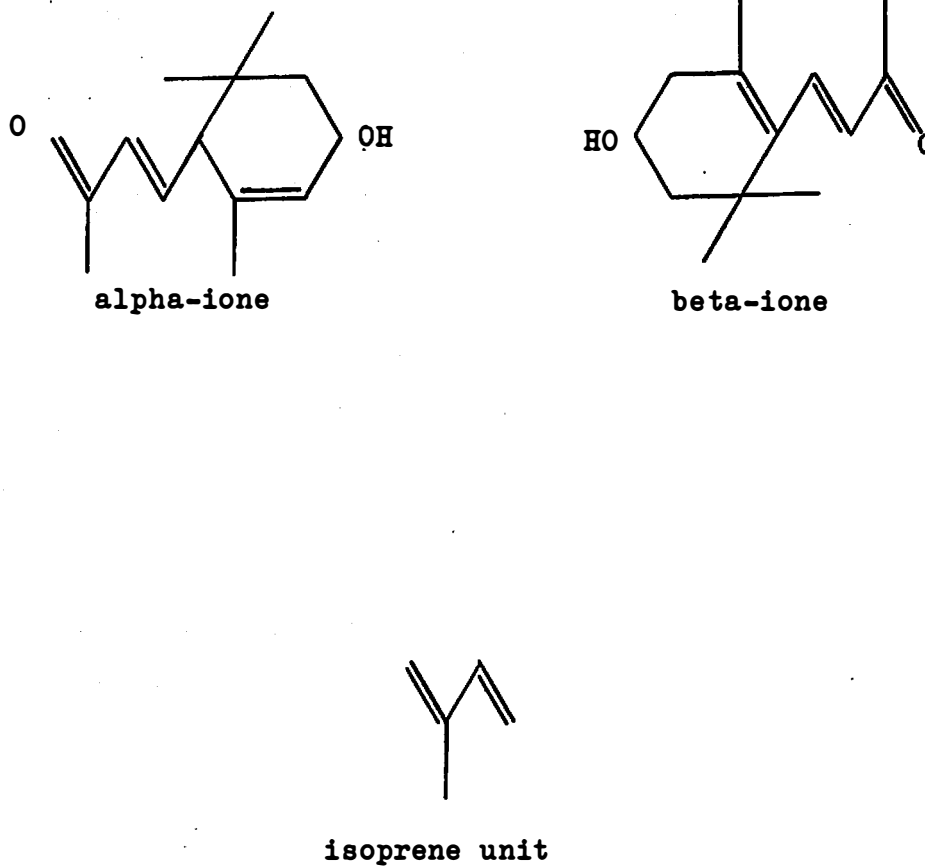


Figure 2. Structure of Alpha-Ione, Beta-Ione and the Isoprene Unit.

70 percent lutein and 30 percent zexanthin, while Scott and Norris (1959) reported cryptoxanthin also present in egg yolks. Several other carotenoids have been isolated in both egg yolks and skin of chickens (Gillam and Heilbran, 1935; Smith and Perdue, 1966; Fritz et al., 1957a; Quackenbush et al., 1965).

Biosynthesis and Metabolism

The biosynthesis of the carotenoids involves the production of a C₄₀ polyene, which undergoes a sequential desaturation followed by cyclization to form the various carotenes (Ciegler, 1965). The xanthophylls are generally thought to be synthesized from their corresponding carotene as the amount of the particular carotenes are inversely related to the amount of the corresponding xanthophylls.

Xanthophyll is formed in plants along with other carotenoids and chlorophyll, but little is known about its metabolic pathway. Goodwin (1958) showed that ¹⁴C-acetate and mevalonate are readily absorbed by excised seedlings and converted into xanthophyll and not into carotene. He also showed that ¹⁴CO₂, on the other hand, is incorporated with a marked preference for beta-carotene. Letendre (1968b) in a conversation with this writer told of a method by which he produced ¹⁴C-xanthophyll by addition of ¹⁴C-mevalonic acid to the media on which he raised alfalfa seedlings.

Isler and Zeller (1957) indicated that eight isoprene units of beta-carotene are derived from acetic acid. They pointed out that these isoprene units were lined head-to-tail except for the

center two units which were lined head-to-head. Ruzicka (1959) suggested that these xanthophylls may result from head-to-tail dimerization of geranyl pyrophosphates.

According to Isler and Zeller (1957), Wolf and coworkers in 1956 produced an activated isoprene unit from acetic acid from coenzyme A and Grob in 1956 used this activated isoprene unit to trace the origin of 26 of the 40 carbon atoms of beta-carotene.

Fox (1953) lists the possible dispositions of xanthophylls as:

- (1) Faecal rejection in chemically unchanged condition.
- (2) Assimilation and storage in chemically unchanged conditions.
- (3) Assimilation and conversion into other carotenoids (e.g. astaxanthin 'canary-xanthophyll,' fish xanthophylls, A-vitamins, etc.).
- (4) Assimilated portions consumed oxidatively.

Function

Gillam and Heilbran (1935) listed several vitamin A active xanthophylls in egg yolk, but did not list xanthophyll (lutein) as vitamin A active. Smith and Perdue (1966) found alpha-carotene in the skin and shanks of chickens on a diet free of carotenes. Xanthophyll, however, has been assumed to have no vitamin A activity by most investigators (Steenbock, 1919; Euler et al., 1934; Bohren et al., 1945, Ganguly et al., 1953). However, since the carbon structures of both the pro-vitamin A, alpha-carotene, and xanthophyll are identical (Figure 1, page 4), other investigators have attempted unsuccessfully to show that xanthophyll does have vitamin A activity

(Palmer and Kempster, 1919a; Plimmer et al., 1923; Willimott and Moore, 1927; Virgin and Klussman, 1932; Rydom, 1933; Kemmere et al., 1947; Callison, et al., 1951).

Temperton and Dudley (1947) were able to decrease chick mortality and increase growth rate by adding xanthophyll to a starter diet deficient in carotenes and vitamin A. They suggested that the physiological effects of xanthophyll, although complimentary to those of vitamin A, were independent in action. Moore (1957) concluded that xanthophyll had no essential role except possibly in vision and might even interfere with vitamin A storage by interfering with enzyme systems necessary for the metabolism of carotenes. Fritz et al. (1958) reported that xanthophylls had no effect on weight gains, feed conversion or finish of broilers.

Absorption, Transportation, Storage and Loss

Palmer (1915) reported that the chicken absorbed carotene and xanthophylls which he found in the egg yolk, body fat and blood serum of hens. He could find no difference in the level of xanthophylls in the blood or the pigmentation of the yolk when the hens were fed xanthophyll-free diets if he supplied carotene to the diet. He noted that there were species differences in storage of carotenoids, with the cow primarily storing carotene in milk and body fat and in blood serum.

Animals were further classified by Goodwin (1950) on their ability to store carotenoid as (1) those storing both carotenes and xanthophylls such as man, (2) those storing primarily carotenes

such as cattle, (3) those storing primarily xanthophylls such as poultry and (4) those storing very little carotenes or xanthophylls such as swine.

Fox (1953) used a similar breakdown:

- (1) 'Carotene animals' (Zechmeister) selectively assimilate and store only or chiefly the hydrocarbon type of lipochrome (e.g. horse, cow, certain invertebrates).
- (2) Conversely, 'xanthophyll animals' store only polyene alcohols, rejecting carotenes in the faeces or possibly converting some of them into xanthophylls (e.g. domestic hen and other birds, most fishes and invertebrates).
- (3) 'Non-carotenoid animals' store little or none of these pigments, whether by quantitative voidance or by complete degradation in the body (e.g. swine, carnivorous and certain other mammals, a few invertebrates).
- (4) 'Non-selective animals' readily assimilate and store both oxygenated and hydrocarbon types of lipochrome (e.g. frog, man, octopus).

Goodwin (1965) reported that the chicken does not reject carotene as Fox (1953) indicated, but that carotene is converted into vitamin A in the wall of the intestine.

Palmer and Kempster (1919b) found the xanthophylls in the epidermis of chickens to be separated from the fat. It was found to be located in rather large granular masses lining blood vessels in the derma, but it was found primarily in the deeper portion of the epidermis. The loss of pigment from the skin was found to occur first in the outermost layers by oxidation.

Loss of pigment in hens was reported by Blakeslee and Warner (1915) to be correlated to egg production. They suggested that the xanthophylls were immobilized from the epidermis and deposited in yolk. However, Palmer and Kempster (1919b), using a staining technique, found the loss to occur first from the outer epidermal layers, then the middle and inner layers. They found that hens

in production could not replace the color even when fed high levels of xanthophylls, but Douglas (1966) found that pullets in production could deposit xanthophylls in their beak, eye ring and vent when fed diets high in this pigment.

Bohren et al. (1945) reported that xanthophylls are simply absorbed in the intestine, transported by the body fluid and laid down in the skin and shanks.

A deficiency of either thyroxin (Goodwin, 1954) or bile salts (Irvin et al., 1941; Letendre, 1968a) have been shown to be associated with a decrease in the absorption of xanthophylls.

Although it is generally accepted that xanthophylls are absorbed intact (Wilson, 1962), Letendre (1968a) suggested that hydrolysis may be necessary for the uptake of xanthophylls from the gut as their esters were found in the blood while they were in the free form in the skin and in an unesterified state in plants (Bickoff et al. 1954a; Quackenbush et al., 1961).

Krinsky et al. (1958) reported that carotenoids are primarily concentrated in the beta-lipoprotein in the plasma. Wilson (1962) concluded that xanthophylls are absorbed by lipoprotein for active transport.

Factors Affecting Xanthophyll Pigmentation

The problem concerning xanthophyll pigmentation was divided into three phases by Bunnell and Bauernfeind (1958) at the XI World's Poultry Congress as:

- (1) the production of adequately pigmented broilers and fryers by feeding carotenoid containing rations;

- (2) the production of market eggs of acceptable yolk color by feeding laying hens rations adequate in carotenoids;
- (3) the production of chicks of good yellow shank color by feeding breeder flocks rations of proper carotenoid content.

At this same meeting Fritz et al., (1958) pointed out that pigmentation per se is dependent on several factors other than the carotenoids present in the feed.

Genetic. The genetic control of pigmentation of poultry and eggs has been studied for many years (Bateson, 1902; Hurst, 1905; Dunn, 1925; Hutt, 1949; Parker et al., 1925). Bateson (1902) reported that the variation between white and yellow skinned chickens was the result of a single autosomal gene (Ww) where white skin (W) was dominant to yellow skin (w). Farnsworth and Nordskog (1955) and Collins et al. (1955) presented evidence of breed and strain differences in pigmentation when fed the same diet.

Moyer and Collins (1960) found differences in shank pigmentation within a strain and suggested that it would be possible to select for "high" and "low" lines within a strain. This suggestion resulted in the development of "high" and "low" lines by Letendre et al. (1968). Letendre (1968a) suggested that the difference between the "high" and "low" lines was due to differences in carotenoid metabolism.

Sex. Contradictory data were found regarding the effect of sex on pigmentation. No significant differences were found by Squibb et al. (1953b, 1955) in the level of carotenoids in blood serum between males and females fed the same diet. However, Douglas (1966) showed that foot and skin pigmentation scores and the level

of blood xanthophylls of males were higher than those of females.

Feedstuffs and feed additives. It has long been known that a relationship exists between the xanthophylls in feed and the pigmentation of poultry and eggs (Thudicum, 1869; Schunk, 1903; Wills-latter and Escher, 1911; Palmer and Kempster, 1919c). Even though Williams et al. (1963) found very small quantities of beta-carotene in the blood serum, adipose tissue, liver, egg yolks and skin, it is generally agreed that beta-carotene is not stored or layed down in the yolk by the domestic fowl (Fox, 1953).

The principle sources of xanthophylls in poultry diets are yellow corn, dehydrated alfalfa meal and corn gluten meal. The value of yellow corn as an egg yolk pigments was pointed out by Palmer (1915), and an analysis of yellow corn by Williams et al. (1963) showed that the xanthophylls in yellow corn were zeaxanthin, the major pigment, and cryptoxanthin, the minor pigment. Dehydrated alfalfa meal analyzed by Bickoff et al. (1954a) contained 40 different carotenoids, 87 percent of which was xanthophyll, cryptoxanthin, zeaxanthin, violaxanthin and neoxanthin.

Many comparisons have been made between the various feedstuffs as to their efficiency as pigmenters. Although xanthophylls in corn are generally considered to be more completely utilized by chickens than those in alfalfa meal and corn gluten meal (Ratcliff et al., 1959; Ratcliff et al., 1962; Williams et al., 1963), it takes more corn to produce the same pigmenting effect due to the lower level of xanthophylls in corn. Holleman and Sullivan (1959) found corn, alfalfa and added pure xanthophyll all effective pigmenters.

Using a new method of measurement to determine the level of feed xanthophylls, yellow corn, alfalfa meal and corn gluten meal were shown to be equally good pigmenters per unit of xanthophylls (Koehler et al., 1967). Kuzmicky et al. (1968) confirmed their finding.

According to Ratcliff et al. (1962) and Day et al. (1963) new strains of corn were developed at Mississippi State University which contain about 18 milligrams of xanthophylls per pound or about two times that of "normal" corn. If this corn becomes generally used in poultry rations it would supply most of the need for xanthophylls in the diet of broilers and of hens producing "table eggs."

Other green meals have been compared to alfalfa meal as to their pigmenting value. Coastal bermuda grass was found to be as good or better than dehydrated alfalfa meal (Barnett and Morgan, 1959; Wheeler and Turk, 1961 and 1963; Wilkinson, et al., 1968; Wilkinson and Barbee, 1968). Clover meal was shown superior to alfalfa (Ratcliff et al., 1961; Ratcliff et al., 1962). Algae were reported to be similar to alfalfa (Grau and Klein, 1957; Moorehouse, 1961; Madiedo and Sunde, 1962; Madiedo and Sunde, 1964).

Many green meals probably unheard of by the average poultryman have been studied. Alfalfa has been compared to aquatic plants with variable results (Black, 1953; Høie and Sannan, 1960; Jensen, 1963), peanut vine meal (Cottier et al., 1965), ramie, banana leaf, desmodium, kikuyu grass (Davis et al., 1947; Squibb et al., 1950, 1953a, and 1953b) and lettuce meal (Schaible et al., 1954).

In order to find more concentrated sources of pigments several investigators fed, and found as good pigments, the petals of aquatic flowering plants (Cregar et al., 1963) and marigolds (Brambila et al., 1962 and 1963). Various materials containing red pigments such as pimento, paprika, mud, tomatoe paste and lobster shells produced undesirable red yolks when fed at high levels, but were found to be satisfactory at lower levels with a diet containing yellow corn (Brown, 1930; Mackay et al., 1963; Nelson and Baptist, 1968). Pigment concentrates made largely of marigold pigments have been used with success (Douglas, 1966).

Runnels (1970) told the author of experiments he did several years ago about feeding broccoli to broilers. He found the broccoli to be a good pigmenter, but at high levels it imparted a broccoli taste to the meat.

The variation found in evaluating the various feeds is probably due to losses during dehydration and/or storage due to oxidation (Knowles et al., 1968) and isomerization (Bickoff et al., 1954a). Bartov and Bornstein (1967) found that storage reduced the rate of utilization of xanthophylls of yellow corn and was related to an increase in the free fatty acid content of the corn.

All commercial-type poultry diets contain certain nutritive and non-nutritive additives, including certain vitamins, trace minerals and antioxidants. All of the additives discussed here are nutrients except the antioxidants which are added to reduce spoilage or oxidative breakdown of the nutrients in the feed.

The deleterious effects of vitamin A on pigmentation have

been noted by several experimenters (Rubin and Bird, 1941; Sunde, 1962; Dua et al., 1965; Dua et al., 1967). Kivimäe et al., (1965) found that at non-therapeutic levels, the amount of vitamin A in the diet varied independently of the pigmentation. March and Biely (1964) found no effect on egg yolk color using therapeutic levels of vitamin A; however, they were feeding diets very low in xanthophylls in as much as they contained only five percent yellow corn and no other source of xanthophylls.

Vitamin K active compounds have been shown to increase pigmentation (Griminger and Fisher, 1960), reduce pigmentation (Mitchell, 1961), and to have no effect on pigmentation (Smidt et al., 1965). Other authors have reported suppressing effects on pigmentation due to meat scraps, fish meal and soybean oil meal (Culton and Bird, 1941), and cod liver oil and manganese (Hammond and Harshaw, 1941; Goldhaber et al., 1950).

Added dietary fat has not had a consistent effect on pigmentation of broilers. Dietary fat added at the five percent level increased pigmentation (Day and Williams, 1958), but three percent added fat had no effect (Donaldson and Gordon, 1960). Douglas (1966) found increased pigmentations with 3.7 percent added fat.

Antioxidants have produced inconsistent effects on the pigmentation value of feeds. Studies of antioxidants, such as N,N' diphenyl-p-phenylenediamine (DPPD) and 2,6 di-tertiary-butyl-4-methyl phenol (BHT), in prevention of loss of vitamin E and vitamin A (Bunnell et al., 1955; Matterson et al., 1955a) led to

the study of their use to increase pigmentation (Matterson et al., 1955b). Wilgus (1954), Matterson et al. (1955b), Potter et al. (1956), Matterson et al. (1956) and Fritz et al. (1957b) reported that DPPD gave significant increases in pigmentation. However, Williams et al. (1960) could find no such increase, while Harms et al. (1958) showed that the addition of DPPD to broiler rations significantly depressed pigmentation.

Potter et al. (1956), Elrod et al. (1958) and Ratcliff et al. (1961) reported only slight improvement in pigmentation with BHT but Fritz and Warton (1957) and Day and Williams (1958) found no improvement in pigmentation due to BHT.

Ethoxyquin has been shown to be especially beneficial with increases of up to 100 percent in the pigmenting value of feed (Harms, 1960; Waldroup et al., 1960; Ratcliff et al., 1961; Anjaneylu et al., 1961; Madiedo and Sunde, 1964; Bartov and Bornstein, 1966).

Availability of xanthophyll. The availability of dietary xanthophylls to the chicken has been studied through the use of naturally occurring pure and synthetic xanthophylls. Peterson et al. (1939) concluded that the high efficiency of yellow corn was due to zeaxanthin, but Williams et al. (1963) suggested that it was due to the small cryptoxanthin content which gave a richer color.

Marusich et al. (1960) tested several synthetic carotenoids (zeaxanthin, capsanthin, isozeaxanthin, isozeaxanthin diacetate, violaxanthin, canthaxanthin, isozeaxanthin dimethyl ether and beta-apo-8'-carotenal) as yolk pigmenters. With the exception of violaxanthin and beta-apo-8'-carotenal all pigments produced orange to

orange-red yolks, depending on the level in the feed. Little effect was noted from feeding violaxanthin while beta-apo-8'-carotenal produced a light yellow yolk.

Tortuero (1968) found that alfalfa meal and red xanthophylls gave more intensely colored yolks than either alfalfa meal or red xanthophylls alone.

Canthaxanthin was also used successfully as a pigments by Marusich and Baurenfeind (1962), Farr et al. (1962), Camp et al. (1963), Rauch (1965) and Douglas (1966). Williams (1963) reported that the principal xanthophylls of yellow corn (zeaxanthin) and alfalfa (xanthophyll) were equally well utilized when supplied in purified form. Zeaxanthin and cryptoxanthin, but not neoxanthin or violaxanthin, were found to be effective pigmenters (Kuzmicky et al., 1969).

Disease. Bird (1953) reported that both coccidiosis and respiratory diseases hindered pigmentation in chickens. Mitchell (1961), Mitchell et al. (1961) and Bletner et al., (1966), found depigmentation and low levels of blood serum xanthophylls 12 days after inoculation of chicks with sporulated Eimeria maxima oocysts.

Douglas (1966) found similar losses of pigment two to three weeks after inoculation of chicks with either E. maxima or E. necatrix. Increasing the level of xanthophyll could not overcome this effect, but intramuscular injection of xanthophylls improved the pigmentation, suggesting that the primary effect was prevention of absorption from the gut. Other investigators found decreased pigmentation with field cases of coccidia (Fritz et al., 1957b;

Ratcliff et al., 1961; Mitchell, 1961; Fritz, 1962; Barnett and Stephens, 1963; Day et al., 1963; Couch et al., 1963; Stephens, 1965).

Squibb et al. (1955) indicated that capillaria infestations could result in "blond" or "platinum" yolks in commercial flocks. Very light colored or "platinum" yolks were produced by hens that were neither fed high vitamin A nor infected with either coccidia or capillaria (Berg et al., 1963). The occurrence of "platinum" yolks was eliminated by addition of antibiotics or furazolidone to the layer diet. Feeding of fecal material from affected birds produced the condition in normal birds (Berg et al., 1963; Nelson and Baptist, 1968).

Barnett and Stephens (1963) fed feces of birds producing pale yolks to birds producing dark yolks in hopes of infecting them with intestinal microbes responsible for the difference in yolk color. They were unable to show a significant change in yolk color, but did find a difference in the intestinal microflora and recovered microbes of various kinds, which were associated with pale or dark yolks.

Feed intake. Little research on the effect of feed intake as related to pigmentation has been reported. Several workers have suggested that the poorer pigmentation of egg yolks and broilers during certain periods, especially during the summer months might be due to lower feed intake (Fritz et al., 1957b; Kingan and Sullivan, 1964; Smidt et al., 1965). Douglas (1966), however, found that reducing feed intake of broilers by as much as 50 percent did not significantly decrease pigmentation and a reduction of 70 percent

in the feed intake resulted in increased pigmentation.

Temperature. A search of the literature disclosed no reports measuring the effect of temperature on egg yolk pigmentation. Some investigators have noted a decrease in yolk pigmentation or a decreased efficiency of xanthophyll utilization during the summer months (Farr et al., 1962; Carlson et al., 1964; Kingan and Sullivan, 1964; Marrett et al., 1968).

Milligan and Winn (1964) reported that the pigmentation was lower in chicks maintained in rooms with temperatures of 80° to 100° F. than in rooms maintained at 46° to 70° F. Douglas (1966) showed that ambient temperatures of 90° F. resulted in chicks with lower pigmentation scores than when chicks were kept at temperatures of 70° to 80° F. He collected evidence to show this was not due to reduced feed intake.

Age. No reported experiments were found on the effect of age of the hen on egg yolk pigmentation, but several reports have indicated that there are special requirements for xanthophylls of chicks during the first and second half of the growing period.

During the first week after hatching, the chick normally has stores of xanthophylls in the unabsorbed yolk to supplement its diet (Davis and Kratzer, 1958). Mann (1946) reported that chicks only a few days old could not utilize xanthophylls, but Douglas (1966) showed that chicks in the first few days after hatching could utilize xanthophylls. He also showed that their pigmentation at four weeks was dependent on their diet, and not the diet fed their dams as was earlier observed by Hammond and Harshaw (1941).

It has been reported that good pigmentation can be obtained by feeding xanthophylls only in the last half of the growing period (Fritz et al., 1957a; Mitchell et al., 1961; Combs and Nicholson, 1963). Couch et al. (1963) decreased this time to the last 2.5 to 3 weeks with satisfactory pigmentation. Others have reported data to support the opposite point of view that the most desirable pigmentation results from feeding the same level of xanthophylls during the entire eight- or nine-week period (Day and Williams, 1958; Bartov and Bornstein, 1967 and 1969).

Amount of xanthophylls required. The previously mentioned factors affect the dietary requirements of chickens for the xanthophylls. Day et al. (1963) concluded from a review of the literature that the requirements of xanthophylls in the diet varied by type of diet. The information in the papers reviewed indicated that the requirement for satisfactory pigmentation of broilers varied from 6 to 10 milligrams of xanthophylls per pound of feed. The requirements for layer diets producing table eggs varied from 5 to 8 milligrams per pound of feed, and if the eggs were to be used by egg breaking plants the requirements listed were from 30 to 45 milligrams per pound of feed.

Fritz and Wharton (1957) reported maximum pigmentation of the skin of broilers as detectable by a panel of judges to be produced with 25 milligrams of xanthophyll per pound of feed, and that "good average pigmentation" was produced with 12.5 milligrams per pound of feed. House (1957) reported 9.5 to 10.0 milligrams of xanthophyll per pound of diet were necessary for "adequate

pigmentation" of broilers. Fritz et al. (1957b) considered that 12.5 milligrams per pound of diet was required to produce good pigmentation. Yellow corn diets supplemented with 10 percent of either alfalfa meal or corn gluten meal produced egg yolks desired by the egg breaking industry according to Sullivan and Holleman (1962).

Mitchell (1961) found no significant increase in pigmentation of broilers when the level of xanthophyll was increased from 6.37 milligrams to 10 milligrams per pound of diet when fed the first four weeks. If the birds received no xanthophyll for the first four weeks he found that 10 milligrams per pound of diet for the last four weeks resulted in maximum pigmentation.

More recently in a review of the literature Heiman (1966) concluded that 6 to 12.6 milligrams of xanthophylls per pound of diet were required to produce good broiler pigmentation. He pointed out that various investigators listed lower requirements for the first few weeks of 0 to 4 milligrams per pound of starter diet. Day and Williams (1958) reported that xanthophylls are most efficiently utilized at the lower levels.

One of the problems of getting the higher levels of xanthophylls (over 10 milligrams per pound of diet) has been that more concentrated sources of xanthophylls than yellow corn must be used. The most commonly added material is alfalfa meal which is high in crude fiber. Many nutritionists do not wish to add the high levels of alfalfa meal necessary to get higher amounts of xanthophylls as they feel it will result in reduced feed efficiency

and a lower rate of egg production (Bunnell and Bauernfeind, 1958; Rauch, 1965; Kivimäe et al., 1965). However, several investigators report little effect on egg production, feed efficiency, fertility or hatchability with 20 to 25 percent alfalfa in the diet (Jensen and McGinnis, 1952; Farr et al., 1961; Kingan and Sullivan, 1963).

Carry-Over to Egg Yolk Products

The egg-breaking industry has demanded and paid premiums for eggs with dark yolks to be used in products such as mayonnaise, cakes and noodles. In order to produce these eggs several investigators have used sources of xanthophylls which are high in the red-colored pigments such as paprika and red pepper. These pigments resulted in egg yolks that had the necessary dark color, but had a reddish tinge (Brown, 1938). This reddish tinge has been reported to be carried over to sponge cake (Mackay et al., 1963) and to mayonnaise (Carlson et al., 1964).

Several other investigators have used eggs with reddish yolks to make mayonnaise and/or cake that was deeper in color with no carry-over of the reddish tinge (Farr et al., 1961; Farr et al., 1962; Carlson et al., 1962; Mackay et al., 1963; Nelson and Baptist, 1968; Scott et al., 1968).

Techniques of Measurement

During the past 50 years many different methods have been used for the objective measurement of xanthophylls in feedstuffs, tissue and egg yolk, and for the subjective measurement of

pigmentation of egg yolk and epidermal tissues of chickens.

A review of the subjective and objective methods used by German investigators is reported by Scholtyssek et al. (1965, 1966). They explained in detail the theory and methods for measurement of egg yolk xanthophylls by reflectance spectrometry using the Zeiss-Electro-Photometer with three filters (FMX, FMYm and FMZ from Zeiss). In this method color tone, degree of saturation and degree of darkness are measured.

The rehydration method was described by Bickoff et al. (1954b) for analysis of xanthophylls in alfalfa. This method was used by several investigators, with modifications, for analysis of alfalfa and other feedstuffs (Mitchell, 1961; Madiedo et al., 1964; Douglas, 1966; Bornstein and Bartov, 1966).

Kohler et al. (1967) developed a new procedure for analysis of alfalfa xanthophylls. This procedure was designed to eliminate error due to the presence of chlorophylls. Nelson and Livingston (1967) developed a method for the determination of the individual xanthophylls by a thin-layer chromatographic procedure.

Measurement of pigmentation by visual scoring has been widely used. Heiman and Carver (1935) developed a color rotor for determination of egg yolk color. Turner and Conquest (1939) matched yolks with sodium dichromate solution of increasing concentration. This method was modified by Dalby (1948) and Bornstein and Bartov (1966). It has the advantage of being easily reproduced in any laboratory and measurements from year to year may be made with standardized solutions. Ashton and Fletcher (1962) describe in

some detail the problems involved in designing the set of 15 rings referred to as Fletcher Yolk Color Rings, used as color standards for egg yolks.

The Roche Yolk Colour Fan is described in a monograph by Hoffman-La Roche, Inc. (Anonymous, 1965) and is widely used in many countries (Bornstein and Bartov, 1966). This 15-blade fan provides a simple method of measuring yolk color which has good repeatability (Scholtyssek et al., 1966). The method consists of comparing the yolk with the blades of the color fan which vary from a pale-yellow (number 1) to an orange-red (number 15).

Most of these subjective methods, although developed for use in the egg yolk, have been used to determine pigmentation of poultry. Blakeslee and Warner (1915) and Palmer and Kempster (1919c) describe the Bradley color top which first was used to determine pigmentation in the earlobes of hens and egg yolks. Brown (1930) used it to measure shank pigmentation. Maw (1939) compared the color of body fat, skin, and shanks with carotene. Ringrose et al. (1939) matched shank color with one of a series of glass tubes colored different shades of yellow which he had used earlier for determining the degree of egg yolk color.

Several investigators have taken birds from their own experiments as standards and compared others to them (Culton and Bird, 1941; Hammond and Harshaw, 1941; Kidd, 1959; Waldroup et al., 1960). Others have made their own standards (Zervas et al., 1962; Milligan and Winn, 1964; Mitchell, 1961; Collins, 1968).

Chemical determination of xanthophylls in various tissues has

been diverse. Palmer and Kempster (1919b) used a staining technique to determine the location of xanthophylls in the layers of the skin. Mann (1946) saponified the whole carcass for extraction and measurement of xanthophylls. Heiman and Tighe (1943) using discs of shank tissue determined the concentration of pigments colorimetrically. Discs cut from the toe webs of chickens have been used to determine the xanthophylls by an extraction and colorimetric method (Wilgus, 1954; Potter et al., 1956; Ratcliff et al., 1959).

The method of Gallup and Hoefer (1946) was modified by Bunnell et al. (1954) for estimation of liver xanthophylls. Ganguly et al. (1953) reported a total carotenoid determination for liver tissue.

Yaocowitz (1961) suggested the use of the preen oil as an indicator of the effectiveness of pigmentation agents used in broiler diets. This oil can easily be sampled by applying mild pressure to the lobes of the preen gland without damage to the bird for periodic measurements of pigmentation.

Kimble (1939) and Moore (1957) described a method for estimation of vitamin A and total carotenoids of blood plasma. Grau and Klein (1957) developed a method by which serum carotenoid concentration might be measured.

The method of Wilson (1956) which was used to identify non-laying hens has been used by many investigators since Davis and Kratzer (1958) used it to predict pigmentation changes before they occurred. They found, after chicks were started on a new diet, that the level of serum xanthophylls at the end of one week was indicative of pigmentation of the shank three or four weeks later.

The xanthophylls in egg yolk have been measured by several methods. Kahlenberg (1949) described the method of the National Egg Producers Association (N.E.P.A.). The method of Ganguly et al. (1953) was used by Bartov and Bornstein (1969). Most investigators have used the method of the Association of Official Agricultural Chemists (A.O.A.C., 1965) in which units are expressed as beta-carotene equivalents.

Scott et al. (1968) described the "Cornell University method" which is a modification of the A.O.A.C. method which requires that the yolk sample be placed in a 1:1 absolute ethanol:acetone mixture 24 hours before filtering. They also described a proposed Animal Nutrition Research Council (A.N.R.C.) method in which a 1:1 chloroform:acetone mixture is added to the yolk sample, homogenized in a Waring Blender, filtered and read spectrophotometrically at 440 millimicrons. This method was earlier reported by Marusich (1967).

III. EXPERIMENTAL PROCEDURE

General

Source of chickens. All hens used were from a high-producing strain of Leghorn-type hens, hatched and reared at The University of Tennessee Experiment Station, Poultry Unit. The chicks used were males of the same strain as the hens.

Determination of level of xanthophylls in feedstuffs. The rehydration technique described by Bickoff et al. (1954b) was used, with slight modifications, to determine the content of xanthophylls of certain samples of corn, corn gluten meal, alfalfa meal and complete feeds. The modifications were the use of positive air pressure rather than vacuum and the use of Sea Sorb 43 (activated magnesium oxide, manufactured by Fisher Scientific Company) instead of No. 262 Westvaco. The optical density, determined at 445 millimicrons, times the milliliters of eluant divided by the weight of the sample in grams times 231 was assumed equal to the milligrams of xanthophyll per gram of sample (Moster and Quackenbush, 1952).

It was not necessary to know the exact dietary level of xanthophylls to satisfy the objectives of these experiments so they were calculated according to the method reported by Douglas (1966). The samples were periodically analyzed for verification.

Determination of level of xanthophylls in excreta. The level of xanthophylls in excretory material was determined according to the method described by Anjaneyalu (1962). Fifty milliliters of 0.1N ethanolic potassium hydroxide was added to a 25 gram

sample of excreta in a separatory funnel. The mixture was then diluted with 100 milliliters of a 3:1 distilled water:peroxide-free diethyl ether, shaken and allowed to stand until two phases appeared.

The xanthophylls were repeatedly extracted with peroxide-free diethyl ether until the extracts were colorless. The combined ether extracts were then washed several times with distilled water to remove the alkali. The ether fraction was then dried over anhydrous sodium sulfate and the ether removed under reduced pressure.

The yellow residue was dissolved in 25 milliliters of petroleum ether and percent transmittance measured with a Coleman No. 11A spectrophotometer at 440 millimicrons. The xanthophyll content, expressed in milligrams per kilogram of dry cow manure, was determined from a standard beta-carotene curve.

Determination of level of xanthophylls in blood. A 4 milliliter sample of blood was obtained from the wing vein, allowed to clot on a slant at room temperature for three or four hours and refrigerated overnight to separate the serum for analysis according to the method of Wilson (1956). A 1 milliliter sample of serum was then pipetted into 15 milliliters of acetone to precipitate the proteins. The extract was filtered through a Whatman No. 2 filter paper under suction and the percent transmittance of the filtrate measured with a Coleman No. 11A spectrophotometer at 445 millimicrons. The xanthophyll content, expressed in micrograms per milliliter of serum as beta-carotene equivalents, was determined from a standard curve made according to Munsey (1938).

Determination of level of xanthophylls in egg yolk. The

level of xanthophylls in the yolks was determined chemically using the method of the Association of Official Agricultural Chemist (A.O.A.C., 1965). The egg yolks were placed in plastic bags after reading the pigmentation scores and removal of the chalaza and albumen. The bags were marked for later identification and placed in a refrigerator overnight.

A 2.5 gram sample of yolk material was weighed into a 150 milliliter beaker being careful that no albumen was included in the sample. A small amount of acetone (2 to 3 milliliters) was then added and the mixture stirred to a smooth consistency after which more acetone was added to bring the total to about 50 milliliters. The mixture was stirred again and allowed to set about five minutes. The mixture was then filtered through a Whatman No. 4 filter paper and washed with successive small portions of acetone. The filtrate was collected in a 100 milliliter volumetric flask and diluted to volume with acetone. It was found that if too small an amount of acetone was added at first and/or filtered too soon it resulted in a cloudy filtrate. The percent transmittance of the filtrate was determined with a Coleman No. 11A spectrophotometer at 450 millimicrons. The xanthophyll content, expressed as micrograms beta-carotene equivalents per gram of yolk was then determined from a standard curve.

Determination of visual pigmentation score. The visual pigmentation scores were obtained by matching the egg yolk, beak, shank or eye ring with one of the 15 blades of the 1961 "Roche Improved Yolk Colour Fan" (Hoffman-LaRoche, Nutley, New Jersey).

The blades of the fan vary from a pale yellow (number 1) to and orange-red (number 15). The matching was done about 10 inches below a fluorescent desk lamp with two 15 watt, 18 inch daylight bulbs with double strength frosted glass.

Statistical analysis. Statistical examination of the data was made by the analysis of variance technique or linear correlation according to Snedecor (1956) with significant treatment differences determined using the multiple range test (Duncan, 1955).

Experiment 1

This experiment was designed to determine the site of absorption of xanthophylls. It involved the use of a null hypothesis which assumed that removal of the section of the intestine in which xanthophylls are absorbed would result in no xanthophylls being absorbed, and removal of a percentage of the section which is involved in absorption would decrease the absorption by that same percentage. Absorption was measured indirectly by determining the increase in the level of blood xanthophylls and in the pigmentation of the eye ring, beak, and shank.

At one day of age all chicks were randomized, wing banded and vaccinated with an intraocular combination Newcastle-infectious bronchitis vaccine. The chicks were housed in electrically heated battery brooders with raised wire floors in a room maintained at 27° C. for the first week and 21° C. to 24° C. for the next two to three weeks. The brooders were thermostatically adjusted to operate at about 35° C. for the first week and the temperature

lowered about three degrees each week.

At four weeks of age the chicks were moved to growing batteries with raised wire floors and no heating elements. The temperature of the room was maintained at about 21° C. Feed and tap water were available at all times in both types of batteries. The birds were returned to similar batteries after surgery and infra-red heat lamps were used to provide supplemental heat.

In preliminary studies feed and water were removed 12 hours prior to surgery, but the intestine contracted so that it could be handled only with great difficulty. This practice was therefore discontinued and the condition of the intestine was much improved.

The operating room was well lighted, the surgical area cleaned before surgery and the temperature kept at 24° C. to 27° C. All instruments were washed and rinsed in ethyl alcohol prior to each operation.

Day-old chicks were started on a diet free of xanthophylls (Diet 1, Table I) and fed for a period of about six weeks in order to obtain birds with little or no measurable xanthophylls in the blood or pigmentation of the eye ring, beak, or shanks.

Various segments of the intestinal tract were then ligated, bypassed or removed surgically from the chickens in all except the control groups. All chickens except in negative control group were then fed a diet relatively high in xanthophylls (66.1 milligram per kilogram of feed) for two weeks (Diet 2, Table I). The pigmentation and blood xanthophylls were again determined and the increase used to indicate the absorption of xanthophylls.

TABLE I
COMPOSITION AND CALCULATED ANALYSES OF DIETS--EXPERIMENT 1

Ingredients	Diet number	
	1	2
	percent	
Yellow corn	-	55.80
White corn	56.70	-
Soybean oil meal (50% protein)	37.40	25.80
Fish meal (60% protein)	2.00	2.00
Alfalfa meal (17% protein)	-	2.50
Corn gluten meal (60% protein)	-	10.00
Defluorinated rock phosphate	1.50	1.50
Limestone	1.20	1.20
Salt	0.48	0.48
DL-Methionine	0.10	0.10
MnSO ₄ supplement (75%)	0.02	0.02
Micro-ingredient premix A ¹	0.60	-
Micro-ingredient premix B ²	-	0.60
Total	100.00	100.00
Calculated analyses:		
Productive energy Cal./kg.	2006	2092
Crude protein, %	24.99	25.54
Xanthophyll, mg./kg.	-	66.10

¹Micro-ingredient premix A supplied the following amounts per kilogram of diet: 4,171 I.U. of vitamin A, 4.74 milligram of riboflavin, 11.0 micrograms of vitamin B₁₂, 406 milligrams of choline, 40.3 milligrams of niacin, 750 I.C.U. of vitamin D, 7.01 milligrams of pantothenic acid, 11.9 milligrams of aureomycin, 1.00 milligram of menadione sodium bisulfite.

²Same as micro-ingredient premix A except it contained no menadione sodium bisulfite, but contained Pigmentene Yellow Gold (Special Nutrients, Inc., Surfside, Florida) to supply 17.6 milligrams of xanthophylls per kilogram of diet.

Three control groups were utilized in this experiment:

(1) a positive, unoperated control group which was fed the diet high in xanthophylls; (2) a negative unoperated control group which continued to receive the diet free of xanthophylls; and (3) a sham operated control group in which the intestine was severed and sewed back together without the removal of a segment. There were six surgical treatment groups as follows: (1) ligation of the ceca, (2) removal of the duodenum, (3) removal of the jejunum, (4) removal of the ileum, (5) removal of the large intestine, and (6) removal of the section of the jejunum-ileum which is involved in Eimeria maxima infected chickens (Figure 3). (Mitchell, 1961, reported that there was loss of pigmentation in E. maxima infected chickens).

When an operation was to be performed each chicken was placed in a restraining trough and anesthetized by an injection of pentobarbital sodium. The abdominal area was plucked, the skin disinfected with ethyl alcohol and a six to eight centimeter incision extending from the lateral sternal notch to the pubis was made with a scapel to expose the intestine. The incision was held open by wound retractors.

The portion of the intestine to be removed was pulled through the opening and placed on a paper towel, carefully pushing any other parts of the intestinal tract back into the body cavity. The intestine was grasped between the forefinger and thumb of each hand at the site to be incised and the intestinal contents gently milked back from the site.

The blood supply to the jejunum-ileum section is through the mesentery, a skirt-like sheet of tissue by which this section of the

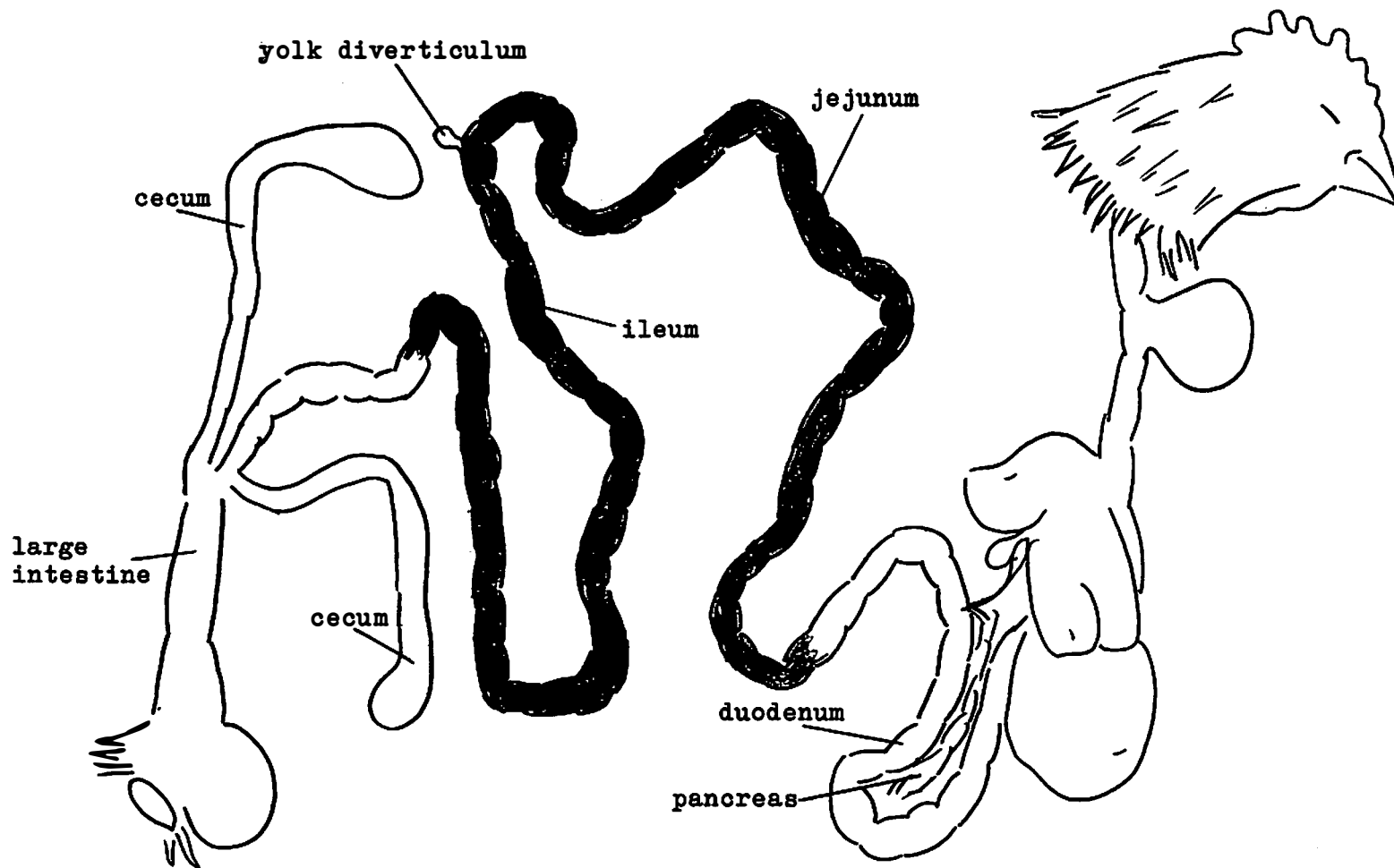


Figure 3. The Section of the Intestine Affected by Eimeria Maxima Infections as Indicated by the Shaded Area.

intestine hangs like the hem of a pleated skirt. The arteries and veins fan out from the "waist" of the mesentery to the intestine at the "hem." Before removal of a section of the jejunum-ileum these blood vessels were first ligated and a section of the mesentery removed with it.

In early trials the bypassed duodenal loop was left in the body, but this resulted in toxemia and extreme morbidity. The duodenum is furnished with a copious supply of blood through many vessels making it difficult to ligate the blood vessels for the removal of the duodenum. Therefore, a fulguration technique, using a Birtcher Hyfrecator, was employed to cauterize these blood vessels before trimming the duodenum from the pancreas. The fulguration technique involved the holding of an electrode one or two millimeters from the blood vessel to be cauterized, causing an electrical arc to travel to the blood vessel.

The severed edges of the intestine were then rejoined by end-to-end anastomosis in such a manner that the lumen remained unobstructed to the flow of the intestinal contents. To facilitate this procedure a piece of macaroni two and one-half to three and one-half centimeters in length, was inserted into the ends of the two intestinal segments to be rejoined. The macaroni also served as a support, and working surface during the suturing procedure similar to a darning egg used for mending socks. The macaroni was quickly absorbed following the operation.

A mattress suture was used to pull the two ends of the intestine together over the macaroni, the intestine was sewn

together using the Schmieden's intestinal suture; and any small portion left unsealed was closed with the inverted interrupted suture (Figure 4). These stitches were employed to keep the serosa (external intestinal walls) of one end of the intestine against the serosa of the other end of the intestine as is required for intestinal anastomosis. The intestine was then dusted with surgical powder and returned to the body cavity. The intestine was not allowed to remain outside the chickens's body any longer than absolutely necessary so as to avoid excessive loss of heat and drying of tissues. The peritoneum, muscles and skin were then sewn together to close the wound. An injection of antibiotic was made immediately following surgery and again on the third postoperative day.

The ceca presented much less of a problem and were successfully ligated with a purse-string suture and left in the body cavity. The large intestine was removed with little problem except for the close working space. The external incision for this operation was made at the mid-line between the pubic bones.

Two weeks postoperative, the health of each chick was rated from 1 to 4 (1-good, 2-fair, 3-poor, and 4-very poor). Each operation was performed on a sufficient number of chicks to assure that three chicks with a health rating of 3 or better would be available from each group for comparison of xanthophyll absorption.

Experiment 2

This experiment consisting of two trials, was designed to determine the relationships between xanthophylls in the feed, the

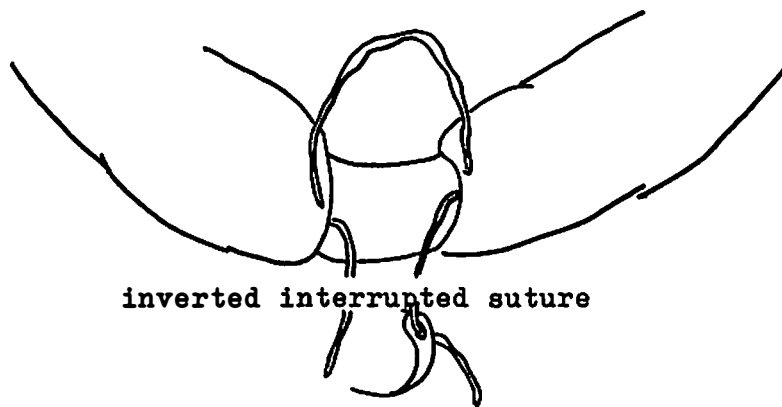
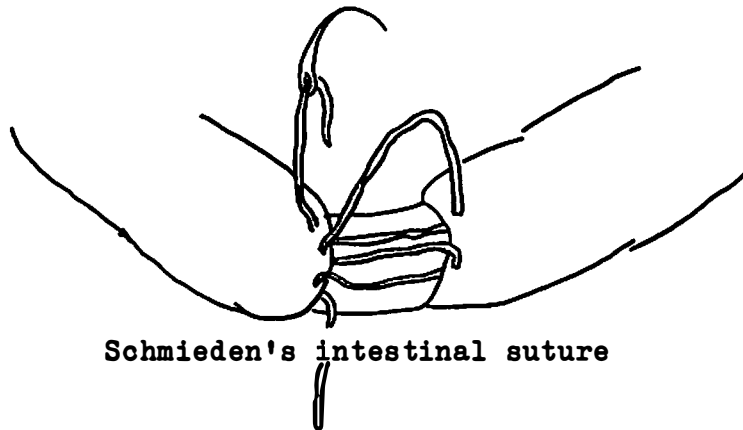
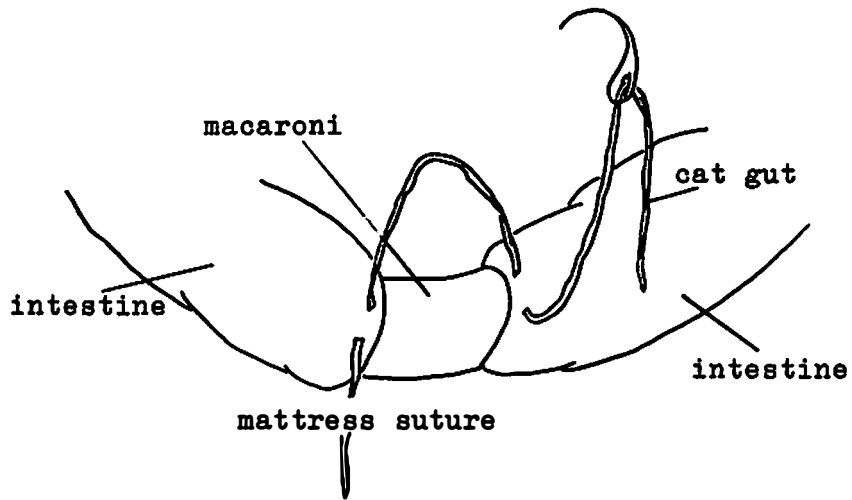


Figure 4. Sutures Used in Surgery Showing the Two Ends of the Intestine with Macaroni Inserted.

color of the egg yolks produced and the color of mayonnaise produced from the egg yolks.

Six Leghorn-type hens per group, housed in individual cages, were fed the diets indicated in Table II. The "layer" diet was the standard diet fed to hens at The University of Tennessee Poultry Unit and contained about 23.0 milligrams of xanthophylls per kilogram of diet. The "white" diet was a similar diet containing no measurable xanthophylls; hens fed it, produced eggs with extremely light colored yolks described as lacking in color. The "yellow," "orange," and "red" diets were formulated by the addition of commercially available xanthophyll concentrates to the "layer" diet to supply 66.1 milligrams of additional xanthophylls per kilogram of diet. The concentrates were blends of naturally occurring xanthophylls supplied by Special Nutrients, Inc., Surfside, Florida.

After the hens had been fed the diets for four weeks, eggs were collected from each hen and the egg yolk color visually determined by matching with a 15-blade Roche Yolk Colour Fan. Equal numbers of yolks from each hen fed a specific diet were pooled by placing the yolks in a plastic bag and marked for identification. The level of xanthophylls in the pooled yolk sample for each treatment was then determined by the A.O.A.C. (1965) (16.023-25) spectrophotometric method for the determination in micrograms of xanthophylls per gram of yolk as beta-carotene equivalents.

The University of Tennessee Department of Food Science and Institution Administration made mayonnaise from the pooled yolk samples and conducted sensory tests to determine which mayonnaise

TABLE II
COMPOSITION AND CALCULATED ANALYSES OF DIETS--
EXPERIMENTS 2, 3, AND 4

Ingredients	Diet	
	White	Layer ¹
	percent	
Yellow corn	-	66.98
White corn	69.70	-
Soybean oil meal (50% protein)	19.28	17.00
Fish meal (60% protein)	2.50	2.50
Alfalfa meal (17% protein)	-	5.00
Defluorinated rock phosphate	1.50	1.50
Limestone	6.00	6.00
Salt	0.50	0.50
MnSO ₄ supplement (75%)	0.02	0.02
Micro-ingredient premix C ²	0.50	-
Micro-ingredient premix D ³	-	0.50
Total	100.00	100.00
Calculated analyses:		
Productive energy Cal./kg.	2072	2017
Crude protein, %	17.35	16.74
Xanthophyll, mg./kg.	-	23.00

¹"Yellow," "Orange," and "Red" diets were formulated by the addition of Pigmentene Yellow Gold, Pigmentene Orange, and Pigmentene Red at the rate of 1.00 gram, 1.20 grams and 1.72 grams, respectively, to one kilogram of the "Layer" diet to supply 66.1 milligrams of xanthophylls per kilogram of diet (Pigmentene supplied by Special Nutrients, Inc., Surfside, Florida).

²Micro-ingredient premix C supplied the following amounts per kilogram of diet: 2,465 I.U. of vitamin A, 4.43 milligrams of riboflavin, 6.50 micrograms of vitamin B₁₂, 441 milligrams of choline, 24.2 milligrams niacin, 2,958 I.C.U. of vitamin D, 4.76 milligrams of pantothenic acid, 1.00 milligrams of menadione sodium bisulfite.

³Same as micro-ingredient premix C except it contained no menadione sodium bisulfite.

samples could be differentiated by color. The mayonnaise contained 78.4 percent corn oil, 10.7 percent vinegar, 6.4 percent egg yolk, 2.1 percent salt, 1.4 percent sugar and 0.8 percent dry mustard. Care was taken that each sample was mixed the same by timing each step. The mayonnaise was then placed in 50 milliliter beakers, covered with a thin plastic wrap and labeled with a code letter. The labeled beakers were then employed in a triangle test for all possible pairs as described by Larmond (1967). In this test the judges were given sets of three samples, two alike and one different and asked to pick the one which was different in color. This experiment was repeated, but in trial 2 mayonnaise from egg yolks from "white" diets was omitted as it had a clearly different, easily identified, mayonnaise color in trial 1.

Experiment 3

Chickens that are allowed to follow cows eat cow manure and grass and produce dark colored yolks. Some of this color comes from the grass, but the cow manure could also be a source of xanthophylls as the cow does not utilize these xanthophylls. There are enzymes and hormones present also in cow manure which could affect the utilization of xanthophyll by the chicken.

This experiment was designed to determine the effect of various levels of cow manure added to the laying diet on the pigmentation of egg yolks and on the level of xanthophylls in egg yolks and blood. Forty-eight Leghorn-type hens were housed in individual cages in eight groups of six hens each and fed a diet free of xanthophylls (Table II, white diet, page 41) for a period of four

weeks to deplete the birds of xanthophylls. At the end of the depletion period egg yolk visual color score, level of egg yolk xanthophylls and level of blood xanthophylls were determined to reveal freedom from xanthophyll.

For two weeks following the depletion period the hens received diets with various amounts of ground, dried cow manure added to the xanthophyll free diet or the standard layer diet. The cow manure for these diets was secured from The University of Tennessee Dairy Unit and was from lactating cows on a grass-legume pasture and grain. The manure was dried for one week on plastic sheets in an empty chicken house with a ventilating fan at the Poultry Unit. The partially dried manure was then removed from the plastic, placed in burlap bags and held in a forced hot air forage drying oven at 65° C. for 24 hours (Oven King, 1960, model number 486120 - 32235-0, Seattle, Washington). The dried manure was then ground in a portable Viking Hammer Mill (Model RS - 81Y - A) to pass through a screen with six millimeter perforations.

After analysis for xanthophylls by the method described in the general procedure, the ground, dry cow manure was added to the diets "white" and "layer" (Table II, page 41) at the rate of 0, 2.5, 5, and 10 kilograms to 100 kilogram of basal as indicated in Table III.

The "layer" diet was the standard diet fed to hens at The University of Tennessee Poultry Unit and contained about 23.0 milligrams of xanthophylls per kilogram of diet. The "white" diet was a similar diet containing no measurable xanthophylls and was the same diet fed during the depletion period. The calculated milli-

TABLE III
COMPOSITION AND CONTENT OF XANTHOPHYLLS OF DIETS--
EXPERIMENT 3

Diet	Composition ¹		Xanthophylls		Total
	Basal ²	Kg. cow manure ³ added to 100 kg. basal	From basal ⁴	From cow manure mg./kg. ⁵	
W + 0	White	-	-	-	-
W + 2.5	White	2.5	-	14.1	14.1
W + 5	White	5.0	-	27.3	27.3
W + 10	White	10.0	-	52.2	52.2
L + 0	Layer	-	23.0	-	23.0
L + 2.5	Layer	2.5	22.3	14.1	36.4
L + 5	Layer	5.0	21.8	27.3	49.2
L + 10	Layer	10.0	20.9	52.2	73.2

¹Diets were formulated by adding the number of kilograms of cow manure indicated to 100 kilograms of the basal indicated.

²Composition and calculated analysis of basal diets given in Table II, page 41.

³Cow manure from lactating cows on pasture was dried and ground.

⁴Calculated analyses.

⁵Analysis by method of Anjaneylu (1962) shows the dried cow manure contained 5.75 milligrams of xanthophylls per kilogram.

grams of xanthophylls per kilogram of diet supplied by the basal, from the added cow manure and in the total diet is indicated in Table III, page 44. Each addition of cow manure increased the total xanthophyll.

After the hens had been fed the diets for two weeks, the egg yolk visual score, level of egg yolk xanthophylls, and level of blood xanthophylls were again determined to indicate the effect of the treatments on pigmentation.

As a further measure of pigmenting efficiency the micrograms of xanthophylls per gram of yolk were divided by the milligrams of xanthophylls per kilogram of diet (Hall et al., 1966).

Experiment 4

This experiment, consisting of two trials, was designed to study the effect of age, temperature, and feed intake on the utilization of xanthophylls by the laying hen. Leghorn-type hens were housed in individual cages at temperatures to simulate summer (hot) and winter (cool) conditions. The "hot" rooms were maintained at an approximate average temperature of 31° C. and 31.2° C. and the "cool" rooms, 22.3° C. and 14.3° C. in Trials 1 and 2, respectively. An attempt was made to equalize the temperature throughout the "hot" room by the location of fans and heaters. The "cool" temperature depended on the temperature of the air pulled in from the outside and the exhaustion of air from the opposite end of the room. All exhaust fans were controlled by time clocks and thermostats so that the temperatures could be maintained. High and low

temperatures were recorded daily in each room. It is accepted by poultrymen that the shell thickness of the egg is markedly reduced at high ambient temperature. In order to determine if the temperatures were high enough to affect the physiology of the hen, the shell thickness was measured by the specific gravity method as described in the 1969 Report of Egg Production Tests, United States and Canada (Anonymous, 1970).

All of the hens received the laying diet (Table II, Diet 2, page 41). This diet contained approximately 23 milligrams of xanthophylls per kilogram of diet. The level of yolk xanthophylls and visual yolk scores were determined at the beginning of the experiment when the room temperatures of all birds were approximately the same. Under each environmental condition hens of two ages were compared. Also in each of the environmental groups and in both age groups birds were given feed under two different regimes. Half of the birds received feed ad libitum, and the other half received feed at 90 percent of the amount of feed eaten the previous week by the birds in the "hot" room. Weekly feed consumption records were kept on each group. Individual daily egg records of all hens were kept throughout the experiment, and only eggs from those hens with similar egg production were used in the study. Eggs from these hens were collected at the beginning of the study and periodically throughout the experiment. The egg yolk color was scored by matching with a 15-blade Roche Yolk Colour Fan and the level of egg yolk xanthophylls determined by spectrophotometric methods (A.O.A.C., 1965). The significant differences in these yolk pigmentation indicators and treatment interactions were determined by analysis

of variance (Snedecor, 1956) and multiple range test (Duncan, 1955).

Trial 1. The experimental design is given in Table IV. Each room contained 15 "old" hens (16 months old at the beginning of the experiment) and 15 "young" hens (12 months old at the beginning of the experiment). Rooms 1 and 2 were maintained at the "cool" temperature, and rooms 3 and 4 at the "hot" temperature. The hens in rooms 2 and 4 had access to the standard laying diet ad libitum. The hens in rooms 1 and 3 were limited to 90 percent of the average daily consumption of the standard laying diet per hen in room 4 (high temperature) the previous week. After two, seven, and twelve weeks eggs were collected and analysed for yolk pigmentation from six hens, from each group, paired on the basis of similar egg production.

Trial 2. The experimental design is given in Table V. Each room contained two groups of six "old" hens (12 months old at the beginning of the experiment) and two groups of six "young" hens (eight months old at the beginning of the experiment). Room 1 was maintained at the "cool" temperature and room 2 at the "high" temperature. The hens in group 2 in each room had access to the laying diet ad libitum. The hens in group 1 in each room were limited to 90 percent of the amount of the average daily feed consumption per hen in group 2 in room 2 (high temperature) the previous week. Eggs were collected and analysed for yolk pigmentation at four, twelve, and twenty weeks from the beginning of the experiment. These eggs were collected from hens paired by egg production so that the eggs used were from hens laying approximately the same number of eggs per period in each group.

TABLE IV
EXPERIMENTAL DESIGN--TRIAL 1, EXPERIMENT 4

Room	Temperature ¹	Feeding regime ²	No. of hens	
			Young ³	Old ⁴
1	Cool (22.3° C.)	limited	15	15
2	Cool (22.2° C.)	<u>ad libitum</u>	15	15
3	Hot (31.4° C.)	limited	15	15
4	Hot (31.5° C.)	<u>ad libitum</u>	15	15

¹Temperature range was 20.0° C. to 25.5° C. in room 1, 18.9° C. to 26.7° C. in room 2, 30.0° C to 34.4° C. in room 3, and 28.9° C. to 34.4° C. in room 4.

²Limited feeding was limited to 90 percent of the amount of the average daily consumption of the hens in room 4.

³"Young" hens were 12 months old at the beginning of the experiment.

⁴"Old" hens were 16 months old at the beginning of the experiment.

TABLE V
EXPERIMENTAL DESIGN--TRIAL 2, EXPERIMENT 4

Room	Group	Temperature ¹	Feeding regime ²	No. of hens	
				Young ³	Old ⁴
1	1	Cool (14.3° C.)	limited	12	12
1	2	Cool (14.3° C.)	<u>ad libitum</u>	12	12
2	1	Hot (31.2° C.)	limited	12	12
2	2	Hot (31.2° C.)	<u>ad libitum</u>	12	12

¹Temperature range was 18.3° C. to 28.9° C. in the "cool" room and 28.3° C. to 35.5° C. in the "hot" room.

²Limited feeding was limited to 90 percent of the amount of the average daily consumption of the hens in room 2, ad libitum feeding regime.

³"Young" hens were eight months old at the beginning of the experiment.

⁴"Old" hens were 12 months old at the beginning of the experiment.

IV. RESULTS AND DISCUSSION

Experiment 1

The percent of the intestine removed and a rating of the health of the birds two weeks postoperative are shown in Table VI. Note that the removal of the section of the jejunum-ileum involved in E. maxima infections amounted to approximately 54 percent of the total intestine. The birds used had health scores of 1 to 3 when examined two weeks postoperative. At this time all of the chickens were eating and drinking. Postmortem examination showed no blockage or leakage of the intestine.

The absorption of xanthophylls which occurred during these two weeks was measured by the visual pigmentation score and the level of blood xanthophylls and is reported in Table VI. As reported in the procedures, all of the chickens were fed a diet free of xanthophylls for six weeks prior to the operation. At the time of the operation their pigmentation ratings were 1, and the level of blood xanthophylls all less than 1 microgram per milliliter.

The visual pigmentation of chicks with ligated ceca was the same as that of those in the unoperated positive control group and statistically did not differ from the sham operated control group. However, the blood xanthophylls of the chicks with the ligated ceca were significantly lower than those of chicks in the unoperated positive control group and significantly higher than those of the chicks in the sham operated groups.

The removal of either the duodenum, jejunum, ileum, or the

TABLE VI

THE PERCENT OF INTESTINE REMOVED, THE RELATIVE HEALTH, PIGMENTATION SCORE AND BLOOD XANTHOPHYLLS IN CHICKENS TWO WEEKS POSTOPERATIVE--EXPERIMENT 1

Operation or section of intestine removed	Relative health of chickens 2 weeks postoperative ¹	Approximate percent of intestine removed	Average visual pigmentation score ^{2,3}	Average xanthophylls mcg./ml. blood ^{2,4}
Unoperated + control	1.0	0	8.6 ^a	22.0 ^a
Unoperated - control	1.0	0	1.0 ^c	0.8 ^f
Sham operated	1.0	0	8.0 ^a	15.8 ^c
Ceca	1.0	22	8.6 ^a	19.9 ^b
Duodenum	1.2	9	4.7 ^b	10.8 ^{de}
Jejunum	1.7	33	3.2 ^b	9.5 ^e
Ileum	2.3	33	4.4 ^b	11.7 ^d
Large intestine	2.3	4	5.8 ^b	9.9 ^e
Jejunum-ileum ⁵	2.3	54	1.0 ^c	0.8 ^f

¹The health of each chicken was rated: 1-Good, 2-Fair, 3-Poor, and 4-Very Poor.

²Averages with different superscripts within a column differ significantly ($P \leq 0.01$).

³Averages of beak, eye ring and shank pigmentation of three chickens on each treatment as measured with a 15-blade Roche Yolk Colour Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

⁴Level of blood xanthophylls measured by the method of Wilson (1956).

⁵Not the complete jejunum-ileum section, only that portion affected in E. maxima infections as indicated in Figure 3, page 36.

large intestine resulted in chicks with lower pigmentation scores and level of blood xanthophylls than those of the sham operated control. Only the removal of that section of the jejunum-ileum that would be affected by E. maxima resulted in chicks with no significant absorption of xanthophylls when compared with chicks in the negative control group.

The section of the jejunum-ileum removed is the same section as the "middle section of the small intestine" found by Renner (1965) to be the site of most active absorption of tallow, lard and soybean oil in the chick. However, in humans, rats, dogs, and hamsters the jejunum is the site of most active absorption of fat (Frazer, 1943; Turner, 1958; Johnston, 1959; Borgstrom et al., 1962). This difference between the chicken and mammals may be caused by the pancreatic and bile ducts entering the small intestine at the distal end of the duodenum in the chicken while in mammals these ducts enter at the proximal end. Fat and possibly xanthophylls may require some preparation by these digestive secretions prior to absorption.

It is therefore concluded that most of the absorption of xanthophylls takes place in the area of the jejunum-ileum in which E. maxima infections occur and very little xanthophylls are absorbed in the duodenum, ceca, and large intestine. It is also concluded that loss in pigmentation in cases of severe E. maxima infection is due to a lack of xanthophyll absorption and not an increase in oxidation of this pigment nor due to use of xanthophyll by the coccidia. The slight decrease in absorption in the chickens with either the duodenum or large intestine removed might have been

related to their morbidity.

Experiment 2

Average visual pigmentation data and level of xanthophylls in the egg yolks are given in Table VII. The yolks produced by hens fed the "white" diet were the lightest in color followed in order of increasing visual intensity by the "layer," the "yellow," the "orange," and the "red" diets. The total level of xanthophylls in the egg yolks was greatest in the eggs from the hens fed the diets containing the yellow, orange, and red concentrates. However, no significant differences were found in level of xanthophylls between these three treatments. These findings were not unexpected as the level of xanthophylls in the diets was the same in each of these three treatments even though the colors or kinds of xanthophylls were different.

The summary of the triangle test for determination of color differences in mayonnaise made from egg yolks produced by hens fed the various diets is given in Table VIII. The mayonnaise made from yolks of eggs from hens fed the "white" diet was very light in color and several of the judges commented that it looked like salad dressing. As can be seen in Table VIII, all judges were able to distinguish this mayonnaise from all other samples in the first trial, thus the "white" treatment was eliminated from the second trial.

The mayonnaise made from the yolks of eggs from hens fed the "red" diets had little or none of the objectionable reddish

TABLE VII
 AVERAGE PIGMENTATION AND LEVEL OF XANTHOPHYLLS IN EGG
 YOLKS USED IN MAKING MAYONNAISE--
 EXPERIMENT 2

Diet ¹	Yolk visual score ^{2,3}	Xanthophylls mcg./g. yolk ^{2,4}
White	1.0 ^a	1.2 ^a
Layer	9.0 ^b	31.7 ^b
Yellow	11.5 ^c	49.3 ^c
Orange	13.0 ^d	49.4 ^c
Red	14.5 ^e	52.5 ^c

¹Composition and calculated analyses shown in Table III, page 44.

²Averages with different superscripts within a column differ significantly ($P \leq 0.01$).

³Measured with a 15-blade Roche Yolk Colour Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

⁴Yolk xanthophylls, level measured in beta-carotene equivalents in micrograms per gram by A.O.A.C. (1965) methods.

TABLE VIII

SUMMARY OF TRIANGLE TEST FOR COLOR OF MAYONNAISE MADE
FROM EGG YOLKS PRODUCED BY HENS ON VARIOUS DIETS--
EXPERIMENT 2

Comparison ¹	Number correct	
	Trial 1 22 judges	Trial 2 15 judges
White - Layer	22***	-
White - Yellow	22***	-
White - Orange	22***	-
White - Red	22***	-
Layer - Yellow	22***	15***
Layer - Orange	22***	15***
Layer - Red	22***	14***
Yellow - Orange	11	8
Yellow - Red	20***	15***
Orange - Red	16***	10**

¹Comparison is between mayonnaise samples produced from egg yolks from hens on the diets indicated. See Table III (page 44) for composition and calculated analyses of diets.

²***indicates 0.1 percent level of significance and ** indicates 1 percent level of significance.

color reported by Carlson et al. (1964) and Nelson and Baptist (1968). Note that some of the judges failed to distinguish between the mayonnaise made from yolks from hens fed the "red" and "yellow" or the "red" and "orange" diets.

There was no significant color difference found between the mayonnaise produced from the yolks from hens fed the "yellow" and the "orange" diets as indicated by the inability of the judges to distinguish between these samples. Color acceptability of these samples was not judged objectively, but when asked, the judges indicated that all were acceptable. The author and one of the judges tasted the mayonnaise samples and could detect no differences in flavor.

The addition of red concentrate to a standard "layer" diet gave the greatest carry-over effect on the color of mayonnaise. The addition of yellow, orange, and red concentrates to the "layer" diet resulted in visual yolk color scores of 11.5, 13.0, and 14.5, respectively, but there were no significant differences found in the level of xanthophylls per gram of yolk from these three samples. Mayonnaise made from the "yellow" and "orange" treatments could not be consistently distinguished from each other by the panel of judges, while that made from the "red" treatment could be distinguished from mayonnaise made from both "yellow" and "orange" treatments.

Egg yolks may appear different, but this is no assurance that this color difference will carry-over to products such as mayonnaise. The color of the mayonnaise depends not only upon the amount of xanthophylls present, but also upon the kind of xanthophylls.

It is indicated that the commercial producer of eggs for breaking plants could add less of the red concentrates than yellow or orange to the standard yellow corn laying diet to produce the visual color desired. The present practice of buying according to N.E.P.A. number rather than visual score should be changed as the color difference from yellow, orange and red concentrate is not identifiable through chemical analysis. Measuring by visual score would be more meaningful as the consumer is interested in how the product looks (visual score) and has no means of chemically measuring the product.

Experiment 3

A summary of the effect of feeding various levels of cow manure to hens is given in Table IX. There was a positive linear correlation between the amount of cow manure added and the micrograms of xanthophylls per milliliter of blood ("white" diet, $r = 0.975$; "layer" diet, $r = 0.959$), the micrograms of xanthophylls per gram of yolk ("white" diet, $r = 1.00$; "layer" diet, $r = 0.990$), and the yolk visual score ("white" diet, $r = 0.955$; "layer" diet, $r = 0.979$).

There was a negative linear correlation between pigmenting efficiency and the amount of cow manure added to both the "white" diet ($r = 0.939$) and the "layer" diet ($r = 0.924$), as can be seen in Figure 5 which also shows the linear regression curves.

The bar graph in Figure 6 shows the relation between the xanthophylls in the diet, xanthophylls in the yolk, and the pigmenting efficiency. The effect of increased amounts of cow

TABLE IX
SOME EFFECTS OF FEEDING VARIOUS LEVELS OF COW MANURE--
EXPERIMENT 3

Diet ¹	Xanthophylls			Yolk visual score ^{2,5}	Pigmenting efficiency ⁶
	mg./kg. diet	mcg./ml. blood ^{2,3}	mcg./g. yolk ^{2,4}		
W + 0	0	0.62 ^a	2.3 ^a	1.2 ^a	-
W + 2.5	14.1	1.48 ^b	9.9 ^b	4.8 ^b	0.70
W + 5	27.3	2.76 ^d	17.4 ^c	7.3 ^c	0.63
W + 10	52.3	3.93	26.4 ^d	9.3 ^d	0.50
L + 0	22.9	3.04 ^d	28.9 ^d	10.3 ^{ef}	1.26
L + 2.5	36.4	2.95 ^d	24.9 ^d	9.8 ^{de}	0.68
L + 5	48.5	4.88 ^e	28.3 ^d	10.8 ^{fg}	0.58
L + 10	73.2	6.26 ^f	40.9 ^e	11.5 ^g	0.56

¹Composition and xanthophyll content of diets given in Table III, page 44.

²Averages with different superscripts within a column differ significantly ($P \leq 0.01$).

³Blood xanthophyll level measured by method of Wilson, W. O., "Identifying Non-Laying Chicken Hens," Poultry Science 35:226-227, 1956.

⁴Yolk xanthophyll level measured in beta-carotene equivalents by A.O.A.C. methods ("Official methods of Analysis," 10th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1965).

⁵Measured with a 15-blade Roche Yolk Color Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

⁶Calculated as micrograms of xanthophyll per gram of yolk divided by milligrams of xanthophyll per kilogram of diet. (Hall, G. M., P. W. Waldroup, J. L. Fry, C. B. Ammerman and R. H. Harms, "A Comparison of the Pigmenting Value of Alfalfa Meals Differing in Protein and Xanthophyll Content," Poultry Science 45:639-641, 1966).

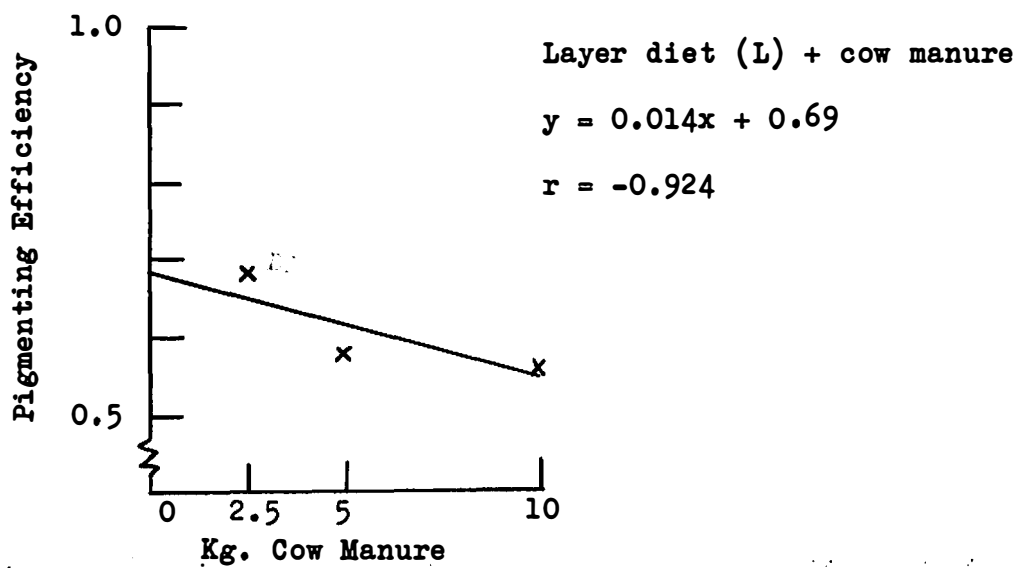
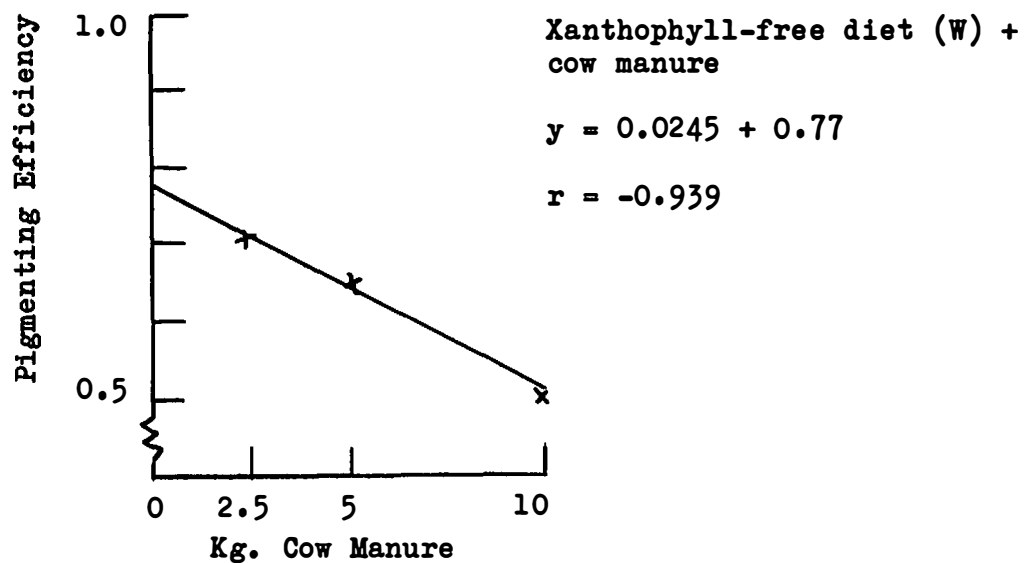


Figure 5. Linear Regression and Linear Correlation of Pigmenting Efficiency and Amount of Cow Manure in the Diets.

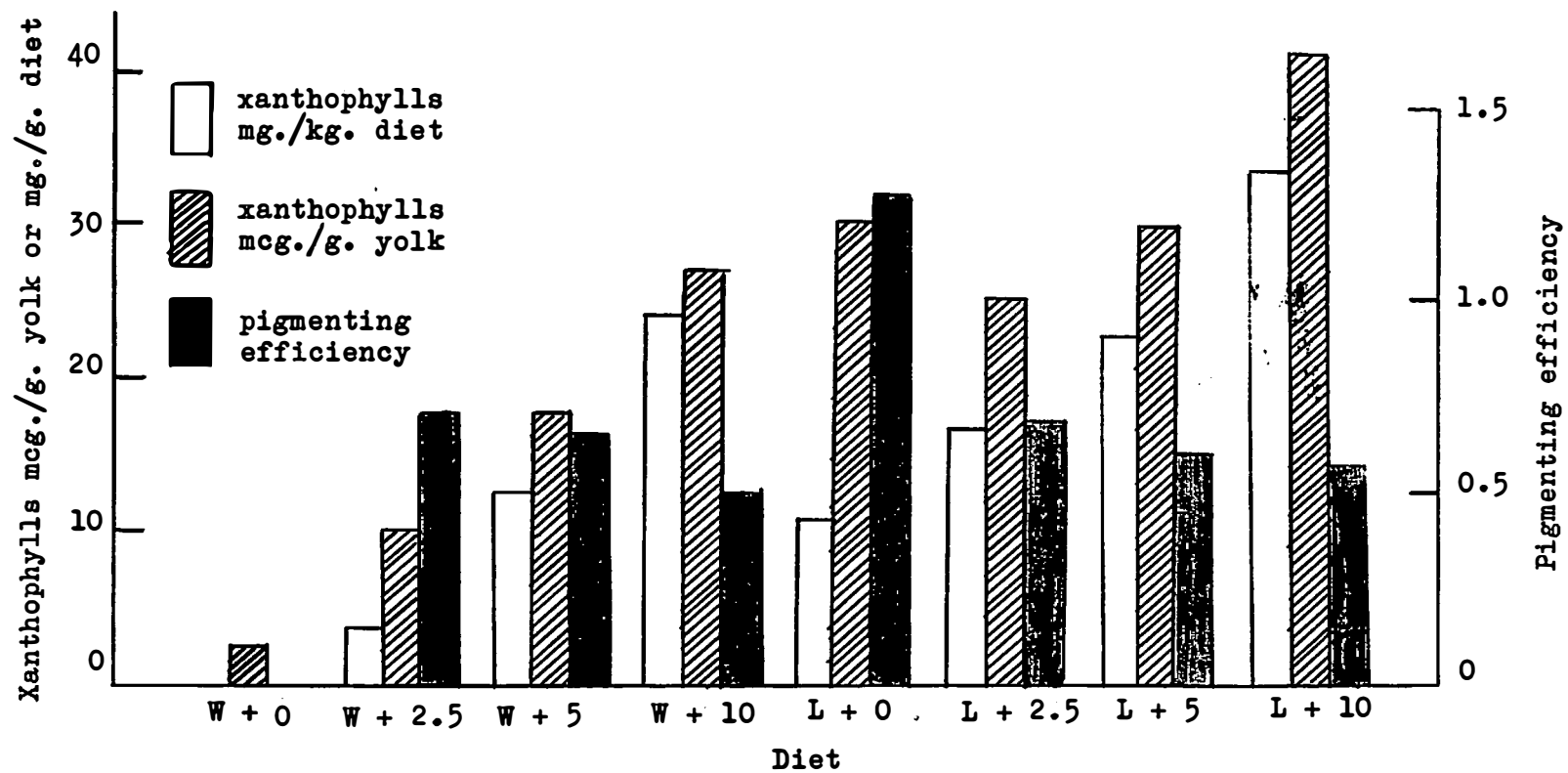


Figure 6. Some Effects of Various Dietary Levels of Cow Manure.

manure added is apparent in the increased level of xanthophylls in the yolk although the total xanthophylls in the diet are utilized less efficiently. The greater efficiency and unexpectedly high utilization of xanthophylls in the layer plus no cow manure (L + 0) is shown in the graph. Although cow manure was a good source of xanthophylls, it was not efficiently utilized by the hen as a source of xanthophylls.

Experiment 4

An analysis of variance test showed a significant difference in the data between trial 1 and trial 2, therefore, the two trials were analysed separately. This difference between trials probably resulted from the differences in rooms used in the two trials, the higher average temperature in the "cool" rooms in trial 1 than in trial 2, the fewer number of weeks between observations in trial 1 than in trial 2 and perhaps in the age of birds used.

The average specific gravity score (shell thickness) of eggs in trial 1 was 0.7 and 2.2 in the "hot" and "cool" rooms, respectively, and in trial 2 were 2.0 and 3.0 in the "hot" and "cool" rooms, respectively. This difference in the shell thickness indicates that the temperature in the "hot" rooms was high enough to affect the metabolism of the hens.

Trial 1. A summary of the effect of age, temperature and feeding regime on the level of yolk xanthophylls and yolk visual scores in trial 1 is given in Table X, and the analysis of variance in Table XI.

TABLE X
EFFECTS OF AGE, TEMPERATURE, AND FEEDING REGIME ON LEVEL
OF YOLK XANTHOPHYLLS AND VISUAL YOLK SCORES--
TRIAL 1, EXPERIMENT 4

Main effect	Means ¹	
	Yolk xanthophylls ² mg./g.	Yolk visual score ²
Young	40.84	8.79
Old	40.28	8.64
Cool	42.36 ^a	8.83 ^x
Hot	38.75 ^b	8.60 ^y
Limited	40.72	8.65
<u>Ad libitum</u>	40.39	8.78
2 weeks	40.25 ^y	9.17 ^a
7 weeks	43.56 ^x	8.58 ^b
12 weeks	37.86 ^z	8.40 ^c

¹Significant differences are indicated between member of the same group (i.e. young and old; cool and hot; limited and ad libitum, feeding regime; 2, 7 and 12 weeks) by superscripts x, y, and z for significance ($P \leq 0.05$) and a, b, and c for significance ($P \leq 0.01$).

²Yolk xanthophyll level as measured by the method of A.O.A.C. (1965).

³Measured with a 15-blade Roche Yolk Colour Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

TABLE XI
ANALYSIS OF VARIANCE OF LEVEL OF YOLK
XANTHOPHYLLS AND YOLK VISUAL SCORES--
TRIAL 1, EXPERIMENT 4

Source	Degree of freedom	Mean square ¹	
		Yolk xanthophylls ²	Yolk visual score ³
Age (A)	1	11.33	0.84
Temperature (T)	1	470.17**	2.01*
AT	1	36.20	0.84
Feed (F)	1	4.07	0.56
AF	1	155.00*	1.17
TF	1	70.00	3.06**
ATF	1	7.29	0.56
Weeks (W)	2	394.14**	7.76**
AW	2	85.69**	2.84**
TW	2	50.38	2.29**
ATW	2	66.95	0.22
FW	2	44.68	1.02
AFW	2	62.61	0.05
TFW	2	13.85	0.65
ATFW	2	8.66	0.40
Error	120	24.24	0.37
Total	143	-	-

¹Significant differences are denoted by
*Significant ($P \leq 0.05$)
**Significant ($P \leq 0.01$).

²Yolk xanthophyll level measured by the method of A.O.A.C. (1965).

³Measured with a 15-blade Roche Yolk Colour Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

According to spectrophotometric determinations and yolk visual scores there was no difference in the yolk pigmentation from the "young" and "old" hens at the beginning of the experiment. When the data from the three periods were combined and analysed, no significant differences ($P > 0.05$) were found in the indicators of pigmentation between "young" and "old" hens (Table X, page 62).

The levels of yolk xanthophylls and the yolk visual scores were significantly greater ($P \leq 0.01$ and 0.05 , respectively) from the "cool" rooms than from the "hot" rooms (Table X).

The level of yolk xanthophylls was not significantly different ($P > 0.05$) on the two feeding regimes (Table X).

Some significant interactions appeared in the analysis of variance as shown in Table XI, page 63. A significant ($P \leq 0.05$) age-feed interaction was found for the level of yolk xanthophylls. "Old" hens on limited feeding had more yolk xanthophyll (41.48 micrograms per gram) than those given feed ad libitum (39.07 micrograms per gram), but the opposite was true with the "young" hens (limited, 39.97 micrograms per gram; ad libitum 47.71 micrograms per gram). A significant ($P \leq 0.01$) interaction between temperature and feeding regime was noted. The yolks from the hens in the "hot" rooms on the ad libitum feeding had greater numerical visual scores (8.81) than those on the limited (8.39) feeding regime, but in the "cool" rooms the differences were not significant ($P > 0.05$).

There was a significant increase ($P \leq 0.05$) in yolk xanthophylls between the second and seventh week and a significant decrease ($P \leq 0.05$) on the twelfth week (Table X). The visual yolk

score showed a significant ($P \leq 0.01$) decrease for each successive measuring period. The reason for the increase at seven weeks is not apparent and is perhaps due to biological variation. Since there were no differences in visual scores at the beginning due to age it is difficult to understand why there was a drop as the birds became older.

A significant ($P \leq 0.01$) age-week interaction resulted when the yolk xanthophylls and the yolk visual scores from the "young" hens at 12 weeks was less than at four weeks, but no differences ($P > 0.05$) in yolk xanthophylls or yolk visual scores from the "old" hens as the trial proceeded. A significant interaction ($P \leq 0.01$) with temperature and weeks resulted in the lowest yolk visual score (8.29) at 12 weeks in the "cool" rooms and at seven weeks (8.25) in the "hot" rooms.

Trial 2. A summary of the effect of age, temperature and feeding regime on the level of yolk xanthophylls and yolk visual scores in trial 2 is given in Table XII and the analysis of variance in Table XIII.

According to spectrophotometric determinations and yolk visual scores there was no difference in the yolk pigmentation from the "young" and "old" hens at the beginning of the experiment. When the data from the three periods were combined and statistically analysed no significant differences ($P > 0.05$) were found in the indicators of pigmentation between "young" and "old" hens (Table XII).

The level of yolk xanthophylls was significantly greater ($P < 0.01$) from hens in the "cool" room than from hens in the

TABLE XII

EFFECTS OF AGE, TEMPERATURE, AND FEEDING REGIME ON LEVEL OF
YOLK XANTHOPHYLLS AND VISUAL YOLK SCORES--
TRIAL 2, EXPERIMENT 4

Main effect	Means ¹	
	Yolk xanthophyll ² mg./g.	Yolk visual score ³
Young	52.70	9.10
Old	52.19	9.01
Cool	55.29 ^a	9.17 ^x
Hot	49.59 ^b	8.94 ^y
Limited	54.22 ^a	9.10
<u>Ad libitum</u>	50.67 ^b	9.01
4 weeks	54.59 ^x	9.23 ^a
12 weeks	51.49 ^y	9.27 ^a
20 weeks	51.25 ^y	8.67 ^b

¹Significant differences are indicated between members of the same group (i.e. young and old; cool and hot; limited and ad libitum feeding regime; 4, 12, and 20 weeks) by superscripts x, y, and z for significance ($P \leq 0.05$) and a, b, and c for significance ($P \leq 0.01$).

²Yolk xanthophyll level as measured by the method of A.O.A.C. (1965).

³Measured with a 15-blade Roche Yolk Colour Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

TABLE XIII
ANALYSIS OF VARIANCE OF LEVEL OF YOLK
XANTHOPHYLLS AND YOLK VISUAL SCORES--
TRIAL 2, EXPERIMENT 4

Source	Degrees of freedom	Mean square ¹	
		Yolk xanthophylls ²	Yolk visual score ³
Age (A)	1	9.30	0.25
Temperature (T)	1	1169.64**	1.78*
AT	1	102.68	0.25
Feed (F)	1	453.69**	0.25
AF	1	100.67	1.78*
TF	1	17.50	0.69
ATF	1	27.91	0.11
Weeks (W)	2	165.92*	5.47**
AW	2	35.66	0.90
TW	2	197.32*	0.63
ATW	2	104.19	0.06
FW	2	77.97	0.77
AFW	2	16.60	0.92
TFW	2	147.74*	0.63
ATFW	2	21.43	0.17
Error	120	46.29	0.27
Total	143	-	-

¹Significant differences are denoted by
*Significant ($P \leq 0.05$).
**Significant ($P \leq 0.01$).

²Yolk xanthophyll level measured by the method of A.O.A.C. (1965).

³Measured with a 15-blade Roche Yolk Colour Fan. The blades varied in color from pale-yellow (number 1) to orange-red (number 15).

"hot" room. The yolk visual scores were significantly greater ($P \leq 0.05$) from the "cool" room hens than from the "hot" room hens (Table XII, page 66).

The level of yolk xanthophylls was significantly greater ($P \leq 0.01$) from hens on the limited feeding regime than from the ad libitum feeding regime (Table XII).

Some significant interactions appeared in the analysis of variance as shown in Table XIII, page 67). A significant ($P \leq 0.05$) age-feed interaction was obtained for the yolk visual scores. "Old" hens on limited feeding had a greater yolk visual score (9.17) than those given feed ad libitum (8.86), but no significant difference was found ($P > 0.05$) in feeding regimes with the "young" hens.

The yolk pigmentation in general decreased significantly ($P \leq 0.05$) with successive measuring periods. The temperature-week interaction shows that this was true only in the "cool" room and that the yolks from the "hot" room resulted in an increase during the last period (Table XII). Between the second and third measuring period (at about 14 weeks) the hens in the hot room molted and went through a short rest period, that is, they ceased to lay. It is possible that the hens were able to store xanthophylls during this period to be later deposited in the egg yolks as is typical of molting hens.

The temperature, feeding regime and weeks interaction indicated that the yolk xanthophyll levels from the "cool"-limited regime and the "cool"-ad libitum regimes at four weeks to be significantly greater ($P \leq 0.05$) than other combinations (Table XIII, page 67).

In both trials the hens in the "cool" rooms and the hens on the limited feeding regime produced egg yolks with the greatest pigmentation. Although not significant, the "young" hens produced eggs with numerically higher pigmentation scores than "old" hens for both trials. The optical observations did not show differences in yolk pigmentation as well as did the chemical determination.

Based on these data it appears that age is not a major factor in causing the light yolk during periods of high temperature. Temperature is the most important factor studied, with the hens in the "cool" winter-like temperatures producing eggs with the greatest levels of yolk xanthophylls and the largest yolk visual scores. The limiting of feed failed to cause a decrease in yolk color as is generally thought, but resulted in deeper pigmented yolks as measured by yolk xanthophyll level in trial 2. Douglas (1966) found similar results when he produced more deeply pigmented broilers on a limited feeding regime.

V. SUMMARY

A total of four experiments were conducted to determine the location of the site of absorption of xanthophylls, to determine the relationship of egg yolk color produced by various feed xanthophylls to the color of mayonnaise, and to study the effect of the level of dietary cow manure, age, ambient temperature and feed consumption on xanthophyll pigmentation of hens and egg yolks.

Increases in the level of blood xanthophylls and visual pigmentation of xanthophyll depleted hens were used to indirectly measure the absorption of xanthophylls. Surgical removal of either the duodenum, jejunum, ileum or large intestine resulted in a slight but significant decrease in absorption of xanthophylls when compared to the sham operated chicks and ligation of the ceca resulted in a slight increase. Only the removal of that section of the jejunum-ileum affected by E. maxima resulted in chicks with no significant absorption when compared to chicks fed a diet free of xanthophylls. It is concluded that most absorption of xanthophylls in the chick takes place in this middle section of the jejunum-ileum.

Mayonnaise was made with yolks from hens fed diets containing no xanthophyll, 23 milligrams of xanthophyll per kilogram and the latter diet plus 66 milligrams of xanthophyll per kilogram from yellow, orange or red concentrate. Visually, the egg yolks appeared different, but there were no significant differences found between the yolks from hens fed the diets with the added concentrates.

A triangle test identified the color differences in mayonnaise

samples. Mayonnaise made from the egg yolks from hens fed the xanthophyll free diet was much lighter and readily distinguished from all other samples. There were no significant color differences between mayonnaise made from egg yolks from hens fed the "layer" plus yellow concentrate and "layer" plus orange concentrate.

All pigmenting concentrates fed resulted in deeper color of mayonnaise than resulted from the "layer" diet alone, with the red concentrate having the greatest carry-over effect. The color of mayonnaise depends on the kind and amount of xanthophylls present.

Dried cow manure was added at the rate of 0, 2.5, 5, or 10 kilograms per 100 kilograms of diets containing 0 and 23 milligrams of xanthophyll per kilogram of diet to determine the effect on blood xanthophyll levels and the pigmentation of yolks produced by hens on these diets. There was a high positive linear correlation between the amount of cow manure added and the amount of xanthophyll in the blood. There was a high negative linear correlation between pigmenting efficiency and the amount of cow manure added to the diet. Although cow manure was a good source of xanthophylls, it was not efficiently utilized by the hen as a source of xanthophylls.

Visual yolk color and yolk xanthophyll levels were used to determine the effect of age, temperature and amount of feed consumed on yolk pigmentation. Both young and old hens were housed in rooms designed to simulate winter and summer temperatures. Half of the hens of each age group at each temperature had access to feed ad libitum and the other half received feed limited to 90 percent of that consumed in the hot rooms by hens on the ad libitum regime.

The hens in the "cool" rooms produced egg yolks with the greatest pigmentation. A four months difference in age was not a major factor in causing light colored yolks. However, egg yolk color generally became lighter as the hens grew older. In one trial limited feeding resulted in deeper pigmented yolks as measured by yolk xanthophyll levels. It is concluded that the reduction of the yolk xanthophyll levels during the summer is associated more with high temperature per se, and less with aging and a drop in feed consumption as generally assumed.

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