SOME PHYSICAL AND CHEMICAL ASPECTS OF MOTTLED EGG YOLKS

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ABSTRACT

SOME PHYSICAL AND CHEMICAL ASPECTS OF
MOTTLED EGG YOLKS

by Denny Angelo Silvestrini

Mottled egg yolks, chemically induced, were obtained from two groups of 12 White Leghorn hens which were fed diets supplemented with 0.0125 and 0.001 percent Nicarbazin, respectively, and control eggs from a third group of 12 White Leghorns which was fed a basal ration. During the first three weeks of the experiment, all eggs laid were collected, broken-out and scored for degree and incidence of mottled yolks and albumen quality. All other eggs laid were stored for various periods at 35°F. and broken-out and scored for incidence and severity of mottled yolks.

Paper electrophoresis analyses were employed to study and separate the proteins of the mottled and normal yolks. After separation and isolation of the lipoproteins, lipovitellin and lipovitellenin, from both normal and mottled yolks, the lipids were extracted from the lipoproteins and analyzed on silicic acid chromatography columns. Gas chromatography was used to separate the fatty acids of the phospholipids and triglycerides of the lipoproteins obtained from silicic acid column chromatographic analyses. Amino nitrogen, total nitrogen, phosphorus, choline and cholesterol determinations were carried out on the phospholipids and triglycerides obtained from the lipovitellin and lipovitellenin of mottled and normal yolks.

A direct relationship was found between the number of mottled yolks and length of time hens were fed the supplemented diets. Storage of eggs from hens fed the drug caused an increase in degree and number

of mottled egg yolks. Twenty-five percent fewer eggs were laid by hens fed 0.0125 percent Nicarbazin, but drug-supplemented diets fed to hens had no effect on albumen quality.

Electrophoresis and lipoprotein isolation analyses showed that mottled yolks contained less lipovitellin and lipovitellenin but more water soluble protein. A direct relationship was observed between the mottled yolk score and the proportion of ovalbumin in the yolk.

Lipovitellin of mottled and normal yolks contained the same percentage of ether and alcohol extracted lipids but lipovitellenin of mottled yolks contained 6.3 percent more ether extracted lipids than normal yolks.

Triglycerides, cholesterol, cephalins and lecithins in ether extracted lipids from lipovitellin of mottled and normal yolks showed no differences. More lecithins and less cephalins were found in alcohol extracted lipids from lipovitellin of mottled yolks than normal yolks, but no differences in triglycerides and cholesterol were observed between the alcohol extracted lipids of normal and mottled yolks.

Ether extracted lipids from lipovitellenin from mottled and normal yolks contained the same percentages of triglycerides, cephalins and lecithins but mottled yolks contained more cholesterol than normal yolks. Less triglycerides and cholesterol but more cephalins and lecithins were found in alcohol extracted lipids from lipovitellenin of mottled yolks than normal yolks.

Mottled yolks contained more oleic acid in triglycerides of the ether extracted lipids of lipovitellin and in triglycerides of the ether and alcohol extracted lipids from lipovitellenin than normal yolks.

The appearance of mottled areas in yolks from hens fed Nicarbazin was probably due to the presence of varying amounts of water and ovalbumin under the vitelline membrane of the mottled yolks.

SOME PHYSICAL AND CHEMICAL ASPECTS OF MOTTLED EGG YOLKS

By

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INTRODUCTION

The appearance of an abnormality in eggs, referred to as mottled yolks, has been recognized for at least 28 years, but little is known of it's incidence in eggs from farm flocks. Although mottled yolks have been found most frequently in stored eggs, they have also been observed in fresh eggs.

The mottled condition observed on egg yolks has been described as transparent, orange-yellow, oily appearing spots which appear on the surface of the yolks.

Some investigators have shown that slight mechanical pressure on the yolk membrane will produce a condition similar to a mottled area and others found that mottled yolks may be induced by drugs, antioxidants and other compounds. It is still uncertain, however, which factors are responsible for mottled yolks which occur in eggs obtained from hens not fed diets containing specific drugs. Long storage periods and high temperatures have been found to increase the intensity and incidence of mottled yolks.

Nicarbazin[1], a coccidiostat, usually recommended for young chickens, has been fed experimentally to laying hens to produce eggs with mottled yolks. Due to carelessness in mixing feed, the drug has been fed to laying hens on farms and mottled yolks have resulted.

Limited data are available concerning the physical and chemical differences between normal and mottled egg yolks.

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[1] Nicarbazin is a trade name for an equimolecular complex of 4, 4[1]-dinitrocarbanilide (DNC) and 2 - hydroxy - 4, 6-dimethylpyrimidine.

The purposes of this study were to determine differences in egg yolk proteins and lipids between normal and mottled egg yolks; to evaluate the influence of egg storage on the incidence of mottled yolks; and to evaluate the influence of the length of Nicarbazin feeding period on yolk mottling and other egg characteristics.

LITERATURE REVIEW

History of Mottled Egg Yolks

Almquist (1933) first observed mottled yolks in eggs from hens fed cottonseed meal in their diet. Yolks from newly-laid eggs appeared normal but the incidence and degree of mottling increased during storage. Mottled yolks contained more water, more egg white protein and a higher protein-fat ratio than normal yolks. Almquist concluded that mottled yolks were caused by diffusion of egg white protein into the yolk because the vitelline membrane was more permeable than that in normal eggs. Schaible et al. (1936) observed a mottled yolk condition apparently similar to that described by Almquist (1933), but the mottled areas of the egg yolks were not caused by feeding cotton seed meal in the diet of the hens. They found that a mottled area could be produced by slight mechanical pressure on the yolk membrane, as done by pushing with the tip of the finger.

Jeffrey (1945) frequently observed mottled yolks in the eggs laid by Barred Plymouth Rock and Rhode Island Red hens but not in the eggs from Single Comb White Leghorns or Legbars. Another defect similar to mottled yolks was caused by the mixing of fragmented yolk with albumen. Romanoff and Romanoff (1949) reported that mottled yolks were seen in stored eggs more frequently than in fresh eggs.

Nicarbazin, a new coccidiostat introduced in 1954, adversely influenced hatchability, thus was not recommended for adult chickens. Within two years after the inclusion of Nicarbazin in broiler rations the presence of mottled yolks in eggs from hens fed Nicarbazin was discovered and reported by Polin et al. (1956 b).

Polin et al. (1957) concluded that the accidental feeding

of Nicarbazin to layers might result from (1) erroneous use of starter, broiler or grower feeds which contained Nicarbazin and fed to hens, or (2) through contamination of layer rations with such feeds. Mottled yolks could occur in eggs even when the hens did not receive Nicarbazin in the ration. No significant increase in the occurrence of mottled yolks was shown until the level of Nicarbazin in the ration was increased to a minimum of 0.005 percent. Polin and Porter (1956) found similar results and reported further that when levels as high as 0.03 percent Nicarbazin were fed a direct relationship was found between degree of mottling and the percentage of Nicarbazin in the ration.

Polin et al. (1956 a) developed a method of analysis for 4-4^{1}-dinitrocarbanilide (DNC) in the yolks of eggs. The rate at which DNC was deposited in the eggs of hens fed Nicarbazin depended upon the concentration of the compound in the blood and tissues and upon the rate of yolk deposition.

Weiss (1957) showed that Nicarbazin fed at a level of 0.0125 percent for 10 to 15 days to small groups of White Rock and White Leghorn pullets and to hybrid hens caused a "whitening of the tinted eggs" after 3 days treatment. Egg production was reduced an average of 8 percent and egg weight an average of 5 percent from control levels. Shell thickness and albumen scores were not affected. One out of nine White Leghorn hens fed Nicarbazin laid fresh eggs with severely mottled yolks.

Polin et al. (1958) reported that 0.007 percent Nicarbazin in the ration of laying hens decreased egg size, by reducing the yolk size. When eggs from hens fed a minimum of 0.005 percent Nicarbazin were stored for 7 to 10 days, significantly more mottled yolks were found than in normal eggs. Extremely mottled yolks were associated with lower albumen quality, but no effect on albumen quality was evident in eggs

which were only slightly mottled.

Severely mottled yolks were observed in eggs stored for 5 or more days at room temperature (Miller et al. 1957). These investigators reported that an increase in level of dietary fat did not increase the number of severely mottled eggs.

Baker et al. (1957) reported that laying hens fed Nicarbazin produced eggs with "blemished" or mottled yolks. When a level of 0.0125 percent Nicarbazin was fed, nearly all of the eggs were severely mottled. At lower levels of Nicarbazin (0.006 to 0.009 percent) only a few severely mottled yolks were observed and only a minimum of 0.0015 percent Nicarbazin was required to increase the incidence of mottled yolks. Storage for 1 to 2 weeks increased the incidence of severely mottled yolks from hens fed 0.006 percent Nicarbazin in the ration. Egg production was reduced markedly by feeding 0.0125 percent Nicarbazin to Leghorns and by feeding 0.007 percent Nicarbazin to the heavy breed hens. Egg size was also reduced by feeding a minimum of 0.006 percent Nicarbazin in the diet. The heavy breeds produced white-shelled eggs when fed 0.009 percent Nicarbazin while a level of 0.003 percent had no effect on shell color, but produced some mottled yolks.

The results of McLoughlin et al. (1957) agree with those of Weiss (1957) and Baker et al. (1957) in that heavy breed hens laid white-shelled eggs after the hens had been fed 0.0125 percent Nicarbazin for 3 days. During the second week of treatment, hens fed Nicarbazin laid only one-half as many eggs as the controls. This difference in egg production was maintained more than a week after Nicarbazin feeding was discontinued. No significant differences were reported in egg weight, shell thickness or interior quality of eggs between those produced by hens fed a control ration and hens fed Nicarbazin.

Miller et al. (1957) showed that holding eggs at room temperature for 3 days resulted in 35 percent slightly mottled yolks and 25 percent moderately mottled yolks. Polin et al. (1956 b) found an incidence of 12 percent mottled yolks in eggs stored at 50°F. Similar results were observed by Baker et al. (1957), McNally and Brant (1958) and Mitchell and Stadelman (1958).

The antioxidants, gallic acid and n-propylgallate, when fed at a level above that which occurred during normal feed consumption, increased the incidence of mottled yolks of eggs stored for 2 to 3 days at 55°F. (McNally and Brant, 1958). Tannic acid fed to hens caused an increase in number of mottled yolks. Dawson (1960) stated that certain worm medicants and ammonia gas have been known to cause mottled yolks.

Chemical Composition of Mottled Yolks

Mottled yolks were reported to contain more water and less solids, lipids and nitrogen than non-mottled yolks according to Polin (1957). He found yolk material intermingled with the inner thin layer of albumen in most eggs with extremely mottled yolks. Polin (1957) agreed with the theory of Baker et al. (1956) that the water passing from the albumen into the yolk appeared to be involved in yolk mottling. Baker et al. (1956) reported that extremely mottled yolks from hens fed Nicarbazin had a more alkaline pH than normal yolks.

Van Tienhoven et al. (1958) observed yolk weight differences between mottled and non-mottled egg yolks which were similar to those reported by Polin (1957). Mottled yolks contained a significantly higher water content than normal yolks. No significant difference was observed between mottled and normal eggs in behavior of the yolk contents to a standard membrane. The lack of difference in ash content between

the mottled and normal yolks would substantiate the theory that yolk composition was not responsible for the added water uptake.

Polin (1960), in a review of "yolk-mottling", reported that the predominant change in mottled yolks was a movement of water from the albumen to the yolk. He stated that "mottling" occurred when yolk material passed into solution because the water, carrying with it buffering salts from the albumen, shifted the pH of the yolk to the alkaline side. Polin (1960) thus described the mottled yolks as "watered-down" yolks.

Three general observations concerning mottled yolks which Baker et al. (1956), Polin (1957) and van Tienhoven (1958) have reported are as follows:

1. Incidence and severity of mottled yolks in eggs laid by hens fed Nicarbazin increased during storage.

2. The mottled areas on yolks produced by hens fed Nicarbazin were associated with a movement of water from the albumen to the yolk and this change accompanied a decrease in the fat content or an increase in the protein-fat ratio of the egg yolks.

3. The change in pH of mottled yolks from acid to alkaline was accompanied by a water movement from albumen to yolk.

Lipoprotein Analyses

Romanoff and Romanoff (1949) reviewed the literature prior to 1949 on yolk composition and reported that yolk was composed of 16.6 percent proteins, 32.6 percent lipids, 1.0 percent carbohydrates, 1.1 percent minerals and other constituents and 48.7 percent water. The yolk lipids contained 62.3 percent neutral fats, 32.8 percent phospholipids, 4.9 percent sterols and a trace of cerebrosides. Yolk fatty acid

fraction was composed of approximately 50 percent oleic, 27 percent palmitic, 11 percent linoleic, 6 percent stearic, and 6 percent palmitoleic, linolenic, clupanodonic and myristic acids.

Yolk protein consisted of three main fractions, lipovitellin, lipovitellenin and livetin. Dumas and Cahours (1842) first studied the egg yolk protein, ovovitellin. Osborne and Campbell (1900) isolated lecithovitellin from egg yolks and found that it contained lecithin, which could be removed with ethyl alcohol, leaving the insoluble vitellin. Chargaff (1942) and Alderton and Fevold (1945) reported different methods for isolating lipovitellin, and later Fevold and Lausten (1946) found that like lecithovitellin was also composed of two lipoproteins, lipovitellin and lipovitellenin. Evans and Bandemer (1957) reported a method for the separation and study of egg yolk proteins by paper electrophoresis.

Lipoprotein Composition

Wittcoff (1951), after a review of literature, stated that lipovitellenin contained approximately twice as much lipid as lipovitellin. Lipovitellenin contained 36 to 41 percent lipids and comprised 40 percent of the total lipoprotein of the egg yolk. When lipoprotein was emulsified in water, the protein was slowly liberated and became increasingly less soluble in salt solutions. The globular nature of the lipoproteins appeared to depend on the phosphatide present and the emulsifying power of the egg yolk appeared to be due to the phosphatide present in complex formation with the protein.

Chargaff (1942) found that lipovitellin prepared by his method contained 18 percent phosphatides which could be removed by extraction with alcohol. Lipovitellin contained 1.5 percent phosphorus, 13.0 per-

cent nitrogen, 0.9 percent sulfur and 3.9 percent ash. Composition of lipovitellin isolated by Alderton and Fevold (1945) agreed well with that of Chargaff (1942). Lipovitellin isolated by Sugano (1957) contained 13 to 14 percent lipid and 2.2 to 2.4 percent phosphorus compared to 16 to 18 percent lipid and 2.3 to 2.4 percent phosphorus in that obtained by Fevold and Lausten (1946).

Lipovitellenin, isolated by Fevold and Lausten (1946) contained 9.9 to 10.2 percent nitrogen and 1.56 to 1.74 percent phosphorus and 36 to 41 percent lipid. Composition of egg lipovitellenin isolated by Sugano (1957) agreed with that of Fevold and Lausten (1946).

Phospholipids in Egg Yolks

As early as 1793 the existence of complex fatty compounds was well established. Thudicum (1884) differentiated lecithin from cephalin by separating the two phospholipids in alcohol. He found that cephalin was insoluble in alcohol but lecithin dissolved readily in alcohol. Folch (1942) reported "cephalin" was actually a mixture of several components, two of which were identified as phosphatidylethanolamine and phosphatidylserine. Another cephalin component identified was inositol.

Levene and Rolf (1921, 1927) developed a method for isolating lecithin by precipitation with cadmium chloride and then washing the precipitate with ether. Pangborn (1941) presented data on an improved quantitative lecithin isolation. Chargaff (1942) isolated lecithin and cephalin from egg yolk, and later Levine and Chargaff (1951) reported further analyses of egg yolk phospholipids. Sinclair (1948) isolated an egg yolk lecithin of good purity that was not contaminated by amino nitrogen.

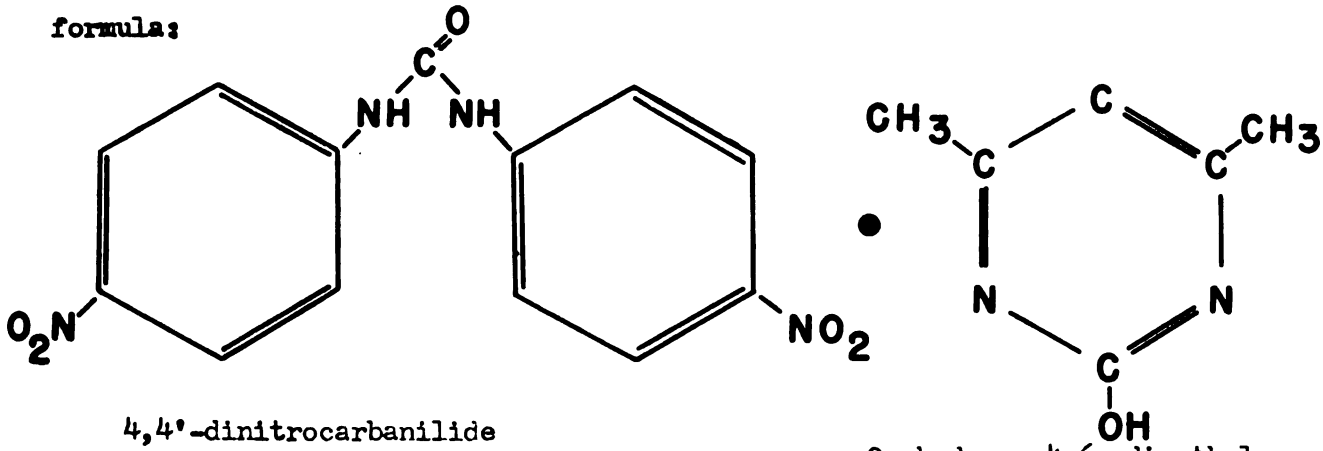
Aluminum oxide and silicic acid columns have recently been

used to separate phospholipids. Hanahan, Turner and Jayko (1951) reported good yield of highly purified lecithin from aluminum oxide columns. Lea and Rhodes (1953, 1954) used cellulose chromatographic columns to separate egg yolk phospholipids. More recently, Lea, Rhodes and Stoll (1955) prepared egg lecithin with a high degree of purity by chromatographic separations on alumina columns. Rhodes and Lea (1957) reported separating egg phospholipids into two fractions, one containing phosphatidylethanolamine and the remaining fraction containing components which phosphatidylcholine predominated.

EXPERIMENTAL PROCEDURE

Source and Quality of Mottled Egg Yolks

Thirty-six (36) White Leghorn hens (18 Dekalb strain and 18 Michigan State University strain) were placed in single bird laying cages. The Michigan State strain was put in the cages on one side of the battery while the other strain was placed on the opposite side of the battery. The hens on both sides of the battery were randomized into three groups; Group 1 was fed the Michigan State University Z-4 Battery all-mash laying ration while Groups 2 and 3 received the same ration supplemented with Nicarbazin (DNC) at levels of 0.001 and 0.0125 percent, respectively. The composition of the Z-4 basal ration was as follows: ground corn, 34.5 percent; ground oats, 20.0 percent; wheat bran, 15.0 percent; flour middlings, 10.0 percent; dehydrated alfalfa meal, 3.0 percent; meat scraps, 3.0 percent; dried skim milk, 2.0 percent; Menhaden fish meal, 2.5 percent; ground oyster shell flour, 5.0 percent; steamed bone meal, 1.5 percent; salt, 0.6 percent; and cod liver oil (2,000 A and 400 D), 0.4 percent. The Nicarbazin added to the basal ration was an equimolecular complex of 4, 4'-dinitrocarbanilide (DNC) and 2-hydroxy-4, 6-dimethylpyrimidine and has the following structural formula:



4,4'-dinitrocarbanilide

2, hydroxy-4,6, dimethyl-pyrimidine

NICARBAZIN

The feed supplements were added after all hens had been fed the basal ration for 2 weeks and feeding started on November 13, 1959 and was continued until March 31, 1960.

During the first 3 weeks after the Nicarbazin feeding had been started, eggs were collected twice daily and broken for fresh egg quality evaluation. All of these eggs were broken-out at intervals of from 1 to 6 days after being laid. All other eggs collected to the termination of the experiment were stored for various periods. Every egg was weighed, broken-out and the height of the albumen measured with an Ames tripod micrometer. The eggs were broken out on a glass plate supported between mirrors so that all surfaces of the egg yolk could be observed. Haugh score values for all the eggs were calculated according to the method of Brant and Shrader (1952).

The yolks were scored for degree of mottling according to a method similar to that of Polin et al. (1957), except that they were scored from 1 to 5, in units of one. The scoring system was established after samples of yolks with different degrees of mottling had been observed. A yolk with a score of 1 was very slightly mottled; however, only on very close yolk examination was the mottled area detected. Yolks with a score of 2 were classified as slightly mottled, easily detectable but not too objectionable in appearance. Yolks with moderate mottling were assessed a score of 3, while severely mottled yolks received a score of 4. Very severely mottled yolks were given a score of 5 and were characterized, in many instances, by being devoid of the bright yellow pigment and appeared orange, oily and transparent. To reduce personal error, all eggs were weighed by one individual and the breaking and quality measurements made by a second individual.

Eggs which were collected after the three-week daily-breaking

schedule were stored at 35°F. until further examination. At intervals during and after the feeding trial, eggs were removed from storage and quality evaluations carried out as previously outlined.

Paper Electrophoresis of Yolk Proteins

The paper electrophoresis procedures of Evans and Bandemer (1956, 1957) were used.

a) Preparation of the egg yolk samples

The yolks were separated from the whites, and any adhering egg albumen was removed from the yolks by rolling them over cheese cloth. The chalaziferous layer and chalazae were removed with the aid of a pair of tweezers. Care had to be taken with severely mottled yolks when removing the albumen and chalazae because the vitelline membranes were weakened due to internal pressure, and they ruptured easily. In general, the samples of yolk were obtained as whole egg yolks, diluted with an equal weight of 10 percent sodium chloride solution. In some cases, a 1 milliliter syringe was used to remove fluid from the mottled areas of the yolk.

b) Method of paper electrophoresis

The Spinco Model R paper electrophoresis apparatus, a ridge-pole type cell, manufactured by the Beckman Spinco Division, was used. A ten microliter sample of egg yolk was applied to each paper strip by means of a special stripper (furnished with Model R) and each sample was run in duplicate on each of two consecutive days. A constant direct current of 5 milliamperes (2.2 to 2.7 volts per centimeter) was applied over the paper strips for 16 hours at room temperature (approx - imately 25°C). Prior to applying the sample to the Whatman Number 3 MM paper strips, the filter papers were allowed to equilibrate in the buffer

solution for one hour. At the end of the 16 hour run, the papers were dried at 125 to 130°C. for 30 minutes. The strips were allowed to cool and were placed in a tray to be dyed over-night (16 hours) at room temperature. After immersion in the dye solution [1], the strips were rinsed in two consecutive rinses with 5 percent acetic acid, each rinse for 6 minutes. Then the paper strips were rinsed in a fixing solution [2] for another 6 minute period. Excess fixative was allowed to drain from the strips. The strips were dried carefully between two sheets of clean paper towelling after which they were oven dried for 15 minutes at 120 to 130°C.

The buffer solution used for the electrophoretic yolk protein analysis was a diethylbarbiturate (barbital) buffer of pH 6.8. The buffer was made by dissolving 10.309 grams of sodium diethylbarbiturate and 1.8419 grams of diethylbarbituric acid and diluting to 1 liter with distilled water. This buffer gave a solution which was 0.05 M in sodium diethylbarbiturate and 0.01 M in diethylbarbituric acid.

c) Paper strip analysis

Some of the stained filter strips were scanned on a Spinco Model RA Analytrol recording scanner and integrator. The distance travelled by each protein was determined by measuring the distance of the recorded peaks from the origin. Distribution of yolk proteins was determined quantitatively after cutting each strip into several parts.

1/ The dye solution contained 0.10 gram of bromophenol blue, 50.0 grams zinc sulfate ($7H_2O$) and 5 percent acetic acid to make 1 liter. In order to speed up solution of the dye, a few milliliters of alcohol were added before dissolving the zinc sulfate in acetic acid.

2/ 3.0 grams of sodium acetate ($3\ H_2O$) were diluted to 1 liter with 5 percent acetic acid.

Each part of the paper strip was placed in a 100 milliliter beaker and then 25 milliliters of 0.01 N sodium hydroxide solution were added in each beaker. The color was extracted for one hour with frequent swirling. After color extraction, the solution was mixed well and the absorbance measured at a wave length of 590 m μ in the Beckman Model B spectrophotometer.

Preparation of Egg Yolk Proteins

a) Lipovitellin

The lipoprotein, lipovitellin, was prepared by a modification of the method of Alderton and Fevold (1945). After removing all adhering albumen and chalazae, 12 yolks were pooled for analysis. Removal of the albumen was aided by rolling each yolk on cheesecloth, and the chalazae were removed with tweezers. The yolks were well mixed with twice their weight in distilled water, and the diluted yolk emulsion was centrifuged in a Servall centrifuge, at full speed, for 30 minutes. The precipitate, lipovitellin, had a waxy, light-yellow appearance. After dissolving the precipitate in a 5 percent sodium chloride solution, the lipovitellin mixture was filtered through cheesecloth to remove foreign materials such as vitelline membranes or chalazae not previously removed. The filtrate was placed in cellophane tubes and dialyzed with distilled water in a walk-in refrigerator (temperature at 2 to 3°C). When dialysis was complete, (no chloride ion detected in dialysate,) the lipovitellin solution was shell frozen, lyophilized, weighed and stored at -30 to -40°C.

b) Lipovitellenin

This yolk protein was prepared by the method described by Evans and Bandemer (1957). The supernatant from the lipovitellin preparation

was dialyzed in a refrigerator at 2 to 3 °C. until the dialysate was free of chloride ions. The pH of the mixture was adjusted to 6.2 with sodium hydroxide and the mixture was centrifuged at room temperature for 30 minutes and allowed to cool in a walk-in refrigerator to 2 to 3 °C. Cooling the mixture solidified the oily lipovitellenin precipitate which had risen to the top of the centrifuge tubes during centrifugation. Cooling the precipitate provided a clear-cut separation of the solid and liquid fractions. The lipovitellenin was a dark yellow, fatty-looking solid. The precipitate was dissolved in 5 percent sodium chloride and dialyzed to remove the chloride ions. After complete dialysis, the lipovitellenin mixture was shell frozen, lyophilized, weighed and stored at -30 to -40 °C.

c) Livetin

The filtrate remaining after centrifugation of the lipovitellenin mixture contained the livetin fraction. This fraction appeared as a milky-yellow, somewhat opalescent-like solution. After complete dialysis, the solution was shell frozen, lyophilized, weighed and stored at -30 to -40 °C. Since the experimental analyses in this study did not involve this protein, no attempt was made to purify it.

The above separation for lipoproteins was used for both normal and mottled yolks. The two lots of yolks were analyzed separately, so as to complete the whole separation procedure in as short a time as possible and thereby reduce loss of lipoproteins due to oxidation or other changes.

Lipid Extraction of Lipoproteins

The lipovitellin and lipovitellenin fractions of both the normal and mottled yolks were used for the extraction of total lipids.

Duplicate 10.0 gram samples of each freeze-dried lipoprotein were extracted for 30 minutes in 125 milliliter glass-stoppered Erlenmeyer flasks by shaking with three 50 milliliter portions of peroxide-free diethyl ether. The extraction was carried out at room temperature and as much as possible under a nitrogen gas atmosphere. Immediately after extraction, the lipids were poured into tared 50 milliliter beakers and the ether evaporated off under nitrogen gas on a controlled 60°C. hot plate. At no time were the lipids in the beakers allowed to become even slightly warm since occasional swirling of the beaker prevented the bottom layer from becoming too warm. When the odor of ether was no longer detected in the lipids, the beaker containing the lipids was placed in a desiccator. With the aid of a vacuum pump the desiccator was evacuated and then the desiccator was placed in a walk-in refrigerator at 2 to 3°C. This desiccator evacuating procedure was continued until the lipid weights remained constant.

The residue remaining from the ether extraction was extracted again, this time using absolute ethyl alcohol. The residue was shaken for 30 minutes at room temperature using three 50 milliliter portions of alcohol and kept as much as possible under a stream of nitrogen gas. The same solvent evaporating system as used above with the ether samples was carried out for these samples. Following evaporation, the beakers were placed in a desiccator and evacuated until the weight of the beakers containing the lipids was constant. The lipid samples were stored at all times in a walk-in refrigerator held at 2 to 3°C.

Silicic Acid Column Chromatography

a) Silicic acid preparation

Mallinckrodt's analytical reagent (100 mesh powder)

silicic acid suitable for chromatographic analysis was used throughout this study. The general column chromatographic procedure used was essentially the same as that outlined by Lea et al. (1955), except that no "celite" was added to the silicic acid slurry. The silicic acid was prepared by drying overnight at 110 to 120°C. and stored in a moisture-proof brown glass bottle. Ten grams of the dried silicic acid were mixed with 25 to 30 milliliters of chloroform-methanol 98:2 (v/v) to make a thin slurry. Settling and draining of the column was aided by applying slight nitrogen gas pressure.

b) Column and apparatus preparation

The columns used in this study measured 19 x 200 millimeters and had a standard taper glass disk (fritted) at the bottom of the column. The top of the column was attached by a cork stopper to a separatory funnel which in turn was connected to a tube from a nitrogen gas tank. When the silicic acid slurry was added to the column, the reservoir funnel was connected and a slight nitrogen pressure used to regulate the flow rate at 1 to 2 milliliters per minute. It was washed with 50 milliliters of the solvent.

c) Addition of sample to column and solvents systems used

After the column was washed with the initial 50 milliliters of chloroform-methanol 98:2 (v/v) and when the level of this solvent reached the surface of the adsorbant, a sample of mixed lipid containing 0.5 to 0.6 gram in 15 to 20 milliliters of chloroform-methanol 98:2 (v/v) was added. The sample was allowed to flow slowly on to the column without disturbing the surface of the adsorbant. The sample adsorbed on the column was washed with 100 milliliter portions of the following solvents: chloroform-methanol (98:2, v/v) to elute triglycerides, sterols and sterol esters; chloroform-methanol (70:30, v/v)

to elute cephalins; methanol-chloroform-water (75:20:5 by volume) to elute lecithins.

The solvent system employed in the chromatography of a mixed phospholipid sample in this study followed the procedure of Rhodes and Lea (1957).

d) Fraction separation and evaporation

After each fraction was collected, the solvents were evaporated to a small volume on a 60°C. hot plate under a stream of nitrogen. The fractions were transferred to tared 30 milliliter beakers and then evaporated to dryness. The beakers, containing the fractions, were placed in a desiccator and the desiccator was evacuated. After the samples had reached constant weight, they were weighed and diluted with chloroform and transferred to glass-stoppered volumetric flasks and stored at 2 to 3°C.

Analytical Methods

a) Amino nitrogen

The quantitative ninhydrin method described by Lea and Rhodes (1954) was used for determining the amino nitrogen content of the phospholipids and triglyceride fractions. Duplicate samples containing 0.3 milligrams of lipid, dissolved in chloroform, were pipetted into 5 X 5/8" pyrex tubes and the chloroform evaporated off under a stream of nitrogen. The residue was taken up in 1 milliliter of methyl cellosolve in which it dissolved quite readily. Two milliliters of ninhydrin solution[3/] were added and each tube shaken and placed in a

3/ Reagent contained stannous chloride, 0.016 gram per milliliter in citrate buffer (pH 5), and ninhydrin, 0.4 gram per milliliter in methyl cellosolve. The solutions were mixed, and allowed to cool. On mixing these two solutions, a brown color appeared which slowly faded to yellow when the reagent was ready for use. This reagent was freshly prepared for each analysis.

water bath at 88°C. for 20 minutes. When the tubes cooled, 7 milliliters of methyl cellosolve were added and the contents of the tube mixed well. The samples were allowed to stand for 10 to 15 minutes and the absorbance of the samples was read on a Beckman Model B Spectrophotometer at 570 m μ. The samples were read against a blank containing the evaporated residue from 0.5 milliliters chloroform.

Each time an amino-nitrogen determination was conducted a standard curve was also run. For purposes of this study, a standard ranging from 0 to 6 micrograms was adequate. The following volumes of the final standard solution[4/] (10 micrograms per milliliter) were added to pyrex tubes: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 milliliter and made up to a one milliliter volume with methyl cellosolve.

b) Total nitrogen

A modified micro-Kjeldahl method, developed in the Agricultural Chemistry Department, Michigan State University, was used.

The sample in chloroform was pipetted into the digestion flask and the chloroform evaporated off with the aid of low heat and nitrogen. The size of sample used to provide accurate and valid results was in the range of 30 to 50 milligrams of lipid. Following the chloroform evaporation, the following substances were added to the digestion flask:

4/ Ten milliliters of 2-amino-ethanol (pH about 13) were weighed into a 100 milliliter beaker. The beaker was placed in a cold water bath and the pH of the 2-amino-ethanol adjusted to about 8.5 using approximately 15 to 20 milliliters of concentrated hydrochloric acid. Hydrochloric acid diluted 1 to 5 with water was added dropwise to the 2-amino-ethanol to adjust the pH to exactly 5. This solution was added to a 100 milliliter volumetric flask and diluted with distilled water. The correct concentration for the standard curve was obtained by further diluting 5 milliliters of the stock solution to 100 milliliters with water and then diluting 0.5 milliliter of this dilution to 50 milliliters with distilled water (10 micrograms amino-nitrogen per milliliter).

1 milliliter 10 percent copper sulfate, 2 milliliters concentrated sulfuric acid, 0.5 gram potassium sulfate and several glass beads to prevent bumping. When all the black (carbon) materials had disappeared and digestion was thought to be almost complete, 3 drops of "superoxal" (hydrogen peroxide) was also added at three different intervals. The blank sample contained the residue from evaporation of chloroform. When frothing appeared to be a problem a drop of capryl alcohol was added to the digestion flask prior to heating. The distillation was carried out in a closed steam distillation system. The receiver flask contained 20 milliliters of 2 percent boric acid and 5 drops of bromo creosol green (0.1 percent bromcreosol green in alcohol). The boric acid solution containing the distillate (ammonia) changed the color of the solution from olive green to a blue shade. Titration of the final distilled solution from blue to green was done with 0.0142 normal sulfuric acid. A sample calculation shows the procedure for determining the nitrogen content.

$$\frac{\text{Milliliters of sulfuric acid}}{1000} \times \text{equivalent weight of nitrogen (14)} = \text{grams of nitrog}$$

$$\frac{\text{Grams of nitrogen}}{\text{Grams of sample}} \times 100 = \text{percent nitrogen}$$

One (1) milliliter of 0.0142 normal sulfuric acid was equivalent to 0.198 milligrams of nitrogen. From this relationship the weight or percent of nitrogen in the original sample was calculated.

c) Phosphorus

The method of Fisk and Subbarow as described by Hawk, Oser and Summerson (1947) was employed to determine the phosphorus content of the triglycerides and phospholipids.

Samples containing 0.7 to 0.9 milligram of lipid were transferred to pyrex test tubes graduated at 10 milliliter, and the

solvent was evaporated under a stream of nitrogen. To each tube was added 0.6 milliliter of 1:1 sulfuric acid and several boiling chips, and the tube was heated on a micro burner until the material appeared charred. The tubes were removed from the microburner and 1 drop of 30 percent hydrogen peroxide was allowed to drop on the reaction mixtures followed by heating again. The hydrogen peroxide additions were repeated until the contents became colorless (usually 5 to 7 drops of hydrogen peroxide were required). After clearing, digestion of the mixture was carried on for 3 minutes. When the tubes had cooled, 3 milliliters of distilled water were added to each tube and then the mixture was again brought to a boil to decompose pyrophosphate.

After the samples had cooled, 1.2 milliliters of 5 normal sodium hydroxide (saturated sodium hydroxide diluted 1:1 with distilled water) were added and the samples were mixed thoroughly. One milliliter of molybdate reagent[5/] was added and the mixture shaken thoroughly. Then 0.4 milliliter of aminonaphthosulphonic acid reagent[6/] was added

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5/ Twenty-five grams of reagent grade ammonium molybdate were dissolved in about 200 milliliters of distilled water. Five hundred milliliters of 10 normal sulfuric acid were placed in a 1 liter volumetric flask, the molybdate solution added anddiluted with washings to the liter mark with distilled water. This solution was stable indefinitely.

6/ One hundred and ninety-five milliliters of 15 percent sodium bisulfite solution were added to a glass-stoppered cylinder and 0.5 gram of 1,2,4-aminonaphtholsulfonic acid was added. Then 5 milliliters of 20 percent sodium sulfite were added and the cylinder shaken until the powder dissolved. (15 percent sodium bisulfite: to 30 grams of sodium bisulfite in a beaker, 200 milliliters of distilled water were added from a graduated cylinder and stirred until the powder dissolved). (20 percent sodium sulfite: 20 grams of anhydrous sodium sulfite were dissolved in distilled water and diluted to 100 milliliters).

and the volume in each tube made to 10 milliliters with distilled water. After the samples were allowed to stand for 10 to 30 minutes, their absorbance was read in the Beckman Model B Spectrophotometer at 660 m μ. A blank containing 1 milliliter of chloroform was treated in the same manner as the samples.

The standard solutions[7] were treated in the same manner as the samples except that to each tube 0.6 milliliter of sulfuric acid (1:1) was added and the solution evaporated on a microburner, until white fumes appeared in the tubes. Then one drop of hydrogen peroxide was added to each tube and the procedure continued as for the samples.

d) Iodine number

The method of Trappe (1938) was employed since it was applicable to micro amounts of lipids. Samples containing 0.5 to 0.8 milligram of lipid were transferred to 50 milliliter glass-stoppered Erlenmeyer flasks. Two milliliters of 0.01 normal bromine solution in sodium bromide saturated methyl alcohol[8] were added to each sample.

7/ Exactly 0.351 gram of pure dry monopotassium phosphate was dissolved in distilled water and transferred quantitatively to a 1 liter volumetric flask. Ten milliliters of 10 normal sulfuric acid were added and the solution was made up to 1 liter with distilled water. This solution contained 0.4 milligrams of phosphorus in 5 milliliters. It was found to be stable indefinitely. Five milliliters of this stock phosphate standard, containing 0.4 milligrams of of phosphorus were added to a 50 milliliter volumetric flask, made up to volume with distilled water and mixed thoroughly. One milliliter of the last solution contained 0.008 milligrams of phosphorus. To establish a standard curve 0, 1, 2, 3, 4, and 5 milliliter portions of this last solution were added to 10 milliliter graduated pyrex test tubes.

8/ Bromine in sodium bromide saturated methyl alcohol was made in the following way: one milliliter of bromine was added to 100 milliliters of methanol saturated with sodium bromide. Then 2.5 milliliters of this were made to 100 milliliters with sodium bromide saturated methanol.

The flasks were stoppered and allowed to stand in the dark for 4 hours with occasional swirling. To each flask was added 1.0 milliliter of 2 percent potassium iodide solution and two drops of freshly prepared, 1 percent starch solution. A solution of 0.002 normal sodium thiosulfate solution[9/] was used to titrate this sample. The blank sample contained 1 milliliter of chloroform. A sample calculation for determining the iodine number is shown below.

$$\text{Iodine number} = \frac{\text{Titration difference} \times 0.2538 \times 100}{\text{Weight of lipid}} \times \frac{\text{Normality of thiosulfate}}{.002}$$

e) Choline

The choline content of the lecithin fraction was determined by the method of Enterman et al. (1944). The determination of choline by this method required at least 20 to 30 milligrams of lipid material per sample. Each sample was transferred to a 50 milliliter beaker and the solution made basic to phenolphthalein with saturated barium hydroxide, and then 25 milliliters more saturated barium hydroxide were added. The beakers were covered with watchglasses and hydrolyzed for 2 hours at about 100°C. on a controlled temperature hot plate. Some reduction in the volume of the samples did occur; however, distilled water was added occasionally to prevent the samples from becoming dry. When hydrolysis was complete, the samples were neutralized with glacial acetic acid and filtered directly into 50 milliliter centrifuge tubes using Whatman No. 42, 7.0 centimeter filter papers. The beakers were

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9/ The sodium thiosulfate solution was made by dissolving 24.83 grams of sodium thiosulfate in distilled water and making to 1 liter, and further diluting 10 milliliters to 500 milliliters with distilled water. The thiosulfate solution was standardized using 5 milliliters of standard potassium iodate (.3568 grams per liter), 1 gram potassium iodide, 10 milliliters of distilled water, 0.5 milliliters of glacial acetic acid and 1 milliliter of 1 percent starch.

rinsed with distilled water and the washings added to the filter paper; however, the combined washings did not exceed 25 milliliters. Five milliliters of a 2 percent solution of reineckate salt in methanol were added to the filtrate and the samples were allowed to stand overnight in a refrigerator at 2 to 3°C. to insure complete precipitation of the choline reineckate. The samples were centrifuged at 2800 revolutions per minute for 15 minutes to pack the precipitate. After decanting the supernatant, the precipitate was washed with cold ethyl alcohol and then centrifuged for the same time as previously outlined. The supernatant was again discarded and the precipitate washed with cold ethanol and centrifuged as before. After centrifugation, the supernatant was decanted and the tube inverted on clean towels to drain the excess ethanol. The washed choline reineckate precipitate was dissolved in a small amount of acetone and then passed through Whatman No. 42 filter paper and collected in 25 milliliter volumetric flasks. After rinsing out the centrifuge tubes with acetone, the washings were also passed through the filter paper. The filter paper was washed with acetone until no choline reineckate precipitate remained on the filter paper. The filtrate was made up to the 25 milliliter mark of the volumetric flasks with acetone and read in the spectrophotometer at 525 mμ. The blank contained acetone only. A standard curve was obtained from results of the following standard preparation. One-tenth gram of a reagent grade choline chloride was made up to 10 milliliters with distilled water giving a solution containing 10 milligrams per milliliter. Then 5 milliliters of this latter solution were diluted to 50 milliliters (1 milligram per milliliter). Aliquots of 1, 2, 3, 4, 5, 6, 7, 8 and 9 milliliters of the 1 milligram per milliliter solution were transferred with a pipette to 50 milliliter beakers. The solutions

were made basic to phenolphthalein with saturated barium hydroxide and the remainder of the procedure was continued as with the lipid samples.

f) Total cholesterol

The method of Schoenheimer and Sperry as cited in Hawk, Oser and Summerson (1947) was employed to determine the total cholesterol content of the triglyceride fraction. Samples containing 3.0 to 5.0 milligrams of lipid were transferred to 15 milliliter centrifuge tubes. After the chloroform was evaporated under nitrogen, three milliliters of acetone-ethanol (1:1) were added to dissolve the residue. Three drops of potassium hydroxide solution (10 grams potassium hydroxide in 20 milliliters of distilled water) were added from a dropping bottle. A stirring rod was added to each graduated centrifuge tube and the contents stirred vigorously. The centrifuge tubes, with the stirring rods, were placed in a preserving jar containing a layer of sand about 3 centimeters deep, previously heated to 45°C. The cover was placed on the jar and then placed in an incubator at 37 to 40°C. for 35 minutes(included was 5 minutes for temperature equilibration). After incubation, the tubes were removed to a wire tube rack and allowed to cool to room temperature. The stirring rods in the tubes were raised momentarily and 3 milliliters of acetone-ethanol were used to wash the rods and sides of the tubes. After mixing, one drop of phenolphthalein solution was added to each sample and then 10 percent acetic acid was added drop by drop until the red color just disappeared and then 1 drop in excess. Three milliliters of digitonin solution (0.4 gram of digitonin was dissolved in 100 milliliters distilled water and the solution filtered) were added to each tube. After a thorough stirring, the tubes, with

stirring rods, were placed in a quart size preserving jar, covered tightly and allowed to stand at room temperature overnight.

The tubes were transferred to a test tube rack and the samples stirred gently to free the particles of precipitate from the tube walls. The rods were removed and placed on a numbered wire rack being very careful not to lose any precipitate particles. The samples were centrifuged for 15 minutes at 2800 revolutions per minute. After decanting the supernatant, the tubes were inverted and allowed to drain for a few minutes on a clean paper towel. The stirring rods were replaced in the tubes and the walls of the tubes washed with 1.5 to 2.0 milliliters of acetone-ether (1:2) from a dropping bottle. The samples were thoroughly mixed, centrifuged for 5 minutes, and after decanting off the supernatant, the precipitate was washed with diethyl ether. The stirring rods were added to the samples and set aside until ready for color development.

A layer of sand about 3 centimeters deep was placed in a shallow pan and heated to 110 to 115°C. in an oven. The tubes containing the washed cholesterol digitonide precipitate were placed in the pre-heated sand and the pan was returned to the oven for 30 minutes. Two milliliters of glacial acetic acid were used to wash the stirring rod and walls of the tube while the tube was still in the hot sand. The samples were stirred well and allowed to stay in the sand for 2 minutes more (no longer). The tubes, including the blank and standard, were placed in a water bath at 25°C. in the dark and allowed to come to temperature equilibrium. Four milliliters of cold acetic anhydride-sulfuric acid

reagent[10] (freshly prepared) were added to each tube, mixed well and returned to the water bath. The tubes were allowed to stand 27 minutes in the dark and read after 30 minutes in the spectrophotometer set at 625 m μ.

No standard curve was required, however, the absorbance of the samples was read against a blank and a standard cholesterol solution[11] containing 0.2 milligrams per milliliter. The blank contained 2 milliliters of glacial acetic acid. Calculation for the cholesterol content of the samples was done by the following procedure:

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times .2 \times \frac{100}{\text{weight of sample}} = \text{milligrams total cholesterol per 100 milliliters}$$

10/ A glass-stoppered, graduated cylinder was placed in a cold room at 2-3°C. along with the acetic anhydride bottle and concentrated sulfuric acid. Twenty milliliters of chilled acetic anhydride and 1 milliliter of concentrated sulfuric acid (a little at a time) were mixed together by placing the stopper on the cylinder and shaking vigorously for a few moments. The reagent was not prepared until ready to be used and at all times kept chilled.

11/ One hundred milligrams of pure cholesterol were dissolved in 50 milliliters of glacial acetic acid by warming on an electric hot plate and stirring, then transferring this mixture with the rinsings to a 100 milliliter volumetric flask. The solution was diluted to 100 milliliters with acetic acid. For making up the working solution, the latter stock solution was warmed to room temperature and 5 milliliters of this were diluted to 50 milliliters with glacial acetic acid (contained 0.2 milligrams per 2 milliliter).

g) Fatty acid methyl esters

Samples from the silicic acid columns were transferred to 100 milliliter beakers and saponified at room temperature by standing overnight in 10 milliliters of ethanol containing 1.5 grams of potassium hydroxide (12 grams potassium hydroxide and 80 milliliters ethanol). Twenty-five milliliters of distilled water were added to dissolve the mixture and then 0.75 milliliters concentrated sulfuric acid in 5 milliliters of distilled water (6.0 milliliters sulfuric acid + 40 milliliters water) were used to acidify the samples. The solutions were allowed to cool and extracted three times in a separatory funnel with peroxide free diethyl ether (using 20 milliliters ether for each extraction). The combined extracts were evaporated in a beaker on a hot plate at 60°C. under a stream of nitrogen. After a large part of the ether had evaporated, the samples were transferred to glass stoppered volumetric flasks and made to volume with ether.

Diazomethane[12/] was added dropwise to 15 to 20 milligrams of the mixed fatty acids in cold ether to convert the fatty acids to the methyl esters, and ether was removed under a stream of nitrogen. The residues were taken up in 100 microliters of pure benzene and transferred to a vial and tightly stoppered until ready for use.

12/ Preparation of Diazomethane: To a distilling flask was added 0.5 gram potassium hydroxide, 0.8 milliliter water and 2.5 milliliters of ethanol. After mixing, the flask was heated to 65°C. Two receivers in ice water were connected through a cold water condensor. One receiver contained 3.0 milliliters of ether. To the distilling flask was added a solution of 2.15 grams of "Diazold" in 13 milliliters of ether. Two more milliliters of ether were added and the rate of distillation was regulated to equal the rate of addition of the "Diazold" solution.

Aliquots of 5 to 10 microliters of fatty acid methyl esters dissolved in pure benzene were injected into the gas-liquid chromatographic apparatus for separation of the fatty acid methyl esters. The determinations were conducted on a Wilkens Aerograph Model A-90-C gas-liquid chromatographic instrument with a Weston, 1 millivolt single point recorder. The fatty acid methyl esters were separated on a diethylene glycol succinate column operated at 200°C. with helium flow rate regulated at 100 milliliters per minute.

The percentages of fatty acids found in each sample were proportional to the areas under the peaks on the graph and the areas were calculated in square millimeters. The area under each peak was measured by multiplying the height of the peak by the width at a point mid-way between the base and top of peak. The percentage of each fatty acid was obtained by dividing the area under each peak by the sum of the areas under all the peaks. Percentage of fatty acids in a sample were calculated as follows:

Fatty acid	Height of curve	Width of curve at mid-point	Area under curve	Percent of fatty acid
	mm.	mm.	sq. mm.	
Myristic	5	2	10	0.4
Palmitic	193	3	675	29.2
Palmitoleic	21	5	105	4.5
Stearic	35	5	201	8.7
Oleic	143	6	926	40.0
Linoleic	53	7	398	17.2
Total			2313	100.0

RESULTS AND DISCUSSION

Photographs of Mottled Yolks

Eggs containing yolks varying in incidence and intensity of mottled areas were obtained from hens fed Nicarbazin and from hens fed a basal ration. Each egg observed was classified and given a visual score of 0 to 5, depending on the presence and appearance of the mottled areas. The typical appearances of a number of mottled egg yolks are shown in Figures 1 to 5. Yolks with mottled scores of 0 to 1 appeared similar, thus no photograph of an egg yolk with a score of 1 is included. Figure 1 is a photograph of a slightly mottled yolk classified with a visual score of 2; Figure 2, a moderately mottled yolk with a score of 3; Figure 3, a severely mottled yolk with a score of 4; and Figure 4, a very severely mottled yolk with a score of 5. A series of four yolks - one normal and three very severely mottled - are shown in Figure 5. One of these mottled yolks was obtained from a commercial farm flock and the others were laid by hens in this experiment.

Incidence of Mottled Yolks

a) Newly laid eggs

A direct relationship was found between the number of mottled yolks and the length of time the birds were fed Nicarbazin-supplemented rations. One-to 2-day old eggs from hens in Group 3 fed for 10 to 11 days were moderately to severely mottled while hens in Group 2 had a smaller percentage of moderately mottled yolks (Tables 1 and 2). At different intervals, all eggs produced during a 2-day

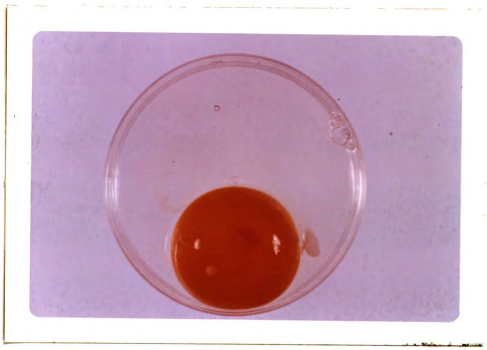

Figure 1. Photograph of an egg with a slightly mottled yolk given a visual mottling score of 2.

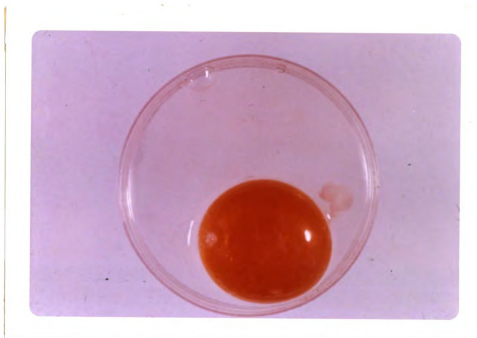

Figure 2. Photograph of an egg with a moderately mottled yolk given a visual mottling score of 3.

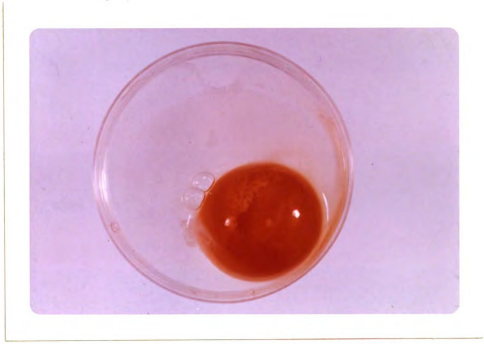

Figure 3. Photograph of an egg with a severely mottled yolk given a visual mottling score of 4.

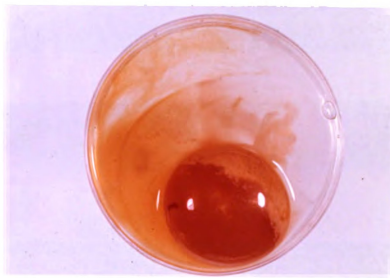

Figure 4. Photograph of an egg with a severely mottled yolk given a visual mottling score of 5.

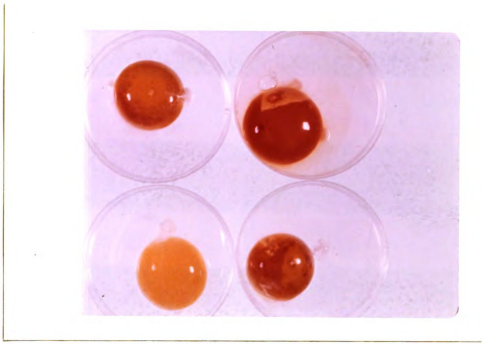

Figure 5. Photograph of 4 egg yolks.

Yolk upper left - very severely mottled obtained in present study.
Yolk upper right - very severely mottled obtained in present study.
Yolk lower left - normal obtained in present study.
Yolk lower right - very severely mottled obtained from commercial flock.

Table 1. Percentage of Eggs in Each Mottled Yolk Classification from Hens Fed Different Levels of Nicarbazin up to 132 Days

Feeding Period		Nicarbazin Supplement									
		Control Mottling Score			.001 percent Mottling Score			.0125 percent Mottling Score			
		0-1	2-3	4-5	0-1	2-3	4-5	0-1	2-3	4-5	
Days	No. eggs	Percent			No. eggs	Percent		No. eggs	Percent		
2-3	15	87	13	0	21	100	0	0	12	92	8	0
4-5	14	100	0	0	11	100	0	0	11	81	19	0
10-11	14	86	14	0	17	76	24	0	13	23	8	69
15-16	13	94	6	0	17	71	29	0	15	27	33	40
18-19	15	80	20	0	15	80	20	0	14	7	50	43
23-24	12	58	42	0	12	50	42	8	15	7	40	53
131-132	13	85	15	0	11	55	45	0	7	43	43	14

Table 2. Percentage of Fresh Eggs[1/] from Hens Fed Nicarbazin to 132 Days with Moderate to Very Severe Mottled Yolk Scores

Time on Feed (days)	Nicarbazin supplement								
	Control			0.001 percent			0.0125 percent		
	Total Eggs	Mottled yolk score of 3, 4, 5		Total Eggs	Mottled yolk score of 3,4,5		Total Eggs	Mottled yolk score of 3,4,5	
	Number	No.	Percent	Number	No.	Percent	Number	No.	Percent
2-3	15		0	21		0	12	1	8
4-5	14		0	11		0	11	1	9
10-11	14		0	17	3	18	13	10	77
15-16	13		0	17	1	6	15	8	53
18-19	15	1	7	15	1	7	14	9	64
23-24	12		0	12	4	33	15	13	87
131-132	13		0	11	1	9	7	4	58

1/ Eggs broken 1 or 2 days after they were laid.

period were broken and the yolks observed and given a mottled yolk score. The incidence and severity of mottled yolks increased in eggs from hens in Group 3 fed the supplemented ration for 10 days or more. Only minor differences were apparent in yolk scores between eggs from hens in Group 1 and Group 2. These results agree with data reported by Polin et al. (1957), Baker et al. (1957), Weiss (1957), and Polin et al. (1958).

b) Storage eggs

In Tables 3 and 4 are reported results which show the influence of storage and drug level in the feed on incidence of mottled yolks. Storage of eggs from hens in Groups 2 and 3 increased the number of mottled yolks compared to the control group, and this increase in number was most noticeable in yolks from hens in Group 3.

When eggs obtained from hens fed Nicarbazin were stored up to 13 months, more mottled yolks were observed (Table 5). Even some eggs from hens fed a basal ration showed a substantial increase in percentage of mottled yolks after storage for long periods. The mottled yolks which were observed in stored eggs from hens of Groups 2 and 3 appeared to be similar to the condition described by Almquist (1933) who reported that "blemished" yolks occurred in storage eggs of hens fed diets containing cottonseed meal or oil. Polin et al. (1957), Weiss (1957), Baker et al. (1957), McLoughlin et al. (1957) and van Tienhoven et al.(1958) have shown that the incidence of mottled yolks may be drastically increased by addition of 0.0125 percent Nicarbazin to the ration of laying hens.

c) Egg production and egg quality

Hens in Group 3 laid 25 percent fewer eggs while those

Table 3. Percentage of Eggs in Each Mottling Score, from Hens Fed Different Levels of Nicarbazin, After Storage at 35°F., Up to 107 Days

Storage Time	Nicarbazin supplement								
	Control Mottling score			.001 percent Mottling score			.0125 percent Mottling score		
	0-1	2-3	4-5	0-1	2-3	4-5	0-1	2-3	4-5
Days	Percent of eggs			Percent of eggs			Percent of eggs		
0	85	15	0	55	45	0	43	43	14
6-7	85	15	0	50	36	14	33	17	50
18-19	100	0	0	82	18	0	17	50	33
49-50	100	0	0	100	0	0	0	25	75
82-83	100	0	0	79	21	0	9	9	82
106-107	82	18	0	74	26	0	0	0	100

Table 4. Percentage of Eggs from Hens Fed Nicarbazin for 6 or More Days with Moderate to Severely Mottled Yolks

Time in storage at 35°F.	Nicarbazin supplement		
	Control	0.001% DNC	0.0125% DNC
Days	Percent	Percent	Percent
0	0	9	55
6-7	5	29	66
18-19	0	10	66
49-50	0	0	88
82-83	0	17	91
106-107	0	0	100

Table 5. Percentage of Eggs with Yolk Mottling Scores from Moderate to Very Severe from Hens Fed Nicarbazin 7 Weeks or More and Stored Up to 13 Months

Time in storage at 35°F.	Time on feed	Nicarbazin supplement		
		Control	0.001% DNC	0.0125% DNC
Months	Weeks	Percent	Percent	Percent
13	7	4	46	92
12	11	3	63	97
11	15	15	73	97
10	19	5	77	98

in Group 2 laid about the same number of eggs as the control group. No difference in albumen quality was found between the eggs of the hens fed a basal ration and those fed the supplemented rations.

The decrease in total number of eggs laid by hens in Group 3 is in agreement with the data reported by Weiss (1957), Baker et al. (1957), and McLoughlin et al. (1957). Others (Weiss, 1957, Baker et al. 1957, and McLoughlin et al. 1957) have also noted no adverse effect on albumen quality of eggs from hens fed Nicarbazin up to levels of 0.0125 percent.

Protein Distribution in Yolks of Normal and Mottled Eggs

Protein distribution data for yolks of normal and mottled eggs as determined by filter paper electrophoresis are presented in Table 6 and Figures 6 to 10.

Lipovitellin content of mottled yolks decreased as the mottled yolk score increased, whereas, the lipovitellenin-lipovitellin ratio increased showing that lipovitellenin increased relative to lipovitellin. The ovalbumin content of the mottled yolks increased until more than 20 percent of the yolk protein was ovalbumin in the severely mottled yolks. Electrophoretic mobility of the lipovitellenin increased as the degree of yolk mottling increased as shown by increased distances between the lipovitellin and lipovitellenin peaks. The distance between the lipovitellin and lipovitellenin in normal yolk was 9 millimeters while the distance between peaks of the mottled yolk was 14 millimeters. The composition of normal yolk protein agreed with data presented by Evans et al. (1958).

Lipoprotein Isolation

Mottled yolks contained less lipovitellin and lipovitellenin

Table 6. Percentage Protein Composition of Egg Yolks with Different Degrees of Mottling, as Determined by Paper Electrophoresis

Protein Fraction	Mottling score				
	0	2	3	4	5
	Percent				
Unidentified protein (a)	14.1	13.3	13.7	16.7	12.6
Lipovitellin (b)	39.1	38.4	33.9	24.9	25.2
Lipovitellenin (c)	38.5	38.5	39.9	37.9	33.2
Livetin (d)	8.3	7.0	8.5	7.5	6.5
Ovalbumin (e)	- -	2.8	4.0	12.9	22.5

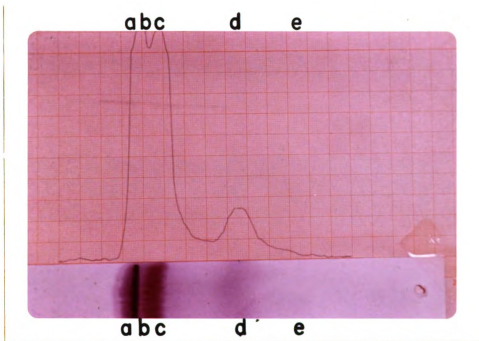

Figure 6. Filter paper strip and Analytrol scanner-integrator graph of the strip for normal egg yolk proteins.

a) water soluble protein
b) lipovitellin
c) lipovitellenin
d) livetin
e) ovalbumin

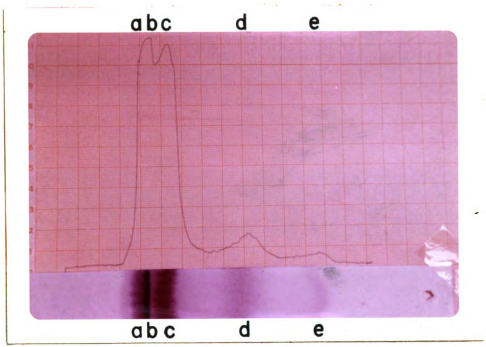

Figure 7. Filter paper strip and Analytrol scanner-integrator graph of the strip for slightly mottled egg yolk proteins.

a) water soluble protein
b) lipovitellin
c) lipovitellenin
d) livetin
e) ovalbumin

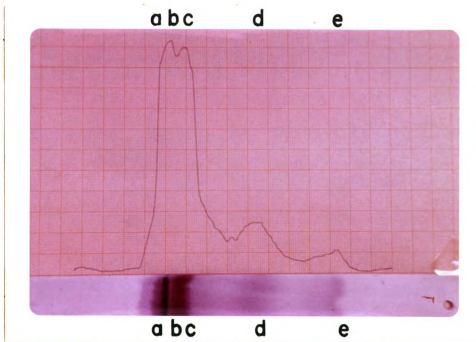

Figure 8. Filter paper strip and Analytrol scanner-integrator graph of the strip for moderately mottled egg yolk proteins.

a) water soluble protein
b) lipovitellin
c) lipovitellenin
d) livetin
e) ovalbumin

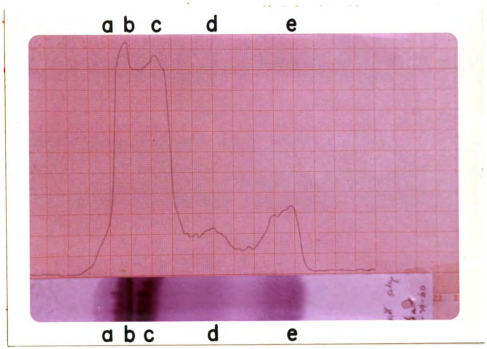

Figure 9. Filter paper strip and Analytrol scanner-integrator graph of the strip for a severely mottled egg yolk proteins.

a) water soluble
b) lipovitellin
c) lipovitellenin
d) livetin
e) ovalbumin

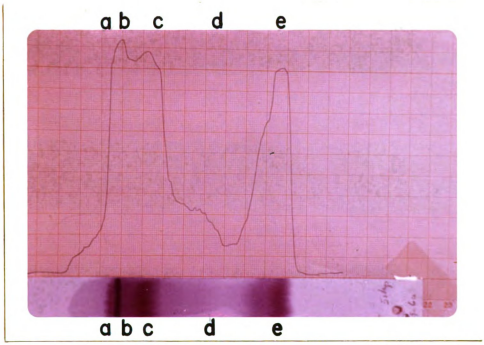

Figure 10. Filter paper strip and Analytrol scanner-integrator graph of the strip for very mottled egg yolk proteins.

a) water soluble protein
b) lipovitellin
c) lipovitellenin
d) livetin
e) ovalbumin

but more water soluble protein than normal yolks (Table 7). Table 8 shows that by calculation of the data, mottled yolks contained 0.92 grams more water per yolk than normal egg yolks. This value was somewhat lower than that reported by Polin (1957). The difference in the percentages of the lipoproteins found in Tables 6 and 7 are explained by the fact that the electrophoresis data in Table 6 measured only the protein part of the lipoprotein while the isolation data in Table 7 included both protein and lipid of the lipoprotein.

Lipoprotein Lipid Extraction

As shown in Table 9, the total lipid extracted from the lipovitellin of mottled and normal yolks was about the same. More of the lipids were extracted by ether than by alcohol from the lipovitellin and lipovitellenin. Lipovitellenin isolated from mottled yolks contained 6.3 percent more lipid than lipovitellenin from normal yolks. Lipovitellin contained about 31.7 percent lipid compared to 21.3 percent in that isolated by Evans and Bandemer (1961). Lipovitellenin isolated from normal yolks contained about 77.4 percent lipid compared to 82.0 percent in that of Evans and Bandemer (1961). Differences in source of eggs and methods of protein isolation and lipid extraction might account for these variations and those reported by Evans and Bandemer (1961).

Lipid Composition of Lipoproteins

No differences were observed between normal and mottled yolks in triglycerides, cephalins and lecithins in the ether extracted lipids of lipovitellin and lipovitellenin (Table 10). The alcohol extracted lipids of lipovitellin of mottled yolks contained less cephalins and more lecithins than that of normal egg yolks.

Table 7. Percentage Yield of Lipoproteins of Normal and Mottled Egg Yolks

	TREATMENT			
	Control		Mottled	
	Whole yolk (percent)	Dried yolk (percent)	Whole yolk (percent)	Dried yolk (percent)
Lipovitellin	11.5	23.6	9.9	21.8
Lipovitellenin	35.5	72.6	31.7	69.4
Water soluble protein	1.9	3.8	3.9	8.7
Total	48.9		45.5	
Water	51.1		54.5	

Table 8. Yield of Lipoproteins in Normal and Mottled Egg Yolks

	Treatment	
	Control	Mottled
	gm. per dozen eggs	
Whole yolk	216.3	223.2
Lipovitellin	24.9	22.3
Lipovitellenin	76.7	70.5
Water soluble protein	4.1	8.9
Water	110.6	121.6

Table 9. Percent Lipid in Normal and Mottled Yolk Lipoproteins

	Lipovitellin		Lipovitellenin	
	Normal	Mottled	Normal	Mottled
	Percent	Percent	Percent	Percent
Ether extract	18.9	17.1	59.7	67.8
Alcohol extract	12.8	14.5	17.7	15.9
Total lipid	31.7	31.6	77.4	83.7
Protein	68.3	68.4	22.6	16.3
Total Lipoprotein	100.0	100.0	100.0	100.0

Table 10. Lipid Distribution from Lipovitellin and Lipovitellenin of Normal and Mottled Yolks

	Triglycerides		Cholesterol		Cephalin		Lecithin	
	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled
				Percent				
				Ether Extracted Lipids				
Lipovitellin	81.0	81.0	5.7	5.4	5.1	4.7	8.2	8.9
Lipovitellenin	84.3	82.8	3.6	4.5	4.9	4.2	7.8	8.6
				Alcohol Extracted Lipids				
Lipovitellin	18.3	18.2	3.1	3.9	42.6	31.9	36.0	47.0
Lipovitellenin	12.3	5.0	2.6	1.7	49.5	52.4	35.6	40.9

No differences were observed between normal and mottled yolks in cholesterol in the ether and alcohol extracted lipids of lipovitellin. More cholesterol was found in the ether extracted lipids of lipovitellenin of mottled yolks than that of normal yolks. Less cholesterol was obtained in the alcohol extracted lipids of this lipoprotein from mottled yolks than that of normal yolks. The cholesterol content of lipovitellenin from normal yolks was similar to that found by McIndoe (1959). The alcohol extracted lipids of lipovitellenin from mottled yolks contained more cephalins and lecithins but less triglycerides than that found in normal yolks. The decrease in cephalins in lipovitellin of mottled yolks was not due to any contamination by triglycerides or lecithins during separation on the columns. Calculation of the phosphorus content showed that the triglyceride fraction did not contain enough phosphorus to account for the difference found in the cephalins.

The amount of nitrogen and phosphorus in phospholipids has been used as a measure of their purity. Theoretically, both cephalin and lecithin should contain approximately 1.7 percent nitrogen and 4.0 percent phosphorus. Inouye and Noda (1958) reported that their sample of egg phosphatidylcholine contained 1.76 percent nitrogen and 3.87 percent phosphorus, whereas, Pangborn (1941) found 1.81 percent nitrogen and 3.82 percent phosphorus for egg lecithin. Egg lecithin prepared by Levine and Chargaff (1951) contained 1.78 percent nitrogen and 3.88 percent phosphorus. Differences in amino nitrogen, nitrogen and phosphorus indicated that separation of the fractions on silicic acid columns was fairly satisfactory (Tables 11 and 12). The choline content of the lecithins of normal and mottled yolks was similar, however, the choline content was about 5 percent higher than that

Table 11. Composition of Lipids from Lipovitellin

	Ether Extract						Alcohol Extract					
	Triglycerides		Cephalin		Lecithin		Triglycerides		Cephalin		Lecithin	
	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled
	Percent						Percent					
Amino Nitrogen	0.0	0.0	1.39	.69	.04	0.0	0.0	0.0	.35	.69	0.0	0.0
Total Nitrogen	0.0	.07	1.39	.63	1.51	1.54	0.0	.07	1.38	1.64	1.46	1.26
Phosphorus	.82	.55	3.53	5.66	3.21	4.34	.64	.91	5.21	3.86	4.34	3.86
Choline	- -	- -	- -	- -	20.31	19.32	- -	- -	- -	- -	21.93	21.56

Table 12. Composition of Lipids from Lipovitellenin

	Ether Extract						Alcohol Extract					
	Triglycerides		Cephalin		Lecithin		Triglycerides		Cephalin		Lecithin	
	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled
	Percent						Percent					
Amino Nitrogen	0.0	0.0	1.47	1.92	0.0	0.0	0.0	0.0	.77	.57	.02	.04
Total Nitrogen	.12	.09	1.53	1.45	1.84	1.63	.46	.34	1.83	1.95	1.95	2.09
Phosphorus	0.0	0.0	2.57	2.72	2.60	3.25	.56	0.0	3.62	3.96	3.81	4.51
Choline	- -	- -	- -	- -	20.01	21.74	- -	- -	- -	- -	22.64	21.05

reported by Hanahan (1954). This difference may have been due to differences in techniques used for the choline determination.

In Tables 13 and 14 are reported the iodine numbers, distribution and degree of unsaturation of fatty acids of lipids extracted from the lipovitellin and lipovitellenin of normal and mottled yolks. The iodine number is defined as the number of grams of iodine absorbed by 100 grams of fat or oil. Since the iodine is absorbed by the double bonds in the molecule, the iodine number is a measure of the degree of unsaturation of the lipid.

It is difficult to explain these data due to the inconsistencies of the observed iodine numbers and calculated iodine numbers obtained from the fatty acid analyses. Furthermore, degree of unsaturation of the fatty acids did not always agree with the iodine number obtained. In general, that fatty acid analyses would be most reliable for evaluating the data on differences between the normal and mottled yolks.

Fatty acid gas chromatographic analyses showed more oleic acid was found in triglycerides of ether extracted lipids of lipovitellin from mottled yolks than normal yolks, but less stearic acid was found in cephalins from alcohol extracted lipids of mottled yolks than normal yolks. Mottled yolks contained more oleic acid in the triglycerides of ether and alcohol extracted lipids from lipovitellenin than normal yolks.

The chemical characterization of the triglycerides and phospholipids may have some merit; however, due to variability in the data, it was difficult to state whether or not the chemical differences which existed between the mottled and normal yolks were significant.

Table 13. Iodine Numbers, Distribution and Unsaturation of Fatty Acids in Lipids of Lipovitellin

	Ether Extract						Alcohol Extract					
	Triglycerides		Cephalin		Lecithin		Triglycerides		Cephalin		Lecithin	
	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled
Iodine No.	71	72	52	41	41	46	90	72	69	59	51	46
				Percent					Percent			
Fatty acids												
Myristic	0.8	0.4	0.0	0.0	0.0	0.0	0.6	0.4	0.0	0.0	0.0	0.0
Palmitic	32.7	25.7	25.6	21.6	40.2	33.7	29.2	24.2	26.0	22.4	39.0	34.5
Palmitoleic	4.8	5.3	3.6	1.4	5.9	2.5	4.1	5.5	1.5	2.5	2.0	3.2
Stearic	9.4	6.4	38.6	42.1	14.5	17.3	9.2	6.8	41.2	29.5	15.3	13.5
Oleic	37.4	46.7	23.7	25.8	27.2	29.6	40.2	45.9	22.3	29.2	27.9	31.9
Linoleic	14.9	15.3	8.5	9.2	12.2	16.9	16.7	17.1	9.0	16.5	15.8	16.9
Saturated	42.9	32.6	64.2	63.8	54.7	50.9	39.0	31.5	67.2	51.9	54.3	49.1
Unsaturated	57.1	67.4	35.8	36.2	45.3	49.1	61.0	68.5	32.8	48.1	45.7	51.9

Table 14. Iodine Numbers, Distribution and Unsaturation of Fatty Acids in Lipids of Lipovitellenin

	Ether Extract						Alcohol Extract					
	Triglycerides		Cephalin		Lecithin		Triglycerides		Cephalin		Lecithin	
	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled	Normal	Mottled
Iodine No.	67	76	51	59	49	50	68	69	69	60	46	47
	Percent						Percent					
Fatty acids												
Myristic	0.5	0.4	0.0	0.0	0.0	0.0	0.6	0.4	0.0	0.0	0.0	0.0
Palmitic	29.1	25.7	20.7	19.2	35.8	35.4	27.6	22.6	28.6	28.1	39.7	38.2
Palmit-oleic	6.5	4.5	1.8	1.9	2.9	3.1	6.1	3.8	2.6	2.2	4.0	2.9
Stearic	9.1	5.5	35.8	35.1	16.3	14.9	10.8	8.4	24.9	22.3	11.5	11.7
Oleic	38.7	46.3	28.6	22.9	29.6	28.9	38.5	47.1	28.3	25.9	30.1	30.3
Linoleic	16.2	17.6	13.2	20.9	15.4	17.7	15.5	17.8	15.7	21.5	14.8	16.9
Saturated	38.7	31.6	56.4	54.4	52.1	50.4	38.9	31.3	53.5	50.4	51.1	49.9
Unsaturated	61.3	68.4	43.6	45.6	47.9	49.6	60.1	68.7	46.4	49.6	48.9	50.1

SUMMARY AND CONCLUSIONS

Thirty-six (36) White Leghorn hens were placed at random in a single hen laying cage battery in three groups of 12 hens each. Group 1 was fed a control ration while Group 2 and Group 3 were fed diets supplemented with 0.001 and 0.0125 percent Nicarbazin, respectively. Nicarbazin was used to chemically induce mottled yolks in eggs from hens fed the drug.

A direct relationship was observed between the time and level of feeding the drug and the incidence of mottled yolks. When eggs from hens in Group 3 were stored 10 to 11 days, a marked increase in the number and degree of mottled yolks occurred and eggs from hens in Group 2 showed a similar trend but fewer mottled yolks were observed. Storage of eggs for 13 months from hens in Groups 2 and 3 caused a further increase in number and degree of mottled yolks and a low percentage of yolks from hens fed the basal ration were slightly mottled. Hens in Group 3 laid 25 percent fewer eggs while hens in Groups 1 and 2 laid about the same number of eggs; however, the albumen quality of the three groups was similar.

Chemical and physical determinations of an exploratory nature were used to ascertain what differences existed between mottled and normal egg yolks obtained in this study. Electrophoresis analyses showed a direct relationship between mottled yolk score and the proportion of ovalbumin in the yolks. Furthermore, the lipovitellenin fraction of mottled yolks showed a change in mobility which did not occur in normal yolks. This protein mobility difference was attributed to a chemical or physical change in the composition of the lipoprotein.

Electrophoretic differences in the lipoproteins observed between normal and mottled yolks were substantiated by similar differences obtained in the percentages of isolated lipoproteins. Mottled yolks contained less lipovitellin and lipovitellenin but more water soluble protein than normal yolks.

Lipovitellin of mottled yolks and normal yolks contained the same percentage of ether and alcohol extracted lipids but lipovitellenin of mottled yolks contained 6.3 percent more ether extracted lipids than normal yolks.

No differences were observed in the triglycerides, cholesterol, cephalins and lecithins in the ether extracted lipids from lipovitellin of mottled and normal yolks. Lipovitellin of mottled yolks contained more lecithin and less cephalin than normal yolks, but no differences were observed in triglycerides and cholesterol between the alcohol extracted lipids of normal and mottled yolks.

Ether extracted lipids from lipovitellenin from mottled and normal yolks contained the same percentage of triglycerides, cephalins and lecithins but mottled yolks contained more cholesterol than normal yolks. Alcohol extracted lipids from lipovitellenin of mottled yolks contained less triglycerides and cholesterol but more cephalins and lecithins than normal yolks.

Amino nitrogen, total nitrogen and phosphorus determinations showed that the separation of the fractions on silicic acid columns was fairly good. Choline determinations indicated that no differences existed between the lecithins of normal and mottled yolks.

Fatty acid gas chromatographic analyses showed more oleic acid was found in triglycerides of ether extracted lipids of lipovitellin

from mottled than normal yolks, but less stearic acid was found in cephalin from alcohol extracted lipids of mottled yolks than normal yolks. Mottled yolks contained more oleic acid in the triglycerides of ether and alcohol extracted lipids from lipovitellenin than normal yolks.

Due to variabilities in some of the chemical determinations, a complete evaluation of the differences between mottled and normal yolks was not possible, however, other results in this study would indicate that definite differences exist between the composition of the lipoproteins and lipids of mottled and normal yolks.

Under conditions of this experiment, the appearance of a mottled area on egg yolks from hens fed Nicarbazin was probably due to the presence of ovalbumin and water. The variation in appearance or intensity of the mottled areas on the yolk was probably caused by the different proportions of water and ovalbumin present under the vitelline membrane of the yolks.

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