Transfer of Protein in Stored Shell Eggs Produced by Hens Fed Crude Cottonseed Oil

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Yolks of eggs produced by hens fed crude cottonseed oil increase in size and become salmon colored during cold storage of the shell eggs. Egg whites decrease in size and become pink. Protein and water migrate from the white to the yolk. This investigation was conducted to see whether all of the protein migrates or there is a selective transfer of one particular white protein. Comparison of the changes in methionine, cystine, and serine in the white and yolk proteins determined by microbiological assay methods with published values for the individual proteins in egg white indicate that ovalbumin selectively migrated from the white to the yolk. The protein transferred contained 79% ovalbumin, 3% conalbumin, 15% ovomucoid, and 3% ovoglobulins, compared to 60% ovalbumin, 14% conalbumin, 14% ovomucoid, and 12% ovoglobulins in the protein of fresh egg white. A knowledge of the nature of the selectively migrating protein in "cottonseed" eggs together with further studies of its properties should lead to ways of preventing this migration and the resulting decrease in egg quality.

T WO TYPES OF DISCOLORATION of eggs from hens fed cottonseed meal or crude cottonseed oil occur when the eggs are stored for 6 months or longer. The small dark colored yolk first described in 1890 by Roberts and Rice (*12*), which turns to olive or chocolate brown after storage (*11*), was shown by Schaible, Moore, and Moore (*15*) to be caused by the gossypol in cottonseed. Sherwood (*17*) also observed another type of discoloration in which the yolks of stored eggs from hens fed cottonseed meal turned salmon in color and the whites turned pink. Schaible and Bandemer (*14*) demonstrated that the pink whites were caused by a reaction of iron from the yolk with conalbumin, and the salmon yolks by the same reaction and a blending of the pink with the natural yolk color. They isolated conalbumin from the yolk.

Sherwood (*17*) observed that "cottonseed eggs" had more yolk and less white than normal eggs, and that the yolk contained less fat and more water than normal egg yolk. Whites of freshly laid eggs have a pH of about 7.6 and yolks a pH of 6.0 or less (*16*). On storage, the pH of the white rapidly rises to 9.0 with the loss of carbon dioxide from the white. Yolk pH also rises. The pH of yolks of stored cottonseed eggs approaches the pH of the whites more rapidly than does that of normal eggs (*18*). Almquist and Lorenz (*1*) observed that protein as well as water increased in the yolks of stored cottonseed or "pink white" eggs.

The purpose of this investigation was to determine whether each of the white proteins is transferred to the yolk of cottonseed eggs, or whether some constituent protein, such as conalbumin, is selectively transferred. The data of Sherwood (*17*), of Almquist and Lorenz (*1*), and of Schaible and Bandemer (*14*) indicate a weakening of the vitelline membrane. Is the membrane so weak that it is no longer selective or does it allow some proteins to migrate from white to yolk more rapidly than others?

Experimental

Laying S.C. White Leghorn hens were kept in laying cages and fed a ration containing 2.5% crude cottonseed oil. The basal ration consisted of ground corn 34.5%, ground oats 20.0, wheat bran 15.0, flour middlings 10.0, dehydrated alfalfa 3.0, meat scraps 3.0, dried milk 2.0, fish meal 2.5, soybean oil meal 2.5, ground oyster shell flour 5.0, steamed bone meal 1.5, salt 0.6, and fish oil (400 D and 3000 A) 0.4. To each 100 pounds of basal ration were added 1250 ml. of crude cottonseed oil. The hens were started on experiment during the summer of 1949. Eggs were saved during November and December from ten hens. Three eggs from each hen were used the day after production and six were placed in cold storage at 0° C. in a walk-in refrigerator. Three eggs from each hen were removed for analysis after 3 months and three more after 6 months of cold storage.

The yolk and white of each fresh hard-boiled (20 minutes) egg were separated. The white was broken up, dried for 48 hours at 40° C., and weighed. The broken up yolk was extracted overnight with 100 ml. of absolute ethyl alcohol at room temperature, and then on the Goldfisch extractor for 4 hours with ethyl alcohol and for 4 hours with diethyl ether. The ether was evaporated and the fat-free protein concentrate weighed. The dried whites from the three eggs of each hen were mixed together, ground in a semimicro Wiley mill, and stored in the refrigerator in stoppered bottles. The three samples of extracted, dried yolk from each hen were also mixed, ground, and stored. Three and 6-month-old eggs were treated in the same way as the fresh ones.

Crude protein (N × 6.25) was determined by the Kjeldahl-Wilforth-Gunning procedure (*2*) on each sample and all amino acid values are presented as percentage of crude egg protein. Half-gram samples of dried white or yolk protein concentrate were hydrolyzed for amino acid assays by autoclaving for 6 hours with 20 ml. of 20% hydrochloric acid. The digests were neutralized and suitable dilutions made for microbiological amino acid assays. Methionine and cystine were determined by assay with *Leuconostoc mesenteroides* P-60 using the medium of Lyman and coworkers (*9*), which contains hydrogen peroxide–treated peptone as the source of most of the amino acids. Serine assays were performed with *Leuconostoc mesenteroides*

P-60 and the medium of Sauberlich and Baumann (*13*).

Results and Discussion

Most of the 6-month-old eggs from the hens fed crude cottonseed oil had typical pink whites and enlarged salmon yolks. However, eggs from one hen did not, and are not included in the averages presented in the tables. Although all the analyses were done on an individual hen basis, only the average for all the hens are presented in the tables. Three-month-old eggs were analyzed as well as the fresh and 6-month-old eggs, and the average values for them are presented, but only the fresh and 6-month-old eggs are considered in the following discussion, because nothing would be gained from a consideration of the 3-month data.

Some loss of protein from the 6-month-old eggs occurred (Table I). The 1.4% loss was about the same as the 1.2% loss from normal eggs after 18 months' storage (*4*). In normal eggs that had been stored for 18 months, 3.9% of the white protein had migrated to the yolk, but the white protein had decreased by 6.1% indicating a loss from the egg of 2.2% of the white protein. On the other hand, there was a transfer of 19.5% of the white protein to the yolk in cottonseed eggs that had been stored for 6 months, and 22.1% of the white protein had gone from the white, indicating a loss from the eggs of 2.6% of the white protein.

Table I. Transfer of Protein (N × 6.25) Between Whites and Yolks of Eggs from Hens Fed Normal Diet (4) and Crude Cottonseed Oil Diet

	Protein Transfer, Gram		
Diet	Fresh	9 mo. old	18 mo. old
Normal			
White	3.11	3.24	2.92
Yolk	2.79	2.74	2.91
Egg	5.90	5.98	5.83
		3 mo. old	6 mo. old
Cottonseed oil			
White	3.33	3.19	2.59
Yolk	2.98	3.06	3.63
Egg	6.31	6.25	6.22

About one fifth of the white proteins of cottonseed eggs migrated to the yolk during 6 months of cold storage and increased the yolk protein to 1.25 times the original weight. Ovalbumin is the predominant protein of egg white. Egg white proteins contain about 60% of ovalbumin, about 14% each of conalbumin and ovomucoid, and about 12% of globulins (*8*). Do all four of these

Table II. Transfer of Methionine in Stored Shell Eggs from Hens Fed Crude Cottonseed Oil

		Methionine			Methionine	
Protein	White Proteins, G.	In white proteins, %	In egg white, g.	Yolk Proteins, G.	In yolk proteins, %	In egg yolk, g.
Fresh eggs	3.331	3.79	0.126	2.982	2.47	0.074
3-mo.-old eggs	3.193	3.76	0.120	3.063	2.60	0.080
6-mo.-old eggs	2.594	3.68	0.095	3.632	2.82	0.103
Protein transferred	0.737	4.20a	0.031	0.650	4.46a	0.029
Ovalbumin		5.4 (4)				
Conalbumin		2.1 (4)				
Ovomucoid		0.9 (4)				
Lysozyme		2.0 (4)				
Ovoglobulin		1.1b				

a Values for percentage methionine in protein transferred were calculated by dividing methionine transferred by protein transferred and multiplying by 100.
b Calculated value. See text for method of calculation.

proteins migrate from the white to the yolk in the same proportions as they are found in the white, or is the migration selective with a larger percentage of one constituent—for example, conalbumin—migrating?

Previous work in this laboratory (*3, 5*) has shown that egg white proteins contain higher concentrations of methionine and cystine and lower concentrations of serine than do the yolk proteins. Ovalbumin, conalbumin, ovomucoid, and lysozyme, one of the globulins, differ widely in their content of methionine, cystine, and serine (*7*). The authors believed that by determining the percentages of the above three amino acids in the protein that migrated from the white to the yolk, they could get a fairly good estimation of which protein or proteins were migrating from the white to the yolk. The assumption was necessarily made that no protein went from the yolk to the white and that no breakdown of protein occurred; this appears reasonable, as no decomposition of egg white proteins was observed after up to 23 months of cold storage of normal eggs (*6*).

The average methionine content of the white protein (N × 6.25) from a 6-month-old egg was 3.68% compared to 3.79% for a fresh egg (Table II), which indicates the selective transfer of a protein containing a higher percentage of methionine than is found in the total white protein. Fevold (*7*) has assembled the available data for methionine in egg white proteins. The better values for ovalbumin, conalbumin, ovomucoid, and lysozyme are listed in Table II. Ovalbumin is the only egg white protein that contains more than 3.79% methionine, which indicates that it is the protein transferred in greatest amount. The total methionine per egg white decreased from 0.125 to 0.095 gram after 6 months of storage, and the protein per egg white decreased by 0.737 gram. The 0.737 gram of protein contained 0.031 gram or 4.20% of methionine, again pointing to a

selective transfer of ovalbumin. If all of the white proteins had migrated at the same rate, the white proteins of the 6-month-old eggs would have contained about 3.79% methionine and so would the protein migrating from the white.

Of the migrating protein, 0.650 gram was transferred to the egg yolk. The methionine content of the yolk proteins increased from 2.47 to 2.82%. The total methionine per egg yolk increased from 0.074 to 0.103 gram (0.029 gram). The 0.650 gram of protein transferred from the white to the yolk contained 0.029 gram or 4.46% of methionine, showing again that the protein transferred to the yolk contained a larger percentage of ovalbumin than did the whole white protein.

Cystine in the egg white protein increased from 2.19 to 2.43% during 6 months of shell egg storage (Table III). The protein transferred from the white, therefore, must be one of low cystine content. Ovalbumin is the only egg white protein that contains less cystine than the whole white protein. Fevold (*7*) listed values for cystine in ovalbumin of from 1.33 to 2.40%. The cystine content of ovalbumin is given in Table II as 1.3% because the lowest value is the only one that will fit these data. The cystine values may not be very good, because they show a greater increase of cystine in the yolk than decrease in the white. There was a slight increase in the cystine content of the yolk proteins. The protein transferred from the white to the yolk contained 0.012 gram or 1.85% of cystine. The data indicate the protein transferred to be predominantly ovalbumin because ovalbumin contains the least cystine (1.3%), whereas the cystine contents of ovoglobulin (2.5%) and conalbumin (3.8%) are somewhat higher and the cystine in ovomucoid and lysozyme is much higher than the 1.85% of the migrating proteins.

The values for the cystine content of the different white proteins may not be correct; different workers have reported

widely divergent values, and the data on the cystine content of the isolated white proteins are still rather limited. The authors propose to investigate the cystine transfer further.

Serine data presented in Table IV were calculated in the same way as methionine. The serine content of white proteins decreased and so did the serine content of yolk proteins. Only ovalbumin of the white proteins contains a higher percentage of serine than do the total white proteins. None of the white proteins listed in Table IV contain as much serine as the total yolk protein. Any transfer of white protein would therefore decrease the serine content of the yolk protein. Protein transferred from the white contained 7.60% serine and that migrating into the yolk 7.50%, values which agree very well.

Fevold (7) listed values for methionine, cystine, and serine in lysozyme, one of the egg white globulins, but gave none for the other globulins. Using the methionine, cystine, or serine contents of ovalbumin, conalbumin, and ovomucoid given by Fevold, the content of that amino acid in egg white protein from fresh eggs obtained in this experiment, and the percentage of ovalbumin, conalbumin, ovomucoid, and ovoglobulins in egg white proteins given by Longsworth *et al.* (8), the percentage of each amino acid in the ovoglobulin fraction can be calculated. If A, C, M, and G represent the percentage of the amino acid in ovalbumin, conalbumin, ovomucoid, and ovoglobulin, respectively, and the values of 60% ovalbumin, 14% conalbumin, 14% ovomucoid, and 12% ovoglobulins in egg white protein are used, the following formula can be written:

$$0.6A + 0.14C + 0.14M + 0.12G = \% \text{ amino acid in egg white proteins}$$

Substituting methionine values from Table II we get:

$$0.6 \times 5.4 + 0.14 \times 2.1 + 0.14 \times 0.9 + 0.12G = 3.79$$

$$0.12G = 0.13$$

$$G = 1.1 = \text{ per cent methionine in ovoglobulin}$$

Substituting cystine values from Table III we get:

$$0.6 \times 1.3 + 0.14 \times 3.8 + 0.14 \times 6.5 + 0.12G = 2.19$$

$$0.12G = 0.30$$

$$G = 2.5 = \text{ per cent cystine in ovoglobulin}$$

Substituting serine values from Table IV:

$$0.6 \times 8.2 + 0.14 \times 6.3 + 0.14 \times 4.2 + 0.12G = 7.44$$

$$0.12G = 0.85$$

$$G = 7.1 = \text{ per cent serine in ovoglobulin}$$

The values calculated have been placed in the tables.

The data have definitely shown that ovalbumin is the protein transferred from the white to the yolk in largest amounts, but it has not indicated the percentage of ovalbumin and other white proteins transferred, except that the migrating protein contains more than 60% of ovalbumin. A formula can be set up for the migrating protein similar in nature to the one that was used for calculating the globulin content of total egg white proteins. Let A, C, M, and G equal the number grams of ovalbumin, conalbumin, ovomucoid, and ovoglobulin in 1.00 gram of migrating protein. Then:

$$A + C + M + G = 1.00$$

and for methionine

$$5.4A + 2.1C + 0.9M + 1.1G = 4.46$$

And for cystine

$$1.3A + 3.8C + 4.0M + 2.5G = 1.85$$

And for serine

$$8.2A + 6.3C + 4.2M + 7.1G = 7.50$$

We now have four unknowns and four equations. It should be possible to obtain values for A, C, M, and G that will fit all four equations. Solving for each one by determinants, $A = 0.79$, $C = 0.03$, $M = 0.15$, and $G = 0.03$, the protein transferred from the white to the yolk contained 79% ovalbumin, 3% conalbumin, 15% ovomucoid, and 3% globulins. Ovalbumin was selectively transferred and conalbumin and the ovoglobulins were selectively retained in the white.

The data obtained demonstrate a definite transfer of protein from the white to the yolk during storage of shell eggs produced by hens fed crude cottonseed oil. Analysis of the proteins for methionine, cystine, and serine indicates that the protein transferred was predominantly ovalbumin. Conalbumin was also shown by Schaible and Bandemer (14) to migrate from the white to the yolk. In the present experiment conalbumin comprised but 3% of the protein transferred to the yolk, and only 0.022 gram of conalbumin passed into the yolk of each egg from the white on the average. More conalbumin might be transferred with longer storage. Perhaps only a small amount of conalbumin gives much color with iron to give the salmon yolks.

White and yolk proteins from both fresh and 5-month-old eggs of one hen were analyzed for 17 amino acids by the chromatographic procedure of Moore and Stein (10), using a sulfonated polystyrene resin column. This was a preliminary study to determine if changes occurred in the concentration of amino

Table III. Transfer of Cystine in Stored Shell Eggs from Hens Fed Crude Cottonseed Oil

| | | Cystine | | | Cystine | |
Protein	White Proteins, G.	In white proteins, %	In egg white, g.	Yolk Proteins, G.	In yolk proteins, %	In egg yolk, g.
Fresh eggs	3.331	2.19	0.073	2.982	1.61	0.048
3-mo.-old eggs	3.193	2.32	0.074	3.063	1.57	0.048
6-mo.-old eggs	2.594	2.43	0.063	3.632	1.64	0.060
Protein transferred	0.737	1.36	0.010	0.650	1.85	0.012
Ovalbumin		1.3 (4)				
Conalbumin		3.8 (4)				
Ovomucoid		6.5 (4)				
Lysozyme		8.0 (4)				
Ovoglobulins		2.5ᵃ				

ᵃ Cystine content of ovoglobulins was calculated. See text for description of calculations.

Table IV. Transfer of Serine in Stored Shell Eggs from Hens Fed Crude Cottonseed Oil

| | | Serine | | | Serine | |
Protein	White Proteins, G.	In white proteins, %	In egg white, g.	Yolk Proteins, G.	In yolk proteins, %	In egg yolk, g.
Fresh eggs	3.331	7.44	0.248	2.982	9.06	0.270
3-mo.-old eggs	3.193	7.37	0.235	3.063	9.01	0.276
6-mo.-old eggs	2.594	7.38	0.192	3.632	8.77	0.319
Protein transferred	0.737	7.60	0.056	0.650	7.50	0.049
Ovalbumin		8.2 (4)				
Conalbumin		6.3 (4)				
Ovomucoid		4.2 (4)				
Lysozyme		7.0 (4)				
Ovoglobulins		7.1ᵃ				

ᵃ Serine content of ovoglobulin was calculated. See text for description of calculations.

acids other than methionine, cystine, and serine. An increase or decrease in the concentration of many amino acids in the white was accompanied by the opposite in the yolk, which indicates that proteins rich or poor in one amino acid were selectively transferred from the white to the yolk.

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ACARICIDE DETERMINATION

Microdetermination of the Acaricide Ethyl p,p'-Dichlorobenzilate (Chlorobenzilate)

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Two analytical methods are presented for the microdetermination of the acaricide ethyl p,p'-dichlorobenzilate (Chlorobenzilate) in the presence of citrus extractives. Both methods are based upon the hydrolysis of Chlorobenzilate in the extractive mixture to p,p'-dichlorobenzilic acid, which is then selectively oxidized to p,p'-dichlorobenzophenone. This ketone is determined either by its absorption at 264 $m\mu$ or by the absorption of its 2,4-dinitrophenylhydrazone in alcoholic alkali at 510 $m\mu$. Both methods are reproducibly sensitive to about 15 γ of Chlorobenzilate in admixture with 3 grams of citrus extractives. These procedures involve a two-stage cleanup of accessory extractives and are, therefore, very specific.

THE COMPOUND ethyl p,p'-dichlorobenzilate [2-hydroxy-2,2-bis(4-chlorophenyl)ethyl acetate, Chlorobenzilate, also known as Compound 338, Geigy 338, or G-23992] is a general acaricide against citrus mites and is proving useful in the control of the citrus bud mite, Aceria sheldoni (Ewing) (6). Carefully purified Chlorobenzilate possesses the following physical properties:
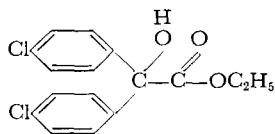
Molecular weight, 325.2
Boiling point, 141° at 0.1 mm.
Refractive index, n_D^{20} 1.5727.

A_s (95% ethyl alcohol), 661 at 266 $m\mu$, 17,940 at 225 $m\mu$.
A_s (2,2,4-trimethylpentane), 741 at 266 $m\mu$, 19,620 at 230 $m\mu$.
(See Figure 1 for detailed ultraviolet absorption characteristics.)

As with all new insecticides and acaricides, the magnitudes and locales of existing residues of Chlorobenzilate on and in citrus fruits must be established before commercial usage on citrus will be permitted. Two chemical methods are available for the microdetermination of Chlorobenzilate residues on and in foodstuffs: a nitration procedure proposed by Harris (5), and an adaptation of a total chlorine method suggested by Gunther and Blinn (3).

Extensive isolative procedures are required for successful use of the nitration

method in the presence of citrus extractives, while the total chlorine method is nonspecific for Chlorobenzilate.

Chlorobenzilate may be hydrolyzed in a homogeneous system to afford p,p'-dichlorobenzilic acid, which is easily oxidized quantitatively to p,p'-dichlorobenzophenone by chromic anhydride in glacial acetic acid. In this manner, the hydrolysis step eliminates all interfering substances not soluble in alkali; the oxidation of the alkali-soluble material provides further cleanup, in that only the resultant water-insoluble ketones persist through the remainder of the determination. The final desired ketone can be selectively determined by its absorption at 264 $m\mu$ or by the absorption of its 2,4-dinitrophenylhydrazone in alcoholic alkali (λ_{max}. 510 $m\mu$) if addi-