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Received for review June 18, 1973. Accepted November 28, 1973. Mention of brand names is for identification only and does not imply recommendation by the U. S. Department of Agriculture.

Physicochemical Appraisal of Changes in Egg White during Storage

Helen H. Koehler

Polyacrylamide gel electrophoresis was used to separate egg white proteins of eggs stored 5, 10, or 18 weeks at 0°, 13°, or 22°. Three fractions appearing to undergo greatest change were recovered and compared by physicochemical means to similar fractions from fresh eggs. Fraction 1 was shown to be conalbumin and fraction 2 to be ovoglobulins; fraction 3 was also ovoglobulin. Increased electrophoretic mobilities of all components resulted from increase in negative charge of the proteins during storage. Electrophoretic bands and densitometric tracings were least sharp for fractions 1 and 2. Immunological and sedimentation studies and molecular weight determinations indicated that alteration such as spatial unfolding (denaturation) had occurred in fraction 1, while in fraction 2 a new, closely related but smaller molecule had split off the principal fraction. Fraction 3 was only slightly changed. Higher storage temperature had a greater effect in causing these changes in egg white proteins than did longer storage at lower temperatures.

Storage of shell eggs causes alterations in the gel structure of egg white proteins. A series of long-term storage studies was undertaken to gain information about the location and types of changes occurring.

The second of these studies (Koehler and Jacobson, 1972) evaluated changes during storage in terms of flavor deterioration. Flavor changes in yolks and whites were judged organoleptically after long-term storage and, although the yolk developed musty and rancid off-flavors, the principal deteriorative changes occurred in the flavor of the white.

In the experiments reported here, electrophoresis was used to separate the protein components of egg white. Three of the separated components were selected to be recovered for further investigation. Densitometer scanning of electrophoretic bands, immunological and sedimentation studies, and molecular weight determinations were carried out in attempts to clarify the nature of changes occurring during storage.

Techniques of electrophoresis used to separate proteins of egg white have been reviewed by Chang *et al.* (1970). They used polyacrylamide gel and published electrophoretic patterns and densitometric tracings of native and heat-treated egg white.

Electrophoresis has been used to study egg white deterioration resulting from various treatments (Baker and Manwell, 1962; Croizier and Sauveur, 1967; Donovan *et al.*, 1970, 1972; Evans and Bandemer, 1956; Evans *et al.*, 1958; Feeney *et al.*, 1963; Kloos and Schmidt, 1967). None of these investigations involved extraction and testing of separated protein components, although two different hypotheses were advanced (Donovan *et al.*, 1972; Feeney *et al.*, 1964) as to causes of the thinning of egg white. Study of separated components may help to understand the nature of changes undergone by individual proteins.

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EXPERIMENTAL PROCEDURE

Eggs used were laid by two white Leghorn hens of the same strain selected from the flocks of the Washington State University Department of Animal Sciences. Eggs were stored at 0°, 13°, or 22° and were tested fresh and after 5, 10, or 18 weeks of storage; these temperatures were chosen for comparison with a previous study in this laboratory. Two whites representing the same treatment were composited and strained through four thicknesses of cheesecloth to obtain outer thin white for all samples (Baker and Manwell, 1962). It is recognized that although study of the fresh white involved only outer thin white, after storage sampled thin white included part of what had previously been thick white. Measurement was made of pH of each composite.

Electrophoresis. Polyacrylamide gel was employed to facilitate recovery of protein components. An adaptation was devised for using the large vertical Büchler (starch-gel) mold to enable recovery of larger quantities of components. The procedure of DeVillez (1964, 1971) was used, modifying the buffers to pH 8.5 as follows: Tris-citrate stock solution, 62 g of Tris and 16 g of citric acid in 1000 ml of water; borate stock solution, 118 g of boric acid and 12 g of lithium hydroxide in 1000 ml of water; gel buffer,

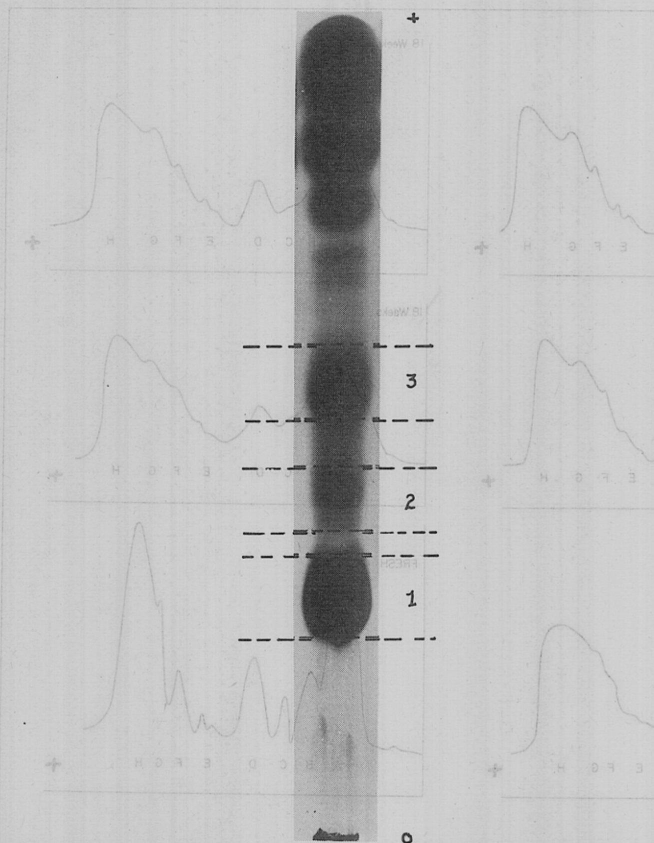

Figure 1. Polyacrylamide electrophoresis separation of fresh egg white proteins showing fractions chosen for recovery and investigation. Fraction 1 is conalbumin; fractions 2 and 3 are globulins.

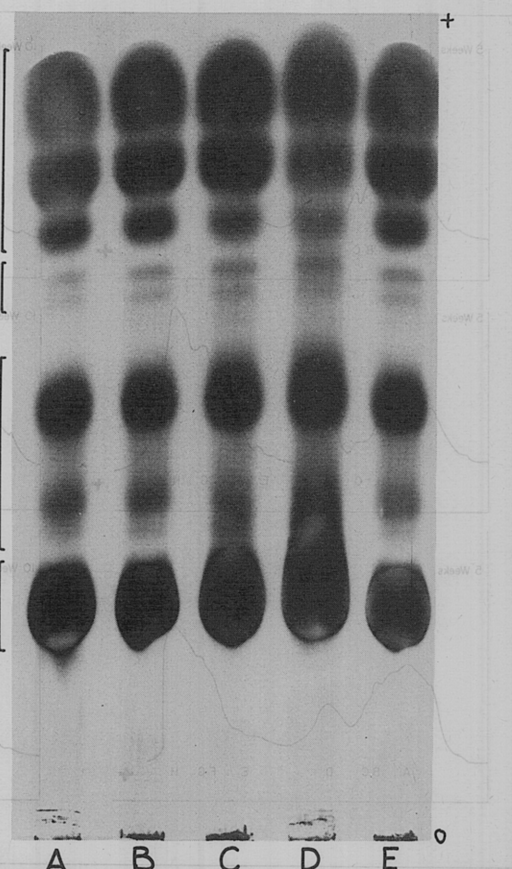

Figure 2. Polyacrylamide gel electrophoretic separations of egg white proteins from: (A) fresh egg; (B) eggs stored 10 weeks at 0°; (C) eggs stored 10 weeks at 13°; (D) eggs stored 10 weeks at 22°; and (E) fresh egg. Proteins corresponded to Baker and Manwell's designations as follows: (1) conalbumins; (2) mucin-globulin fraction comprised of (reading upwards) preconalbumins, globulins, and mucoid; (3) postalbumins; and (4) albumins.

36 ml of Tris-citrate stock solution and 4 ml of borate stock solution in 400 ml of water; chamber buffer, borate stock solution 1:10 in water. An 8% gel was poured into a mold carefully put together and sealed with petroleum jelly. Seven-sample slot-formers were obtained. Sample size was 50 μl of undiluted outer thin white. Electrophoresis at 200 V and 45 mA for 24 hr at 1° resulted in good separation.

Comparisons were made of electrophoretic patterns of egg white protein fractions and of patterns of commercially purchased crystalline conalbumin, ovoglobulin, ovomucoid, and ovalbumin (all from Sigma Chemical Co.).

Separated components were recovered by sectioning unstained strips as indicated by stained marker strips cut from each edge of the gel (Radhakrishnamurthy *et al.*, 1963). The stain was 0.1% Amido Black in 7% acetic acid. Three components appearing to undergo greatest change as indicated by smearing of electrophoretic bands were chosen for further study and designated as fractions 1, 2, and 3 (Figure 1). These three fractions were recovered from whites of fresh eggs or eggs stored 5 or 10 weeks at 22° or 18 weeks at 13°. Cut fractions were separately ground in a size C Teflon pestle tissue grinder with 0.9% NaCl, gel particles were removed by centrifugation, and the supernatant was dialyzed against distilled water to remove nearly all the NaCl. The fractions were lyophilized and their pH was measured.

Densitometer Scanning. Acrylamide gel for densitometer scanning was made ⅛ in. thick, electrophoresis time was shortened to 20 hr at 200 V and 35 mA, and 25-μl samples of undiluted egg white were put into slots 1, 3, 5, and 7. Gels were stained for 1 min in 0.1% Amido Black in 7% acetic acid, and then destained in five changes of 7% acetic acid, constantly agitated on a rotating platform (Eberbach Rotator). Dye absorption was measured by scanning the strips in a densitometer apparatus described in detail by Lawrence *et al.* (1970). The scanning response was recorded for a gel strip from each treatment after each storage period in the study.

Immunodiffusion and Immunoelectrophoresis. One white of an egg from each of the two hens was combined to form the sample. Double diffusion was used to compare fresh and stored forms of fractions 1, 2, and 3, and to compare these fractions from fresh and stored eggs with crystalline conalbumin, ovoglobulin, ovomucoid, and ovalbumin (all from Sigma Chemical Co.). Agar (1% Difco Bacto Agar) in Tris-chloride buffer with ionicity 0.15 and pH 7.4 (Crowle, 1961) was set in trays of microscope slides. Six wells around a center well were cut with a punching device. The appropriate antiserum was put in the center well. Rabbit antisera to fresh fractions 1, 2, and 3 were made by Antibodies, Inc., Davis, Calif. Antisera to fractions 1 and 2 separated from eggs stored 10 weeks at 22° were prepared by Washington State University College of Veterinary Medicine, Department of Veterinary Pathology.

Immunoelectrophoresis involved 1% agar with veronal buffer of pH 8.6 and 0.05 ionic strength to compare fractions from fresh and stored eggs. After electrophoretic separation (Gelman apparatus, 250 V, 9 mA per tray) antisera to the stored fractions were applied in troughs cut lengthwise between the electrophoresed fresh and stored fractions.

Analytical Ultracentrifugation. Sedimentation studies (Schlieren system) were made of the fractions from fresh white and of those from eggs stored 10 weeks at 22°. Sedimentation coefficients (S_{20w}) were calculated from analyses made by personnel of Washington State University Department of Chemistry using a Beckman model E analytical ultracentrifuge at a rotor speed of 56,000 rpm and a

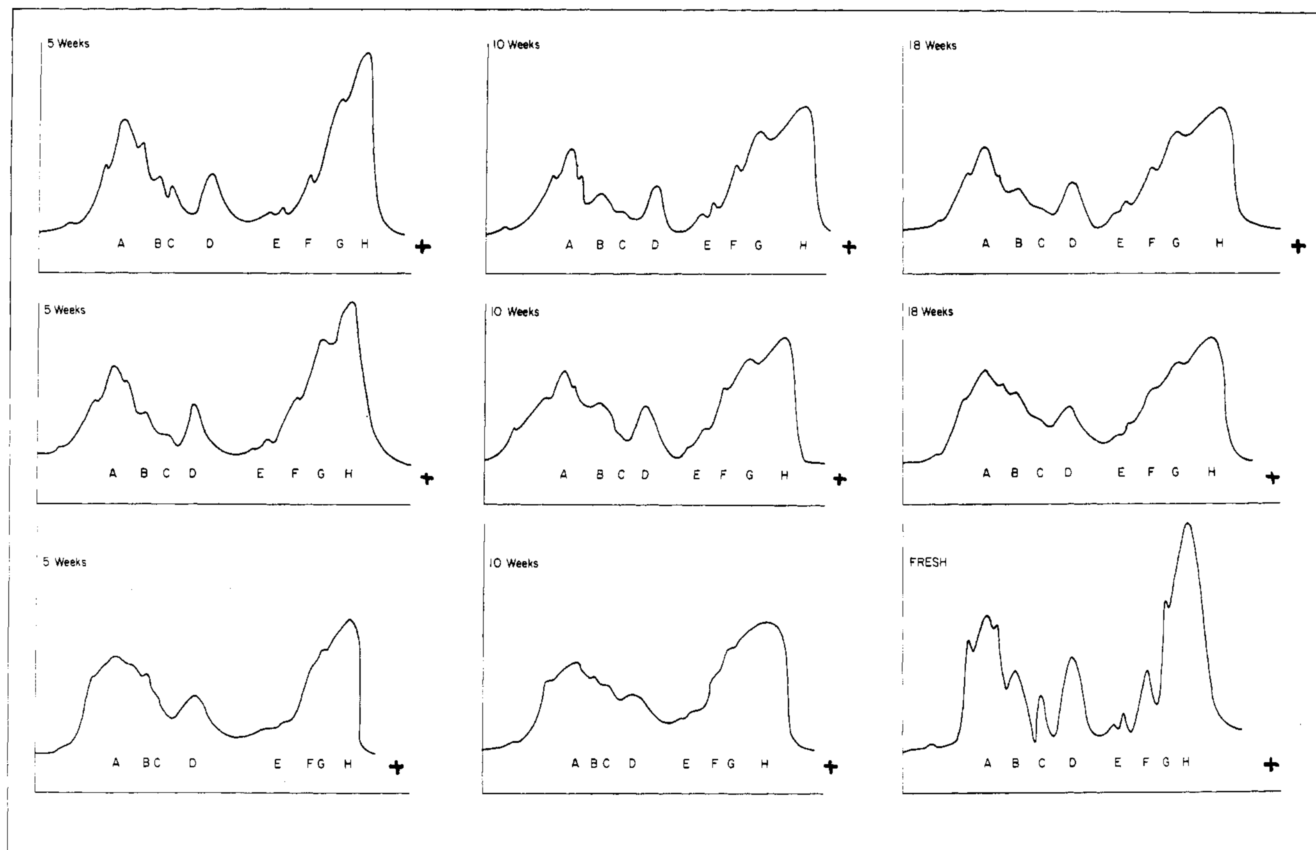

Figure 3. Densitometric tracings of polyacrylamide gel patterns of egg white proteins from fresh and stored eggs (top row, from eggs stored at 0°; center row, from eggs stored at 13°; bottom row, from eggs stored at 22°, and from fresh eggs).

temperature of 4°. Protein concentration was 1% in 0.01% NaCl.

Molecular Weight Determinations. The method of Weber and Osborn (1969) using sodium dodecyl sulfate (SDS) was followed, except that the tube adaptation for the Büchler vertical electrophoresis system was used. For each gel, protein aliquots were prepared as directed, using 50 or 100 μl of protein solution. For these solutions, 0.0015 g of each crystalline protein was dissolved in 1.0 ml of 0.01 *M* phosphate buffer (lysozyme, conalbumin, ovoglobulin, ovomucoid, and ovalbumin all from Sigma Chemical Co.; human serum albumin from Nutritional Biochemicals Corp.). Aliquots of 0.5 ml of fresh or stored fractions 1, 2, and 3 were mixed with 1 ml of phosphate buffer. Crystalline protein determinations were replicated four times; fresh and stored fractions were replicated three times.

RESULTS

Electrophoresis. Separation of components of white from fresh and from stored eggs is shown in Figure 2. Comparison with electrophoretic mobilities of crystalline conalbumin, ovoglobulins, ovomucoid, lysozyme, and ovalbumin showed that these bands are as designated by Baker and Manwell (1962). As storage time and temperature increased, bands in the regions of Baker and Manwell's "mucin-globulin fractions" and "conalbumins" became less clear and more diffuse, being least discrete in white from eggs stored at 22° for 10 weeks. Of the three fractions chosen for further investigation, fraction 1 was shown by immunological comparisons with crystalline proteins to be principally conalbumins but to contain a small amount of another protein. Fraction 2 was shown to be ovoglobulins. Fraction 3 reacted immunologically with crystalline ovoglobulins, and comparison with electrophoretic patterns of Chang *et al.* (1970) suggests that it is one of several unidentified globulins.

In all electrophoretic separations, protein components exhibited increased mobilities toward the anode as egg storage temperature and time increased. Therefore, at the conclusion of the storage period, a new separation was made to compare mobilities of proteins from eggs stored for 10 weeks at 0°, 13°, or 22° with those from eggs stored for 18 weeks at 0° or 13°. All components moved fastest in the strips representing 10 weeks at 22°, followed in order by 18 weeks at 13°, 10 weeks at 22°, 18 weeks at 0°, 10 weeks at 0°, and (somewhat farther back) fresh white. In a similar comparison of the separated, recovered, concentrated fractions from stored egg with those from fresh white, the same phenomenon was seen. Thus, the higher the storage temperature, the faster the separating components moved. High storage temperature had greater effect on increasing mobility of proteins than did longer storage at lower temperature.

Measurements of pH. Fresh white was measured as 8.51 (average of 12 samples, each a composite of three whites). Storage at 0° caused a rise to 9.09 (5 weeks) and 9.12 (18 weeks). Values for eggs stored at 13° were similar: 9.16 (5 weeks) and 9.15 (18 weeks). A greater rise occurred at 22°, readings being 9.39 (5 weeks) and 9.27 (10 weeks).

Densitometer Scanning. Densitometer scans of electrophoretic patterns of egg white proteins from fresh egg and from eggs stored for 5, 10, and 18 weeks at 0°, 13°, and 22° are shown in Figure 3. Although the spacing of the peaks remained the same, the sharpness lessened, the height decreased somewhat, and the amplitude became greater as temperature and length of storage increased. The greatest change in the scan pattern occurred for white from eggs stored for 10 weeks at 22°.

Immunodiffusion and Immunoelectrophoresis. When double diffusion was used to compare fresh fractions 1, 2, and 3 with crystalline conalbumin, ovoglobulin; ovomucoid, and ovalbumin, fraction 1 antiserum reacted strongly with conalbumin and very slightly with ovoglobulin. Fraction 2 antiserum reacted with ovoglobulin. Fraction 3

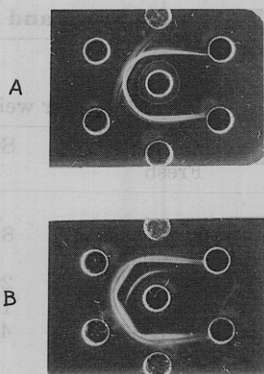

Figure 4. (A) Reaction of fraction 1 (clockwise from bottom well) fresh, stored 5 weeks at 22°, stored 10 weeks at 22°, stored 18 weeks at 13°, against antiserum to fraction 1 stored 10 weeks at 22°; (B) reaction of fraction 2 (clockwise from bottom well) fresh, stored 5 weeks at 22°, stored 10 weeks at 22°, stored 18 weeks at 13°, against antiserum to fraction 2 stored 10 weeks at 22°. Wells at the right were empty.

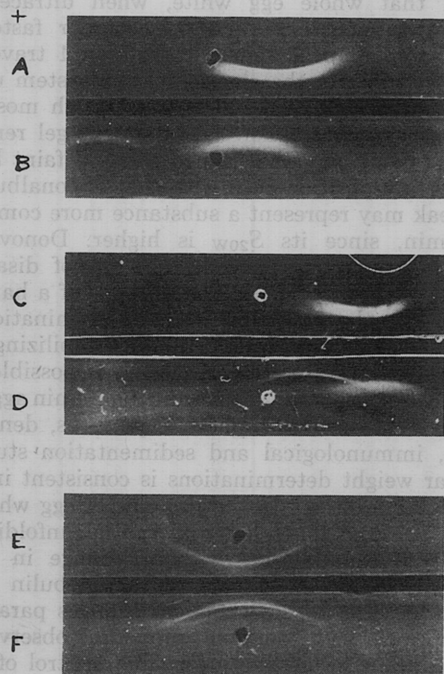

Figure 5. Immunoelectrophoretic reaction of fresh fractions 1, 2, and 3 (A, C, E) and of fractions 1, 2, and 3 from eggs stored 10 weeks at 22° (B, D, F) against antiserum to the stored fractions.

antiserum also reacted with ovoglobulin. Double diffusion (Figure 4) comparing fresh and stored fractions 1 and 2 using antisera to the respective fractions from eggs stored 10 weeks at 22° showed that immunological differences had developed in the 10-week fractions. In fraction 1, the presence of spurs at the junctures of the 10-week sample with those stored 5 weeks at 22° and 18 weeks at 13° suggested spatial unfolding of the molecule or presence of a different component. The faint precipitin line near the center well could represent a dissociated fragment of the conalbumin aggregate not necessarily different antigenically. The appearance of a new line in fraction 2 stored 10 weeks indicated that a new molecule, closely related in structure but of slightly lower molecular weight, had been formed. This new component is also visible in fraction 2 stored 18 weeks at 13°, but in much smaller concentration.

Reaction of antisera to stored fractions 1 and 2 with crystalline proteins compared to similar reactions of antisera to fresh fractions showed changes had occurred in both fractions. In fraction 1, there was a slight reaction

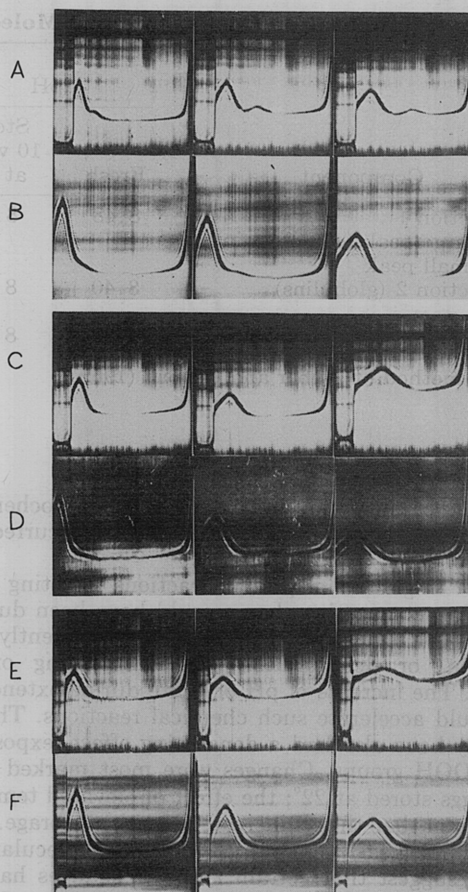

Figure 6. Ultracentrifuge sedimentation patterns of fraction 1 from (A) fresh egg and (B) egg stored 10 weeks at 22°; of fraction 2 from (C) fresh egg and (D) egg stored 10 weeks at 22°; and of fraction 3 from (E) fresh egg and (F) egg stored 10 weeks at 22°, each after 32, 80, and 160 min.

with globulin in addition to the principal one with conalbumin. In fraction 2 an extra globulin line was visible, and there was an indication that some conalbumin had migrated into this fraction. Stored fraction 3 was not tested.

With immunoelectrophoresis, when antisera to fractions 1, 2, and 3 separated from egg whites stored for 10 weeks at 22° were reacted with fresh and with stored fractions 1, 2, and 3, changes in immunological properties of stored fractions were indicated. Figure 5 shows that in each of the three fractions one or more additional precipitin lines appeared in the stored sample which were not present in the fresh.

Analytical Ultracentrifugation. Ultracentrifuge patterns and sedimentation coefficients (S_{20w}) are shown in Figure 6 and Table I. These show a significant decrease in S_{20w} from fresh to 10 weeks' storage for fractions 1 and 2. The decrease for fraction 3 was not significant. The small faster-moving auxiliary peak visible with the large peak of fraction 1 showed an increased S_{20w} after 10 weeks of storage.

Molecular Weight Determinations. Calculated molecular weights for fractions 1, 2, and 3 from fresh eggs and from eggs stored for 10 weeks at 22° are given in Table I. Although there was no change in the molecular weight of fraction 1 and a small but not significant decrease in that of fraction 3, the calculated molecular weight of fraction 2 had decreased 12% (standard deviation 1.2%). A fainter second band in the fraction 2 gels indicated the presence of a compound of very low molecular weight. In the fraction 1 determination, only one band, shown to be conalbumin, was visible; hence no estimation of molecular weight was possible for the small faster-moving peak seen in the sedimentation patterns.

Table I. Sedimentation Coefficients and Molecular Weights of Fractions 1, 2, and 3 Fresh and after Storage

Component	pH		Sedimentation coefficient, S_{20W}		Molecular weight[a]	
	Fresh	Stored 10 weeks at 22°	Fresh	Stored 10 weeks at 22°	Fresh	Stored 10 weeks at 22°
Fraction 1	8.48	8.62				
Large peak (conalbumin)			2.3	1.2	80,900 ± 1960	81,100 ± 2310
Small peak			7.0	7.4	(Not visible)	
Fraction 2 (globulins)	8.40	8.57	2.0	1.3	33,400 ± 350	29,350 ± 350 12,400 ± 120
Fraction 3 (globulins)	8.32	8.40	1.6	1.3	46,200 ± 860	45,000 ± 840

[a] By method of Weber and Osborn (1969).

DISCUSSION

Results of this study indicated that physicochemical alterations in the egg white proteins had occurred during storage of eggs.

Faster migration of protein fractions resulting from increase in net negative charge could have been due to oxidation of the protein, dissociation of covalently bonded hydrogen, or denaturation with unmasking of buried COOH. The increase of pH of white during extended storage would accelerate such chemical reactions. The higher pH may have also had a denaturing effect, exposing buried -COOH groups. Changes were most marked in white from eggs stored at 22°; the effect of elevated temperature was greater than that of increased length of storage.

Immunological, sedimentation, and molecular weight studies suggest that certain types of changes have taken place in the protein. In fraction 1, unmasking of new immunological sites could result from spatial unfolding (denaturation); such a change is indicated by the fact that the molecular weight remained unchanged although the sedimentation coefficient decreased, probably because of an increase in the frictional coefficient. The component represented by the faint line is not necessarily different antigenically and may be a dissociated fragment of the conalbumin aggregate. In fraction 2, the decrease in both the molecular weight and S_{20W} suggests chemical alteration or physical cleavage of the molecule, such as hydrolytic splitting of peptide bonds. Immunological and molecular weight determination evidence indicates the splitting off of a unique molecule. In the molecular weight study, in addition to the principal band of fraction 2, a faint band was present representing a component of very low molecular weight (about 12,400). Denaturation also probably occurred in fraction 2 molecules. With fraction 3, changes in S_{20W} and in molecular weight were not significant, although a new precipitin line was seen in the immunological tests.

It is emphasized, however, that there was no major change in the identity of the protein molecules appearing in each fraction after storage. Immunological tests showed that the same proteins were always recovered from approximately the same relative area of the electrophoretic pattern and that no drastic migrations of components had occurred. Rather, the whole pattern of proteins proved to have moved ahead faster as the result of storage.

Sedimentation coefficients for fractions from fresh whites (Table I) are lower than might be expected for such proteins. Gandhi *et al.* (1968) found an S_{20W} of 2.80 for the principal sedimenting peak of whole egg whites; the small peak was not measured. Osuga and Feeney (1968) reported S_{20W} values of 2.3 for ovomucoids and 5.0 for ovotransferrin (in 0.05 *M* phosphate buffer). A large Donnan effect caused by very low NaCl concentration would reduce the sedimentation coefficient. The low coefficients for fresh fractions could indicate that the proteins had become extended and less compact during the electrophoresis, extraction, and dialysis procedures carried out with all samples.

The appearance of the small auxiliary peak during sedimentation of fraction 1 is interesting. Gandhi *et al.* (1968) reported that whole egg white, when ultracentrifuged, sediments in one large and one smaller faster-settling peak. The small peak found in fraction 1 travels evenly with conalbumin in the electrophoretic system used here. Donovan *et al.* (1970) found that although most of purified ovomucin electrophoresed on starch gel remained in the sample slot and was stained there, a faint band of it migrated to a position even with that of conalbumin. The small peak may represent a substance more compact than conalbumin, since its S_{20W} is higher. Donovan *et al.* (1970) estimated the molecular weight of disaggregated ovomucin to be 1.6×10^5. The absence of a band for the small peak in the molecular weight determination of fraction 1 could have been caused by the solubilizing action of SDS in breaking up the aggregate. It is possible that the small peak represents a part of the conalbumin aggregate.

The evidence of electrophoretic patterns, densitometric tracings, immunological and sedimentation studies, and molecular weight determinations is consistent in pointing to distinct alterations in the proteins of egg white during storage. These changes involved spatial unfolding (denaturation) of conalbumins without change in molecular weight and cleavage of part of the globulin molecules (fraction 2). The physicochemical changes paralleled loss of organoleptic and functional properties observed in previous studies. The necessity for close control of temperature during extended storage to minimize deteriorative changes in quality of shell eggs is evident.

ACKNOWLEDGMENT

The author is indebted to: David J. Hinrichs, Department of Bacteriology and Public Health, Washington State University, for assistance in interpretation of immunological findings; Travis McGuire, Department of Veterinary Pathology, Washington State University, for rabbit antisera; and Henry Krakauer, Department of Chemistry, Washington State University, for advice in evaluation of physicochemical data.

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Received for review July 5, 1972. Resubmitted September 24, 1973. Accepted November 12, 1973. Scientific Paper No. 4000, Washington Agriculture Research Center, College of Agriculture, Pullman, Wash. 99163, project 1807.

Conjugated Compounds in Cow's Milk. II

C. Ronald Brewington, Owen W. Parks,* and Daniel P. Schwartz

An additional 28 compounds existing in cow's milk as conjugates, presumably detoxification conjugates of glucuronic and sulfuric acid, were identified. The compounds included 13 saturated and unsaturated *n*-aliphatic acids, three aromatic acids, four guaiacols, three catechols, three phenols, and aceto- and propiovanillone. The relative amounts of the compounds are given. A comparison of the amount and nature of compounds found in milk and compounds isolated from urine by other workers is discussed.

In a previous investigation (Brewington *et al.*, 1973) the presence and identification in cow's milk of numerous compounds existing as detoxification products of glucuronic and sulfuric acid were demonstrated. This report is a continuation of that work.

EXPERIMENTAL SECTION

Isolation and Enzymatic Hydrolysis of Conjugates. The procedures used were the same as previously described (Brewington *et al.*, 1973), with the exception that double volumes of redistilled methylene chloride were used for extractions.

Fractionation of Free Compounds. The methylene chloride solution of free compounds, obtained after enzymatic hydrolysis, was evaporated on a steam bath under nitrogen to approximately 1 ml and treated at room temperature overnight with 5 ml of a 1.5% solution of sulfuric acid in methanol. Five milliliters of H_2O then were added, and the phenolic compounds and methyl esters formed were extracted with 2 × 20 ml of methylene chloride. The phenolic compounds were separated from the methyl esters by extraction in the cold with 2 × 30 ml of a 5% NaOH solution, which was acidified to pH 1 with concentrated HCl and extracted with 3 × 120 ml of methylene chloride. Both methylene chloride solutions then were dried over Na_2SO_4 and evaporated to a small volume suitable for analysis.

Gas Chromatographic and Mass Spectrometric Methods. The five columns employed for the gas chromatographic analysis of the two methylene chloride fractions obtained are listed in Table I. Column E was programmed from 80 to 190° at 2.5°/min; column C from 50 to 220° at 4°/min; column S from 75 to 220° at 4°/min; column X from 70 to 150° at 4°/min; and column O from 70 to 200° at 5°/min. The instrument used was the Perkin-Elmer 900, equipped with a flame ionization detector and having an injection port and detector temperature of 230°. Certain areas were trapped, as before, and reinjected into a LKB 9000 combination gas-liquid chromatograph–mass spectrometer using the same columns. The operating conditions of the mass spectrometer were the same as previously described (Brewington *et al.*, 1973).

Reference Compounds. The compounds were identified by comparison with the mass spectra and retention times of authentic compounds. 4-Methylguaiacol, 4-ethylguaiacol, and 4-vinylguaiacol were kindly supplied by Dr. Aaron Wasserman, Eastern Regional Research Center, Wyndmoor, Pa. 3-*n*-Propylphenol was prepared by reduction of isosafrole with sodium in ethanol (Cousin and Lions, 1937). Propiovanillone was synthesized by oxidation of 4-hydroxy-3-methoxyphenyl-1-propanol (Pearl, 1956). 4-Allylphenol was made by alkylation of phenol with allyl chloride in the presence of $ZnCl_2$ (Buu-Hoi *et al.*, 1954). Fries rearrangement of catechol diacetate and subsequent reduction with zinc amalgam of the 4-acetylcatechol formed gave 4-ethylcatechol (Miller *et al.*, 1938). All other compounds were purchased commercially.

RESULTS AND DISCUSSION

Table II lists all compounds found to date, the relative amount of each found in the methylene chloride extract and the column(s) used in their isolation and identification. The Carbowax column was the most versatile for the array of phenols present and was particularly useful for the catechol compounds. The EGSS-X and EGA columns were satisfactory for methyl esters. The EGA gave better resolution for the short-chain methyl esters, whereas the EGSS-X column was more suitable for the long-chain methyl esters.

Table III gives mass spectral peaks most useful in diagnosis of those compounds whose spectra are not published or easily located.

A comparison of the amounts of conjugated compounds found in milk by us and in urine (Suemitsu *et al.*, 1970) leads to the conclusion that urine is the preferred medium for elimination of such compounds. However, a comparison of the nature of the compounds present and their relative amounts reveals many differences. One important difference is the absence in urine of aliphatic acids, which occur in appreciable quantities in cow's milk. Milk, on the other hand, contained only the aromatic acids benzoic, phenylacetic, and hippuric, whereas Suemitsu *et al.* (1970) found a host of methoxy and hydroxy derivatives of

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