Ovalbumin was detected in developing chicken eggs. The large majority of these ovalbumin molecules was found to be in a heat-stable form reminiscent of S-ovalbumin. About 83 and 90% of the ovalbumin population was in a heat-stable form in day 14 or stage 40 amniotic fluid and day 18 or stage 44 egg yolk, respectively, whereas ovalbumin in newly deposited eggs was in the heat-unstable, native form. Purified preparations of stable ovalbumin from egg white and amniotic fluid showed a less ordered configuration than native ovalbumin, as analyzed by circular dichroism and differential scanning calorimetry. In addition, mass spectrometric analysis exhibited distinct size microheterogeneity between the stable and native forms of ovalbumin. Immunohistochemical study revealed that ovalbumin was present in the central nervous system and other embryonic organs. These results indicated that egg white ovalbumin migrates into the developing embryo while changing its higher order structure.

Major proteins in albumen or egg white of chicken eggs are synthesized in the tubular gland cells of the magnum, from which they are secreted and coat the egg during its passage within the oviduct (1). The most abundant protein in egg white is ovalbumin, occupying about 55% of the total proteins in newly deposited eggs (2). It consists of three isoforms called A1, A2, and A3, differing in the number of bound phosphate residues (3). Ovalbumin does not act as a proteinase inhibitor, although it belongs to the serpin superfamily (4). The native form of ovalbumin is coagulated easily upon heating, but can be induced according to modification of a previous method (4); 1% aqueous solution of native ovalbumin purified from day 0 egg white (see below) was filtered through a 0.22-μm Millex filter (Millipore) and incubated at 55 °C for 3 days in 50 mM CHES buffer, pH 9.2; under these conditions almost all albumin molecules were expected to assume the S form (6, 14).

In this study, we confirmed that ovalbumin becomes heat stable during migration from egg white to embryonic organs. Examination by circular dichroism (CD) and differential scanning microcalorimetry (DSC) supported the idea that the heat-stable form of ovalbumin has undergone conformational changes. The present report may be the first to characterize extensively the naturally occurring heat-stable form of ovalbumin.

**EXPERIMENTAL PROCEDURES**

**Starting Material**—Fertile eggs of the white Leghorn hen were incubated at 38 ± 0.5 °C at 80% relative humidity. The developmental stages during incubation were assigned according to Hamburger and Hamilton (15). Hatching occurred on day 21 or at stage 46 (where day 0 denotes stage 1, shortly after oviposition). Egg white and yolk were separately withdrawn as described in a previous report (7). Amniotic fluid was taken carefully with a syringe needle from the amnion after the stage when it began to be apparent; if the fluid was thin, it was concentrated by lyophilization before analysis. Serum was withdrawn from an extra-embryonic blood vessel with a syringe needle, avoiding contamination of other material. Serum specimens were also collected from hatched chicks and adults. All specimens were processed to remove insoluble material (7, 8) before using for further experiments.

**Induction of S-ovalbumin**—The stable form of ovalbumin, called S-ovalbumin, was induced according to modification of a previous method (14); 1% aqueous solution of native ovalbumin purified from day 0 egg white (see below) was filtered through a 0.22-μm Millex filter (Millipore) and incubated at 55 °C for 3 days in 50 mM CHES buffer, pH 9.2; under these conditions almost all albumin molecules were expected to assume the S form (6, 14).

1 The abbreviations used are: CD, circular dichroism; DSC, differential scanning microcalorimetry; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; native PAGE, polyacrylamide gel electrophoresis under nondenaturing conditions; PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

*This work was supported in part by grants-in-aid for scientific research by the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.*

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Heat Stability Test—The 1% aqueous solutions of crude or purified ovalbumin were heated at 80, 90, or 97 °C for 10 min, cooled on ice, and centrifuged at 10,000 g for 15 min at 4 °C; the supernatants and/or precipitates were then subjected to further analysis.

Protein Analyses—Protein concentrations were determined by the phenol method as described (15). SDS-PAGE using 12% gels and native PAGE using 8% gels were performed according to the methods of Laemmli (16) and Davis (17), respectively, in 25 mM Tris-glycine, pH 8.0, with or without SDS. Coagulants formed after heating were dissolved in the SDS buffer prior to electrophoresis. The gels were stained with Coomassie Brilliant Blue or subjected to Western blotting with polyclonal antibodies against ovalbumin (Millepore), which were reacted with anti-rabbit goat IgG (see below) previously labeled with peroxidase (18).

Preparation of Antibodies—Antiserum was raised against purified native ovalbumin from day 0 egg white using adult male rabbits and verified by Ouchterlony’s double diffusion test (19). IgG against ovalbumin was purified by Protein A (Bio-Rad) column chromatography and then by ovalbumin-agarose affinity chromatography.

Purification of Ovalbumin—Ovalbumin was purified by the following three steps: 1) precipitation with 50% saturated ammonium sulfate at pH 4.5 followed by solubilization in 50 mM sodium acetate buffer, pH 4.4 (this step was repeated three times); 2) column chromatography with CM-Cellulofine C-200 m (Seikagaku Kogyo) equilibrated with 50 mM sodium acetate buffer, pH 4.4; ovalbumin fractions were eluted with a linear gradient of NaCl from 0 to 0.4 M made up in the same buffer; when only small amounts of protein were available, CM-Cellulofine column chromatography was replaced by affinity column chromatography using anti-ovalbumin IgG agarose (Pharmacia); 3) gel filtration with a column of SW 30000G (Tosu) using 50 mM phosphate buffer, pH 7.2.

CD Spectroscopy and DSC—Far-UV CD spectra (180–250 nm) were recorded at 25 °C with a JASCO 720 spectropolarimeter. Samples were at a concentration of 2.0 μM in 50 mM sodium phosphate buffer, pH 7.4. Data were expressed as mean residue ellipticity (degree cm²/dmol) employing a mean residue molecular weight of 111, which was based on the molecular weight of 42,747 and 385 amino acid residues. The predicted degree of secondary structure was calculated using the program SELCON (20). DSC was carried out with a Microcal calorimeter VP-DSC at a heating rate of 1 °C/min. Protein solutions at 1 mg/ml in 20 mM sodium phosphate buffer, pH 7.4, were degassed before analysis.

Determination of Size Microheterogeneity with a Matrix-assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometer (MALDI-TOF-MS).—This analysis was conducted using a Bruker MALDI-TOF-MS apparatus (REFLEXII) under the conditions specified by the manufacturer, using bovine serum albumin as a calibrant. In brief, 10 pmol/μl aqueous solution of protein was embedded in a light-absorbing “matrix” (2,5-dihydroxybenzoic acid), coated onto a sample plate (target), dried, and excited by laser light. The absolute intensity (putative ion mass) of desorbed proton was plotted in arbitrary units against molecular weight.

Analysis of Partial Amino Acid Sequence—Purified ovalbumin solutions at a concentration of 1 mg/ml were incubated with porcine pepsin (Sigma) at 37 °C under appropriate conditions. The amino acid sequence of some of the peptide fragments obtained was analyzed using an Applied Biosystems 491A sequencer.

Immunohistochemistry—Embryonic organs were dissected out and washed extensively with PBS (10 mM sodium phosphate buffer, pH 7.2, with 154 mM saline) at 4 °C. Then these were fixed with Bouin’s solution, embedded in paraffin, and sectioned at 5 μm thickness at room temperature. Sometimes whole embryos were similarly processed. The sections were deparaffinized, blocked with PBS containing 20% normal goat serum and 1% BSA for 1 h to minimize nonspecific staining, rinsed with PBS, and incubated for 1 h with affinity-purified anti-ovalbumin rabbit IgG (10 μg/ml) suspended in PBS containing 0.1% BSA (BSA/PBS). The sections were washed extensively with PBS and then incubated for 1 h with 8 μg/ml horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce). After washing with PBS as above, the sections were stained by addition of 0.05% diamobenzidine and 0.01% H2O2. The optimum antibody concentration was a dilution of 1:2000. A negative control was performed by using the anti-ovalbumin rabbit IgG preincubated with 100-fold commercial ovalbumin (w/w) as a primary antibody. Western blotting was performed to confirm the ovalbumin distribution in the organs, which were thoroughly washed with PBS and homogenized in 5 volumes of 50 mM HEPES buffer, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA before electrophoresis.

RESULTS

Detection of Ovalbumin in Amniotic Fluid and Embryonic Serum.—To detect ovalbumin, the amniotic fluid and the embryonic serum were prepared daily from developing eggs and subjected to SDS-PAGE. It was possible to collect fluid specimens at later stages of development. After day 10 or stage 36, the amniotic fluid showed several protein bands, including a 45-kDa band that was confirmed to possess ovalbumin by Western blotting with affinity-purified anti-ovalbumin IgG (Fig. 1). The immunological signal was strong after day 14 or stage 40. The embryonic serum also exhibited a signal for ovalbumin at the 45-kDa position after day 10 or stage 36 (Fig. 2). Ovalbumin could be hardly detected in the serum specimens from newly hatched chicks (lane H) and young chicks prior to sexual maturation (data not shown), indicating that serum ovalbumin disappears rapidly after hatching (although laying hen serum was found to contain ovalbumin as seen in lane Ad; the basis of this phenomenon will be considered elsewhere).

Detection of Heat-stable Ovalbumin in Developing Eggs.—We tried to demonstrate that developing eggs contained heat-stable ovalbumin by analyzing specimens before and after heating. We first examined egg white specimens since these were expected to be rich in ovalbumin. Before heating the specimens showed isoform bands A1, A2, and A3 on native PAGE (Fig. 3A); these also showed an ovalbumin signal after Western blotting (Fig. 3B). After heating at 80 °C for 10 min, little or no band was seen (lane 0 of Fig. 3, C and D), indicating that the egg white specimen on day 0 or stage 1, shortly after oviposition, contained pH-coagulative ovalbumin molecules, which were thus in the native, heat-unstable form. Detailed study indicated that a subtle fraction, up to 2%, of day 0 egg white ovalbumin was heat-stable (data not shown). In contrast, the supernatants on days 3–18 or stages 20–44 gave strong ovalbumin bands (lanes 3–18 of Fig. 3, C and D); thus, these contained noncoagulative material comparable to S-ovalbumin. Banding intensity was

![Fig. 1. SDS-PAGE of proteins in amniotic fluid from developing eggs: Coomassie Blue stain (A) and the Western blots with anti-ovalbumin IgG (B).](Image)
the most marked on days 10–12 or stages 36–38. The three ovalbumin isoforms were still present after heating. Moreover, as seen in Fig. 3 (E and F), ovalbumin stable to heating even at 90 °C for 10 min was detected at some stages.

We then analyzed the amniotic fluid (day 14, stage 40), the egg yolk (day 18, stage 44), and the embryonic serum (day 19, stage 45). These also gave noncoagulative protein bands in three isoforms in the supernatants after heating at 80 or 90 °C for 10 min (Fig. 4). These bands were ascertained to contain ovalbumin by Western blotting analysis (data not shown).

**Purification of the Heat-stable Form of Ovalbumin**—Day 11 egg white, day 14 amniotic fluid, and day 19 embryonic serum were each subjected to the purification steps for ovalbumin, in the expectation that the naturally occurring heat-stable form(s) could be isolated. For the egg white and amniotic fluid specimens, after precipitation in 50% ammonium sulfate, DEAE-Cellulofine column chromatography was applied, wherein stable ovalbumin and native ovalbumin were separated from each other on the basis of differential elution in a linear gradient of NaCl (data not shown). Although the peaks were not completely pure, the stable/native ratios could be calculated from the relative peak areas as 3:1 and 5:1 for day 11 egg white and day 14 amniotic fluid, respectively. On the other hand, because the serum specimen contained little protein, after ammonium sulfate precipitation ovalbumin was purified with anti-ovalbumin IgG-agarose in place of DEAE-Cellulofine, whereby it was not possible to separate the two forms to obtain a ratio of stable/native. Each of the heat-stable ovalbumin fractions from egg white and amniotic fluid, as well as the mixture from serum, was subjected to the final purification step by gel filtration through SW 3000G. Upon SDS-PAGE followed by Western blotting (Fig. 5, A and B), all final preparations exhibited a single band. These results showed that the IgG raised for native ovalbumin cross-reacted with stable ovalbumin.

The purified ovalbumin preparations are abbreviated as in Table I. In the subsequent experiments, EWS-11 and AMS-14 (the developmentally occurring heat-stable ovalbumin preparations) as well as S-ovalbumin (the heat-stable one artificially induced by heating the native preparation EWN-0 at pH 9.2) were mainly used, although EWN-0 (sometimes together with another native ovalbumin preparation, EWN-11) was also analyzed. No further investigation was carried out with the preparations whose availability was limited.

**Confirmation of the Heat Stability of the Purified Ovalbumin Preparations**—EWS-11 was heated at 80, 90, or 97 °C for 10 min, centrifuged, and the supernatants and precipitates were analyzed by SDS-PAGE (Fig. 6; see the right half). After heating at 80 °C, all material remained in the supernatant (lane 1) and precipitates were scarcely formed, indicating that ovalbumin was stable. Even after heating at 90 or 97 °C, a large part of materials remained in the supernatants (lanes 2 and 4, respectively). In these cases, however, precipitates were formed (lanes 3 and 5), indicating that some fractions were unstable. The left half of Fig. 6 illustrates the results for EWN-0 as a reference; almost all material was recovered in the precipitates upon heating at 80 or 90 °C (lanes 3 and 5), respectively while no material was seen in the supernatants (lanes 2 and 4), confirming again that EWN-0 was in the native form. AMS-14 gave exactly the same results as those produced by EWS-11 (data not shown).

**CD Spectra and DSC of the Heat-stable Ovalbumin Preparations**—When analyzed for CD spectra, purified preparations of stable and native ovalbumin as well as artificially induced S-ovalbumin from EWN-0 exhibited different patterns (Fig. 7). Particularly noticeable was the difference between the heat-stable ovalbumin (EWS-11 or AMS-14) and the native ovalbumin (EWN-0 or EWN-11). S-ovalbumin gave an intermediate curve. The α-helix and β-sheet contents were calculated from the spectra and are summarized in Table I. EWS-11 and AMS-14 had on average 25% less α-helix than EWN-0 and EWN-11. We conclude that the developmentally occurring heat-stable ovalbumin molecules are different in conformation, i.e. less α-helix and a relatively increased share of β-sheet compared with the native counterparts, and even with the artificially induced S-ovalbumin.

These conclusions coincided well with our results using DSC (data not shown), which indicated that both EWS-11 and AMS-14 gave a major transition at 86.3 °C, whereas EWN-0 gave a major transition at 78.5 °C (see also Table I for a summary of temperature data). All three preparations gave a shoulder at 82.6 °C. The artificially induced S-ovalbumin gave again an intermediate pattern (with a peak at 82.6 °C and shoulders at 78.5 and 86.3 °C) between native ovalbumin and developmentally occurring heat-stable ovalbumin.

**Size Microheterogeneity of the Heat-stable Ovalbumin Preparations**—MALDI-TOF-MS analyses of ovalbumin preparations revealed about 20 major peaks at the Mₐ of 44,000–45,000 (data not shown). EWS-11 and AMS-14 shared the same curve, whereas EWN-0 exhibited a differential curve. EWS-11 and AMS-14 gave the highest peak at an Mₐ of 44,490, which was not present in EWN-0. EWN-0 had the highest peak at an Mₐ of 44,570, which was present in EWS-11 and AMS-14 but with a decreased height. The gap of 80 between these Mₐ data might be attributable to the loss of a phosphate residue in the stable ovalbumin. Exactly the same situation was seen at the peaks of 44,250 and 44,330; again, the difference of 80 might be explained similarly. Although the precise relationship between the change of size and heat stability remains to be clarified, the present data clearly indicated that the heat-stable ovalbumin from developing eggs differed from the native conformer in composition of subcomponents. In addition, the induced S-ovalbumin gave a pattern indistinguishable from the native form of ovalbumin.

**Partial Amino Acid Sequence of the Heat-stable Ovalbumin Preparations**—We were unable to analyze directly the N- and C-terminal amino acid sequences of the present ovalbumin preparations, probably because they are blocked by an acetylated glycine and an imino acid proline, respectively (24). Therefore, to characterize the peptide composition, EWN-0, EWS-11, its supernatant after heating at 80 °C for 10 min, and AMS-14 were each digested with pepsin under conditions appropriate for each preparation and the products were subjected...
to SDS-PAGE. All showed the same peptide patterns (not shown). A mixture of the fastest and the slowest bands from these preparations gave an amino acid sequence of Ala-Ala-Ser-Val-Ser-Glu-Glu-Phe-Arg-Ala-Asp-His-Pro-Phe-Leu-Phe-X-Ile-Lys-His-Ile-Ala-Thr-Asn. This is a bona fide sequence of ovalbumin, corresponding to residues 351–374 (25).

Detection of Ovalbumin in Embryonic Organs—Thin sections of developing embryonic organs were examined for immunohis-}

stochessm staining using affinity-purified anti-ovalbumin IgG. These experiments could be conducted after day 4 or stage 24, and all specimens until hatching gave positive signals for ovalbumin, although not discriminated for the native and stable forms. Fig. 8 illustrates typical results on day 5.5 or stage 28, when the brain system has begun to exhibit extensive organogenesis (13). As shown in panel A, immunostain was most intensive in the central nervous system, although mesenchymal tissues, developing kidney, epidermis, and inner

masses (probably the contents of alimentary canal, which will be considered again below) were to some extent positive. In panel B, signals are seen in the ependymal tissue surrounding the ventricle (V). In panel D, heavy stain was produced in the rhombencephalon (next to another ventricle, V). In panel F, stain was clearly detectable in the developing spinal ganglia (arrowheads). Positive signals were mostly seen in the cytoplasm. There were no positive reactions in controls (panels C, E, and G, corresponding to B, D, and F, respectively), carried out with IgG preincubated with excess amounts of commercial ovalbumin.

The organ distribution was also confirmed by Western blotting. Fig. 9 illustrates the results for specimens on day 18 or stage 44, when embryogenesis is almost complete. Positive signals for ovalbumin were seen in the extracts of many organs including the head, eye, heart (although the signal was weak), liver (showing rather complex banding patterns), intestine, spinal cord, muscle, dermis, and bone (again the signal was

FIG. 3. Native PAGE of proteins in egg white from developing eggs: Coomassie Blue stain (A, C, and E) and the Western blots with anti-ovalbumin IgG (B, D, and F). Egg white specimens were directly analyzed (A and B) or the supernatants recovered after heating for 10 min at 80 °C (C and D) or at 90 °C (E and F) were analyzed. Numerals along abscissa stand for developing age in days. Day 0 represents stage 1, shortly after oviposition, with little or no material in the supernatants after heating (C, D, E, and F). Lane 0+ represents day 0 egg white before heating (in A and B, the same as lane 0). A1, A2, and A3 indicate ovalbumin isoforms due to differential modification with phosphate (see text). OA is defined in the legend to Fig. 1.

FIG. 4. Detection of the heat-stable form of ovalbumin in amniotic fluid (AMF), embryonic serum, and yolk of developing eggs: Coomassie Blue stain after native PAGE. The specimens were analyzed directly (lanes 1) or as the supernatants recovered after heating at 80 °C for 10 min (lanes 2) or at 90 °C for 10 min (lanes 3). EW0 and EW11 show day 0 (stage 1) egg white and day 11 (stage 37) egg white before heating, respectively, run as references. For A1, A2, and A3, see the legend to Fig. 3.

FIG. 5. SDS-PAGE of the purified ovalbumin preparations: Coomassie Blue stain (A) and the Western blots hybridized with anti-ovalbumin IgG (B). Lanes 1, 2, 3, 4, 5, and 6 stand for commercial (native) ovalbumin, EWN-0, EWS-11, EWS-11 supernatant after heating at 80 °C for 10 min, AMS-14, and the serum ovalbumin mixture, respectively. M, markers (see the legend to Fig. 1).
weak). The band intensity for the stomach contents was by far stronger than that for the stomach tissue itself. This is reminiscent of the positive stain in the inner masses on the whole mount thin section presented in Fig. 8A. It is noteworthy that the migration rate of the major band in SDS-PAGE was roughly similar to that of control ovalbumin in all organ extracts including the stomach contents (Fig. 9). The neonate organs were no longer positive for ovalbumin shortly after hatching (data not shown).

**DISCUSSION**

The present study showed that an ovalbumin-like material accumulates in the amniotic fluid, serum, yolk, and organs including the central nervous system of the chick embryo. It is unlikely to be Hsp47, which is another non-inhibitory serpin functioning as a collagen chaperon and with 41% homology to ovalbumin (26), since immunological experiments using poly- and monoclonal antibodies to ovalbumin and Hsp47 reveal no cross-reactivity. Thin sections and organ extracts showed positive signals for anti-ovalbumin IgG (present study) and for anti-ovalbumin monoclonal antibodies. Together with the partial amino acid sequence data obtained here, we conclude that the material detected in embryo was authentic ovalbumin.

Although the chick liver has been reported to be able to express ovalbumin (27), the ovalbumin molecule detected in the present study cannot be the product of de novo synthesis, since no signals for ovalbumin mRNA were seen in the chick liver and other tissues. Thus, the major origin of ovalbumin found in the developing embryo must be egg white. Our preliminary studies also showed that other egg white members such as lysozyme and ovotransferrin were detectable in embryonic organs. This suggests that there may be a direct flow of egg white proteins to the developing embryo.

It is likely that ovalbumin is present in most regions of an

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**Table I**

Abbreviations, recoveries, and some molecular properties of purified ovalbumin preparations

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| Source                | Native or stable | Abbreviation in text | Recovery* | α-Helix content† | β-Sheet content‡ | T m | M r of highest peak |
|-----------------------|-----------------|----------------------|-----------|-----------------|-----------------|-----|-------------------|
| Day 0 egg white       | Native           | EWN-0                | 48        | 27              | 25              | 78.5 | 44,570            |
| Day 11 egg white      | Native           | EWN-11               | 11        | 25              | 26              | —    | —                |
| Day 11 egg white      | Stable           | EWS-11               | 35        | 19              | 32              | 86.3 | 44,490            |
| Day 14 amniotic fluid | Native           | —                    | 10        | —               | —               | —    | —                |
| Day 14 amniotic fluid | Stable           | AMS-14               | 38        | 20              | 33              | 86.3 | 44,490            |
| Day 19 serum          | Mixture          | —                    | 28        | —               | —               | —    | —                |
| Day 0 egg white       | Stable           | S-ovalbumin*         | 27        | 25              | 82.6            | 44,570 |
| Commercial product    | Native           | Native ovalbumin†    | 29        | 25              | 78              | —    | —                |
| Commercial product    | Stable           | S-ovalbumin†         | 29        | 25              | 86              | —    | —                |

*Expressed in the rate per starting material of purification.
†Calculated from CD spectra (Fig. 7), expressed in percentage of total ordered structures.
‡Due to DSC (see text).
§Due to MALDI-TOF-MS (see text).
∥Prepared by the heating of EWN-0 at 55 °C for 3 days at pH 9.2.
‡‡Cited from Refs. 14 and 32.

**Fig. 6.** SDS-PAGE of the purified ovalbumin preparations after heating at different temperatures for 10 min: Coomassie Blue stain. **Right half,** EWS-11. Lane 1, supernatant after heating at 80 °C (no precipitate was formed); lanes 2 and 3, supernatant and precipitate, respectively, after heating at 90 °C; lanes 4 and 5, supernatant and precipitate, respectively, after heating at 97 °C. **Left half,** EWN-0. Lane 1, analyzed without heating; lanes 2 and 3, supernatant and precipitate, respectively, after heating at 80 °C; lanes 4 and 5, supernatant and precipitate, respectively, after heating at 90 °C. M, markers (see the legend to Fig. 1).

**Fig. 7.** CD spectra analyzed for the purified ovalbumin preparations and S-ovalbumin. Mean residue ellipticity was plotted against the wavelength. Five determinations were made for each sample, and the spectrum represents the average. ●, EWN-0; X, EWN-11; ○, S-ovalbumin (induced from EWN-0 at pH 9.2); ■, EWS-11; □, AMS-14. The β-sheet and α-helix contents were calculated, and the results are listed in Table I.

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Y. Sugimoto, unpublished data.
embryo, exhibiting differences in apparent amounts from organ to organ. A previous in vitro culture of embryo explants with 11–13 somites (28) has indicated that radiolabeled exogenous ovalbumin can reach the brain and trunk without degradation, although a large part was digested to amino acids. We propose that at least a fraction of ovalbumin molecules is transported intact from egg white to the embryonic organs, including the brain, probably via the amniotic fluid, embryonic serum, and egg yolk, which are all rich in egg white proteins as reported here and in previous articles (7–12). It is noteworthy that the amniotic fluid can come into contact directly with the embryo via an open channel to the head region, where the neural system is formed. As ovalbumin was detected in the cytoplasm, it is probably incorporated into cells through a specific receptor.

Ovalbumin may be digested by chymotrypsin and other proteinases. In particular, the heat-stable preparations obtained in the present study were more susceptible to chymotrypsin and thermolysin than native ovalbumin.2 A previous study (14) also indicated higher rates of degradation of S-ovalbumin than the native ovalbumin by porcine pancreatic elastase and sub-

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3 S. Ohsako, unpublished data.
Naturally Occurring Stable Ovalbumin

Egg white ovalbumin has also been known to change spontaneously into a heat-stable form during storage of unfertilized eggs at 38 °C (5, 6). This was reproducible in the present study; when newly deposited, unfertilized eggs were kept at 38 °C, proteins stable to heating at 80 °C for 10 min occurred in egg white after 3 days (data not shown). However, the bands appearing in the supernatants of developing eggs after heating at 90 °C for 10 min were never observable in unfertilized eggs allowed to stand at 38 °C for up to 19 days. The conformational alteration taking place in developing eggs may therefore be somewhat different from the spontaneous one seen in unfertilized eggs.

We propose that egg white ovalbumin moves into the embryonic organs by changing its form to less ordered structures fitted to transportation. Ovalbumin may not merely be a storage protein serving as an amino acid source but may also have a more dynamic function in developing organic cells. It will be of importance to verify this inference through extensive analyses of ovalbumin and its related proteins.

ACKNOWLEDGMENTS—We are very grateful to C. Kawano, R. Kobayashi, and H. Uetsuji for secretarial assistance; to Y. Hiraoka of Sibel Hegner Japan Co. for performing DSC analysis; to Dr. M. R. Goldsmith of the University of Rhode Island for critical reading of the manuscript; and to Dr. P. Champon of Université Louis Pasteur, Dr. M. Hirose of Kyoto University, Dr. K. Nagata of Kyoto University, and Dr. M. Eto of Miyazaki University for generous gifts of ovalbumin cDNA, chicken Hsp47, its monoclonal antibodies, and hybridoma cells producing monoclonal antibodies for ovalbumin, respectively.

REFERENCES

1. Burley, R. W., and Vedelma, D. V. (eds) (1989) The Avian Egg Chemistry and Biology, pp. 129–145, John Wiley & Sons, Inc., New York.
2. Osuga, D. T., and Fennec, R. E. (1977) in Food Proteins (Whittaker, J. R., and Tannenhaun, S. R., eds) pp. 209–266, Avi Publishing Co., Westport, CT.
3. Engelsdor, B. (1980) J. Food Sci. 45, 570–573.
4. Guttens, P., Patston, P. A., and Schapira, M. (1993) Biosci. Essays 15, 461–467.
5. Smith, D. B. (1964) Aust. J. Biol. Sci. 17, 261–270.
6. Nguyen, T. L. H., and Smith, C. B. (1984) CSHO Food Res. Q. 44, 44–48.
7. Sugimoto, Y., Saito, A., Kusakabe, T., Hori, K., and Koga, K. (1984) Biochim. Biophys. Acta 992, 400–403.
8. Sugimoto, Y., Hanada, S., Koga, K., and Sakaguchi, B. (1984) Biochim. Biophys. Acta 768, 117–123.
9. McNicole, W. H. (1960) J. Embryol. Exp. Morphol. 8, 47–53.
10. Romanoff, A. L. (1960) The Avian Embryo (Romanoff, A. L., ed) pp. 209–266, pp. 1059–1110, Macmillan Co., New York.
11. Baintner, K., and Feher, G. (1974) Dev. Biol. 36, 272–278.
12. Marshall, M. E., and Deuch, H. F. (1951) J. Biol. Chem. 185, 155–161.
13. Hamburger, V., and Hamilton, H. L. (1951) J. Morphol. 88, 49–92.
14. Huntingdon, J., Patston, P., and Guttens, G. W. (1990) Protein Sci. 4, 613–621.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
16. Lannamaki, U. K. (1960) Nature 207, 680–685.
17. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
18. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.
19. Ouchterlony, D. (1949) Acta Pathol. Microbiol. Scand. 26, 507–515.
20. Sreeraman, N., and Woody, R. W. (1993) Anal. Biochem. 209, 32–44.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Harbor, NY

22. Gannon, F., O’Hare, K., Perrin, F., LePennec, J. P., Benoist, C., Cochet, M., Breathnach, R., Royal, A., Garapin, A., Cami, B., and Chambon, P. (1979) *Nature* **278**, 428–434

23. Kawasaki, E. S., Clark, S. S., Coyne, M. Y., Smith, S. D., Champlin, R., Witte, O. N., and McCormick, F. P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5698–5702

24. Nisbet, A. D., Saundry, R. H., Moir, A. J. G., Fothergill, L. A., and Fothergill, J. E. (1981) *Eur. J. Biochem.* **115**, 335–345

25. Tatsumi, E., and Hirose, M. (1997) *J. Biochem.* (Tokyo) **122**, 300–308

26. Clarke, E. P., and Sanwal, B. D. (1992) *Biochim. Biophys. Acta* **1129**, 246–248

27. Dierich, A., Gaub, M.-P., LePennec, J.-P., Astinotti, D., and Chambon, P. (1987) *EMBO J.* **6**, 2305–2312

28. Hassel, J., and Klein, N. W. (1971) *Dev. Biol.* **26**, 3880–392

29. Sugimoto, Y., Kusakabe, T., Nagaoka, S., Nirasawa, T., Tasuguchi, K., Fujii, M., Aoki, T., and Koga, K. (1996) *Biochim. Biophys. Acta* **1295**, 96–102

30. Saito, Z., and Martin, W. G. (1966) *Can. J. Biochem.* **44**, 293–301

31. Koketsu, M. (1997) in *Hen Eggs* (Yamamoto, T., Tuneja, L. R., Hatla, H., and Kim, M., eds) pp. 99–115, CRC Press, Boca Raton, FL

32. Mellet, P., Michels, B., and Bieth, J. G. (1996) *J. Biol. Chem.* **271**, 30311–30314