Structural Proteins in the Egg-Shell of the Oriental Garden Cricket, *Gryllus mitratus*

By HIROYA KAWASAKI, HITOSHI SATO AND MOTOKO SUZUKI

Department of Biochemistry, School of Dentistry, Iwate Medical College, Morioka 020, Japan

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1. The egg-shell of the oriental garden cricket, *Gryllus mitratus*, contained at least two different types of structural protein in an approximate ratio of 5:1. The major fraction was extracted in a solvent containing dithiothreitol, EDTA and 8M-urea, and was purified to apparent homogeneity as judged by free-boundary electrophoresis and ultracentrifugation. This was designated SH-fraction and its *S*-carboxymethyl derivative (CM-fraction) was also prepared. The minor fraction, insoluble in the solvent, was designated insoluble residue. 2. The major fraction was a phosphoprotein, rich in serine (29.8mol % of the total amino acids) and phosphate (nearly equimolar to serine), and *O*-phosphoserine was identified in its partial acid hydrolysate. The content of cystine was rather low (0.9mol %) in spite of the importance of this amino acid residue in the native form of the protein. The insoluble residue contained only a small amount of phosphorus, and its amino acid composition was clearly different from the major fraction. 3. CM-fraction, a fibrous protein with an average molecular weight of 57 500, behaved as a typical polyanion owing to the high content of phosphate. SH-fraction and CM-fraction were precipitable from their aqueous solutions by the addition of bivalent metal cations, and the precipitation of CM-fraction by Ca^{2+} and Mg^{2+} was studied in detail. 4. When SH-fraction was exposed to air, intermolecular disulphide linkages were formed, yielding a net-like gel that changed its volume with changes in Ca^{2+} , Mg^{2+} and Na^+ . 5. The possible role of this protein fraction in maintaining the integrity of the egg-shell, and a comparison of its composition and properties with other egg-shell proteins and other phosphoproteins, are discussed.

Insects lay eggs in various conditions, and the eggs are exposed to a variety of environmental stresses for various periods of time. Consequently, the protective function of the egg-shell must vary according to the kind and degree of the stress from which the eggs suffer. Although very little is known about the chemical nature of insect egg-shells, a few reports indicate that protein is the major component of the egg-shell of a fruit fly (Wilson, 1960), a house cricket (Furneaux, 1970) and six silkworms (Kawasaki, Sato & Suzuki, 1971).

Following our previous work on the structural proteins of the silkworm egg-shells (Kawasaki *et al.* 1971), we have now studied the egg of the oriental garden cricket, *Gryllus mitratus* Burmeister. Furneaux (1970) reported that the egg-shell of another cricket, *Acheta domesticus* L., was particularly rich in serine and phosphate, and contained *O*-phosphoserine. His findings were confirmed with our material, and we have obtained further information on the kind and chemical nature of the structural proteins in the egg-shell of

G. mitratus. The egg-shell contained at least two different types of structural protein. The main protein fraction, a phosphoprotein, was obtained in a soluble form, and its properties were studied in detail. The minor protein fraction, which contained only a small amount of phosphorus, differed from the main one also in solubility and other chemical properties. These results are presented in this paper with a discussion on the chemical organization of the egg-shell.

EXPERIMENTAL

Material. The oriental garden cricket, *G. mitratus*, was collected in the neighbourhood of our college. The crickets were fed on commercial rat food and supplementary vegetables for a week or two. Moistened quartz sand (150–200 mesh), packed in a large Petri dish, was successfully utilized in place of soil: the females oviposited into the layer of quartz sand. The eggs were separated from the quartz sand by a flotation method with 35% (w/v) sucrose solution, washed with water, air-dried and stored frozen until use.

Preliminary fractionation of the egg. Proteins that were soluble in water and dilute alkali were removed from the egg by a preliminary fractionation. The eggs were finely ground in a mortar. The water-soluble materials were extracted three times with 5–10 vol. each of chilled water by extensive stirring and by centrifugation at 1–3°C (6700g for 10 min). The extracts were combined, concentrated *in vacuo* to a small volume, and added to an equal volume of 0.6 M-trichloroacetic acid. The precipitate was collected by centrifugation, washed with 0.3 M-trichloroacetic acid, ethanol and ether, and then dried *in vacuo* to give the water-soluble protein fraction. The average yield of this fraction was 33.0% of the weight of the eggs.

The water-insoluble residue was then extracted with chilled 10 mM-NaOH as described above. Combined extracts were filtered and neutralized with acetic acid, and then treated as described above. The yield of this alkali-soluble fraction was 1.1%.

The alkali-insoluble residue was washed with water, ethanol and ether before drying. This fraction, the scleroprotein fraction, represented 3.7% of the weight of the egg.

Dissolution and fractionation of the scleroprotein fraction. The scleroprotein fraction (880 mg; moisture, 4.0%) was stirred at 20°C in 30 ml of 8 M-urea–10 mM-dithiothreitol–30 mM-EDTA–0.2 M-tris-HCl buffer, pH 8.6. The substance swelled immediately, and the greater part dissolved in 30 min. After centrifugation at 8700g for 10 min, the precipitate was extracted three times more with 7 ml portions of the solvent.

The insoluble residue was washed with water, ethanol and ether and then dried. The brown powder obtained was designated insoluble residue: yield, 120 mg (moisture, 4.1%).

The extracts were combined and passed through a glass filter to remove a small amount of floating material. The filtrate was then dialysed in 8/32 Visking cellophane tubing against deionized water (5 litres) with stirring at 3°C overnight. After removal by centrifugation of the precipitate (8 mg) that formed during the dialysis, the protein in solution was almost quantitatively precipitated by the addition of 4 ml of 0.9 M-CaCl₂. The supernatant contained only 1 mg of protein. The precipitate was suspended in 30 ml of water. The suspension was chilled in ice-water and adjusted to pH 1.4 with 1 M-HCl. The precipitate was collected by centrifugation at 1–3°C and washed once with 20 ml of water at pH 2 (adjusted with HCl). This procedure removed most of the calcium, but only a small amount (2 mg) of protein material from the precipitate. The precipitate was again suspended in water (20 ml) and dissolved completely by addition of NaOH to pH 8 and by the addition of dithiothreitol (100 μ mol) and EDTA (250 μ mol). The solution was thoroughly dialysed against water under N₂ at 1–3°C, and freeze-dried after removal of the precipitate (26 mg) that formed during the dialysis. The material obtained weighed 605 mg (moisture, 1.5%) and was referred to as SH-fraction.

SH-fraction (350 mg) was dissolved in 25 ml of the same solvent that was used to dissolve the scleroprotein fraction. After keeping the solution at 20°C for 2 h, sodium monoiodoacetate (800 μ mol) was added, and the vessel was kept in the dark for 15 min. Then, 250 μ mol of Na₂SO₃ was added to decompose the excess of mono-

iodoacetate. The reaction mixture was dialysed against water extensively, and finally freeze-dried to give 340 mg (moisture, 1.8%) of white powder. This *S*-carboxymethyl derivative of SH-fraction was referred to as CM-fraction.

Ash-free CM-fraction was also prepared for the purpose of chemical analyses. CM-fraction was quantitatively precipitated from its aqueous solution by the addition of an equal volume of 0.6 M-trichloroacetic acid. The precipitate was washed with 0.3 M-trichloroacetic acid, ethanol and ether and then dried to constant weight *in vacuo* at 110°C over P₂O₅.

Analytical methods. An automatic amino acid analyser (Hitachi KLA-3B) was used for the assay of hexosamines and amino acids. Samples were hydrolysed in an evacuated tube with 6 M-HCl at 100°C for 6, 12, 24 and 48 h. The 6 h hydrolysates were used for the assay of hexosamines and others for amino acids. Table 1 gives the highest values found for each amino acid, which were generally in 24 h hydrolysates except where otherwise stated. Because of the high phosphoserine content of the scleroprotein fraction and the CM-fraction, the usual correction factors for serine destruction cannot be made. Instead, values of serine + phosphoserine + total ammonia – amide groups in 24 h hydrolysates are shown, since control hydrolysates of phosphoserine gave maximum recoveries under these conditions and the two samples did not show any indication for destruction of appreciable amounts of amino acids other than serine during hydrolysis for 24 h. This correction may be incomplete, but the corrected values must represent more than 95% of the amount of serine present in the two samples.

Tryptophan content of proteins was determined independently by the method of Goodwin & Morton (1946).

Total nitrogen was assayed by the micro-Kjeldahl procedure. For the determination of amide, proteins were heated in 2 M-HCl at 100°C for 3 h, and liberated ammonia was measured with Conway's microdiffusion apparatus and by the indophenol method of Katsumata (1953). In some experiments, the amount of protein was measured with the Folin-Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951).

Phosphorus was determined by the method of Fiske & Subbarow as described by Leloir & Cardini (1957). Moisture was measured by heating the samples *in vacuo* at 110°C in an Abderhalden apparatus, and ash by micro-incineration.

The amounts of calcium and magnesium were determined with a Hitachi 207 atomic-absorption spectrometer, and those of sodium and potassium by flame spectrometry. Before the determination of metals in the scleroprotein fraction, dry ashing was carried out by heating the sample in a platinum crucible at 550°C for 7 h. The ash was added with a small amount of 6 M-HCl, dried on a steam bath, and dissolved in water. In the assay of calcium and magnesium, LaCl₃ solution (50 mM in 0.6 M-HCl) was used as the solvent to minimize the interference of phosphate (Willis, 1961).

Physical methods. Free-boundary electrophoresis was carried out in a Tiselius-type apparatus (Hitachi HTD-1) with buffers of *I* 0.2 at 3°C. Owing to the polyelectrolyte nature of CM-fraction, its electrophoretic mobilities were obtained by extrapolating the values calculated from the

descending-boundary patterns to zero concentration of the sample.

Ultracentrifugal analyses were conducted in a Hitachi UCA-1A analytical ultracentrifuge at 20°C. A buffer of pH 5.4, consisting of sodium phosphate buffer (40mM), NaCl (0.2M) and EDTA (1mM), was used as the solvent. The same buffer was also used to determine the partial specific volume and to measure viscosity and specific optical rotation. To measure the sedimentation velocity, samples were centrifuged in a standard single-sector cell at 170 000–206 000g for about 75 min, and observed s_{20} values were corrected to $s_{20,w}$. Since $s_{20,w}$ values were markedly dependent on the concentration of the sample (CM-fraction), the values $1/s_{20,w}$ were plotted against the concentration of the sample to obtain $s_{20,w}^0$. Diffusion patterns of schlieren optics were obtained by centrifuging the sample in a bulb-type synthetic-boundary cell at approx. 8000g for about 3 h and D_{20} values were calculated from the patterns by the maximum ordinate–area method. The values were corrected to $D_{20,w}$ and then extrapolated to zero concentration of the sample to give $D_{20,w}^0$.

Partial specific volume, $\bar{v}$, was obtained by measuring the densities of the protein solutions of known concentration (0.6–1.1 g/100 ml) at 20°C. Molecular weight was calculated by the Svedberg equation from $s_{20,w}^0$, $D_{20,w}^0$ and $\bar{v}$. Viscosity was measured with a capillary viscosimeter of the Ostwald type (water-time about 75 s).

Optical rotation was measured at room temperature with a polarimeter (Hitachi PO-B) provided with a sodium lamp and a cell of 10 cm light-path.

Anion-exchange chromatography for the identification of O-phosphoserine in CM-fraction. A portion of ash-free CM-fraction was hydrolysed in an evacuated tube with 2M-HCl at 100°C for 10 h. The partial hydrolysate was freed from HCl by repeated evaporation *in vacuo*, and was subjected to anion-exchange chromatography as described by Furneaux (1970) with only minor changes in the experimental conditions.

RESULTS

Chemical composition and solubility of the scleroprotein fraction. The chemical composition of the scleroprotein fraction is shown in Table 1. Its general pattern resembled closely that of the egg-shell of *A. domesticus*, reported by Furneaux (1970). Differences between the two analytical results in the relative amounts of the individual constituents may be a reflexion of the species difference and of difference in preparing the materials. Since 10mM-sodium hydroxide was used to prepare the scleroprotein fraction, the contents of metals may not represent those of the egg-shell as such. However, the scleroprotein fraction did contain a fair amount of magnesium that was not detected by Furneaux (1970) in the egg-shell of *A. domesticus*.

The scleroprotein fraction was insoluble at room temperature in 10mM-dithiothreitol, 0.1M-sodium hydroxide, 0.5M-sodium carbonate, 1M-hydrochloric acid, conc. trifluoroacetic acid, 60% (w/v) lithium thiocyanate, 8M-urea and 40mM-EDTA (tetrasodium salt). It was partially soluble in

0.15M-sodium sulphide and in 0.2M-sodium hydroxide. When it was first treated with 40mM-EDTA, the solubility in the sodium sulphide or sodium hydroxide increased significantly. This observation led us to find an effective solvent, which dissolved most of the scleroprotein fraction in mild conditions. The solvent consisted of 8M-urea, 10mM-dithiothreitol, 30mM-EDTA and 0.2M-tris-HCl buffer, pH 8.6. Omission of any of the reagents other than tris buffer made the solvent completely ineffective. Dithiothreitol could be replaced by other disulphide-cleaving reagents such as cysteine, thioglycolic acid and sodium sulphide or by 1M-sodium hydroxide, but these were far less effective than dithiothreitol.

In the scleroprotein fraction, SH-fraction was the main component and insoluble residue the minor one, their ratio being approx. 5:1. The sum of the yield of the two protein fractions was 84% of the scleroprotein fraction on moisture-free basis, but about 96% of the total nitrogen was recovered in the two fractions. Taking into account the amount of protein that was removed during the purification procedure, it was assumed that the scleroprotein fraction contained about 11% of non-nitrogenous materials which were not identified.

Purity of the protein fractions. The examinations for purity by free-boundary electrophoresis and by ultracentrifugation were carried out mainly on the CM-fraction. It behaved as a single-boundary peak in these tests, and some of the representative patterns are given in Figs. 1 and 2. Owing to the high negative charge of CM-fraction, its electrophoretic patterns showed hypersharp ascending-boundary peaks and broad descending-boundary peaks at the concentrations usually used for purity check. On dilution, the ascending- and descending-boundary peaks became more alike, and peaks still remained single. When the purity of SH-fraction was examined at neutral and weak-alkaline conditions in the presence of a slight excess of dithiothreitol, the sample was also found to move as a single-boundary peak in ultracentrifugation (Fig. 2) and in electrophoresis.

It should be mentioned, however, that the protein gave heterogeneous patterns in the electrophoresis with 0.1M-sodium veronal-HCl–0.1M-NaCl buffers, pH 8.6–10.2 and *I* 0.2: three or four fast-moving minor peaks appeared in addition to the main peak which also tended to separate into two during prolonged electrophoresis. This phenomenon was not observed with other buffers of the same ionic strength and of similar pH values. CM-fraction moved as a single-boundary peak in sodium phosphate buffer, pH 9.2 (Fig. 1), NaHCO₃–Na₂CO₃–NaCl buffer, pH 9.1, tris-HCl–NaCl buffer, pH 9.8, and glycine–NaOH–NaCl buffer, pH 9.8. The samples that were recovered after electrophoresis in

Table 1. *Chemical composition of the scleroprotein fraction and of the two protein fractions obtained from the fraction*

Values are expressed as $\mu\text{mol}/100\text{ mg}$ of sample on a moisture-free basis. Ash-free CM-fraction, prepared as described in the Experimental section, was used for the chemical analyses. Analytical methods are also described in the Experimental section.

Constituent	Scleroprotein fraction	CM-fraction	Insoluble residue	Egg-shell of <i>A. domesticus</i> *
Glucosamine	3	0	22	
Galactosamine	0	0	0	
Lysine	8	6	19	6.7
Histidine	11	12	22	6.7
Arginine	17	18	27	12.0
Aspartic acid	36	49	36	37.6
Threonine	10	10	21	7.6
Serine (+phosphoserine)	151†	237†	50	176.0
Glutamic acid	75	105	35	83.0
Proline	25	13	107§	11.4
Glycine	24	21	66	17.5
Alanine	22	16	70	13.1
Cystine‡	5	12	4	8.5
Valine	56§	77§	31§	74.5
Methionine	1	Trace	6	0.5
Isoleucine	25§	37§	14	26.6
Leucine	19	22	30	17.0
Tyrosine	7	6	21	4.4
Phenylalanine	7	6	20	4.6
Tryptophan	13	17	12	
Amide	27	34	35	
Phosphate	160	246	14	194.0
Calcium	70			316.2
Magnesium	53			
Sodium	162			122.5
Potassium	21			18.0
Ash (%)	24.5	0	7.0	27.8
Total N (%)	9.1	11.6	11.3	9.0
N recovered (%)	98	97	100	

* Results of Furneaux (1970), shown for comparison.

† Corrected for incomplete hydrolysis and for destruction during hydrolysis as described in the text.

‡ In the CM-fraction, this stands for *S*-carboxymethylcysteine.

§ Values for 48 h hydrolysis.

the veronal buffers by dialysis against water followed by freeze-drying showed only single peaks when re-examined with other buffers. Ultracentrifugal patterns of CM-fraction in the veronal buffers showed a single-boundary peak without any indication of aggregation of molecules. Thus, the protein seems to interact with sodium 5,5'-diethylbarbiturate reversibly in an electric field. The nature of the interaction is unknown at present and, therefore, there is still some uncertainty about the homogeneity of the major fractions.

We were unable to find a suitable solvent to dissolve the insoluble residue, and the purity remains to be checked.

Chemical compositions of CM-fraction and insoluble residue. The chemical compositions of the

two protein fractions obtained from the scleroprotein fraction are shown in Table 1.

As in the scleroprotein fraction, the major amino acid of CM-fraction was serine and other relatively abundant amino acids were glutamic acid, valine and aspartic acid. The phosphate content was very high and the amount was nearly equivalent to that of serine. Threonine content was low in contrast with serine. The content of *S*-carboxymethylcysteine was rather low in spite of the importance of the disulphide linkage in the native form of this protein fraction. Only a trace amount of methionine was found and there were no hexosamines.

The insoluble residue had a chemical composition quite different from that of CM-fraction. The contents of serine and phosphate were far less than

Fig. 1. Free-boundary electrophoresis patterns for CM-fraction. Electrophoresis was carried out at 3°C with buffers of I 0.2. Ascending-boundary peaks are at the left side. (a) 1.4% solution of CM-fraction in sodium phosphate buffer, pH 9.2, 30 min at 3.86 V/cm; (b) 0.6% in sodium phosphate buffer, pH 9.2, 25 min at 3.86 V/cm; (c) 1.6% in phosphate buffer (a mixture of 0.2 M- KH_2PO_4 and 67 mM- Na_2HPO_4), pH 6.1, 30 min at 3.70 V/cm; (d) 1% in 0.1 M-sodium acetate-0.1 M- NaCl buffer, pH 4.5, 25 min at 3.54 V/cm.

Fig. 2. Ultracentrifugal patterns for CM-fraction and SH-fraction. Samples were centrifuged in a standard single-sector cell at 20°C. (a) 1.1% solution of CM-fraction in 40 mM-sodium phosphate buffer, pH 5.4, -0.2 M- NaCl -1 mM-EDTA was centrifuged at about 170 000g. The picture shown was taken at bar-angle 75° 131 min after reaching the set speed (51 200 rev./min); (b) 1% solution of SH-fraction in 40 mM-sodium phosphate buffer, pH 8.0, -0.2 M- NaCl -1 mM-EDTA-1.5 mM-dithiothreitol was centrifuged at approx. 206 000g. The picture was taken 114 min after reaching the set speed (55 430 rev./min). Bar angle was 75°.

those in CM-fraction. Instead, non-polar amino acids such as proline, alanine and glycine were abundant. It contained more basic amino acids and less acidic ones than did CM-fraction. It also contained significant amounts of glucosamine, indicating that this protein fraction was of glycoprotein nature or contaminated with a glycoprotein or with chitin. Hydroxyproline was not detectable in this protein fraction.

Identification of O-phosphoserine. A partial acid hydrolysate of CM-fraction was subjected to anion-exchange chromatography as described by Furneaux (1970) and the elution pattern (Fig. 3) was similar to that obtained by him with the hydrolysate of the egg-shell of *A. domesticus*, except that peaks 1 and 3 were not shown in his figure.

The constituent of peak 2 was identified as O-phosphoserine by co-chromatography with the authentic compound under the same conditions as in Fig. 3 and by one-dimensional paper chromatography with four different solvent mixtures, butan-1-ol-acetic acid-water (4:1:2, by vol.), butan-2-one-2-methylpropan-2-ol-dimethylamine-water (25:20:2:10, by vol.), methanol-formic acid-water (80:13:7, by vol.) and phenol-water-ammonia (155:43:2, by vol.). The substance in peak 4 was assumed from results of Furneaux (1970) to be phosphoserylphosphoserine, and the identity of the dipeptide was checked by the dinitrophenylation of the substance. After hydrolysis of the dinitrophenylated substance with 6 M-HCl at 100°C for 16 h, the amounts of DNP-serine and serine were nearly equimolar when corrected for destruction during hydrolysis.

The substance in peak 1 contained only serine as the amino acid component, and the serine/phosphate molar ratio was 1.8. The substance was treated with sodium metaperiodate at pH 7.0 for 10 min at 20°C. After decomposing the excess of metaperiodate by the addition of sodium bisulphite, the oxidation product was hydrolysed with 6 M-HCl at 100°C for 24 h. The amount of serine found in the hydrolysate was 47% of that found in the hydrolysate of the intact substance, indicating that about one-half of the serine in the substance had unsubstituted amino and hydroxyl groups. Thus, peak 1 was probably serylphosphoserine. The constituent of peak 3 was found to be orthophosphate.

Molecular parameters of CM-fraction. CM-fraction, which consisted of 89% of protein and 11% of sodium on a moisture-free basis, was studied for various molecular parameters, and in the examinations the concentrations of the sample were expressed on the basis of dry weight of the sodium salt. The results (Table 2) indicated that CM-fraction was a fibrous, negatively charged polyelectrolyte of medium molecular weight. High laevorotatory specific optical rotation may indicate

Fig. 3. Column chromatography of a partial hydrolysate of CM-fraction on Dowex 1 (X8; 200–400 mesh; formate form). Ash-free CM-fraction (30.9 mg) was hydrolysed in an evacuated test-tube with 1 ml of 2 M-HCl at 100°C for 10 h. After removal of HCl, the sample was applied to the column (1 cm × 30 cm). A gradient elution was obtained by using 200 ml of 0.1 M-formic acid in the mixing chamber and 0.25 M-pyridine formate (pH 4.5) in the reservoir, and after collecting 100 fractions, 0.25 M-pyridine formate in the reservoir was replaced by 2 M-pyridine formate, pH 4.5. Elution was carried out at 20°C, and the flow rate was approx. 0.44 ml/min. Fractions were collected (2.1 ml) and 0.2 ml portions of each fraction were analysed for ninhydrin-positive material (●) and for phosphate content (△). The amounts of the ninhydrin-positive materials are expressed as *O*-phosphoserine.

Table 2. *Molecular parameters of CM-fraction*

CM-fraction was in the form of a sodium salt, comprising 89% protein and 11% sodium on a moisture-free basis.

Sedimentation coefficient, $s_{20, w}^0$	3.26 S
Diffusion coefficient, $D_{20, w}^0$	$2.76 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$
Partial specific volume at 20°C, $\bar{v}$	0.50 ml/g
Molecular weight*	57 500
Frictional ratio*, f/f_0	3.43
Intrinsic viscosity, $[\eta]$	0.58 dl/g
Specific optical rotation, $[\alpha]_D^{15}$	$-82.7^\circ \pm 1.5^\circ$ (1.07 g/100ml of a buffer†)
Electrophoretic mobility	
at pH 9.2‡	$-13.6 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$
at pH 6.1	$-12.6 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$
at pH 4.5	$-10.5 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$

* Calculated from $s_{20, w}^0$, $D_{20, w}^0$ and $\bar{v}$.

† Sodium phosphate (40 mM)–NaCl (0.2 M)–EDTA (1 mM) buffer, pH 5.4.

‡ Buffers used were as in the legend to Fig. 1.

the poor α -helix content of CM-fraction in solution according to Szent-Gyorgyi & Cohen (1957).

Precipitation of CM-fraction by the addition of bivalent metal cations. SH-fraction and CM-fraction were precipitable from their aqueous

solutions by the addition of bivalent metal cations. Precipitation of SH-fraction with Ca^{2+} was utilized as a purification procedure (see the Experimental section). Since Ca^{2+} and Mg^{2+} were actually present in the scleroprotein fraction (Table 1),

their effects in precipitating CM-fraction were studied in detail. The results are shown in Fig. 4 and Table 3.

Fig. 4(a) shows that CM-fraction was completely precipitated by the addition of Ca^{2+} when the ratio added Ca^{2+} /mol of phosphate in the protein was more than 1.5. In the presence of a large excess of Na^+ , more Ca^{2+} was required for complete precipitation. Analysis of the precipitates for the contents of Ca^{2+} and phosphate revealed that for precipitation the amount of Ca^{2+} required to bind to the protein was at least 1.23 in molar ratio to phosphate, regardless of the presence or absence of added large excess of Na^+ . Ca^{2+} could bind to the protein as much as 1.36 times the amount of phosphate when it was present in a large excess, although this fact would be partly due to the incomplete removal of the mother fluid from the precipitates.

Fig. 4(b) shows that Mg^{2+} was less effective than Ca^{2+} . A Mg^{2+} /P ratio of 8 still failed to result in complete precipitation, and the presence of a large

excess of Na^+ affected the precipitation to a far greater extent than with Ca^{2+} . The complete precipitation of the protein required an amount of Mg^{2+} about 14-fold the amount of phosphate (Mg^{2+} /P ratio in the precipitate 1.32), but only 50% of the protein was precipitated under these conditions in the presence of Na^+ . It should be noted, however, that the ratio Mg^{2+} /mol of phosphate in the precipitates was 1.25–1.32, similar to the values with Ca^{2+} .

Table 3 shows the results of experiments where both Ca^{2+} and Mg^{2+} were added to the aqueous solution of CM-fraction. The addition of Ca^{2+} in equimolar amount to phosphate merely precipitated 12% of the protein (Fig. 4a) and less than twofold Mg^{2+} caused no precipitation (Fig. 4b). However, the addition of an equimolar amount to phosphate of the two cations precipitated 79% of the protein, and the addition of Ca^{2+} of the equimolar amount and Mg^{2+} of 1.5 times the amount resulted in complete precipitation. Thus, although Mg^{2+} was far less effective than Ca^{2+} , these two

Fig. 4. Precipitation of CM-fraction by the addition of (a) Ca^{2+} or (b) Mg^{2+} . Portions (0.5 ml) of an aq. 0.5% solution of CM-fraction (pH 7.0, adjusted by the addition of NaOH), containing $5.45 \mu\text{mol}$ of phosphate bound to the protein, were placed in test-tubes. Various amounts of CaCl_2 or MgCl_2 were added in the absence (○) or in the presence (●) of 100 μmol of added NaCl , and the total volume was made up to 1 ml with water. The amounts of the bivalent cations were expressed as the molar ratio to the amount of phosphate. The test-tubes were allowed to stand at 20°C for 30 min and were centrifuged at $1900g$ for 10 min. Samples of the supernatants were analysed for the content of protein with the Folin-Ciocalteu reagent. The precipitates were washed once with 1 ml portions of water, without vigorous shaking, to remove the rest of the mother fluid. Ca^{2+} or Mg^{2+} bound to the precipitates was extracted three times with 0.3 M-trichloroacetic acid, by using a total volume of 2 ml for each test-tube. The extracts were diluted with water and subjected to the assay for the cations with an atomic absorption spectrometer. The residues of extraction were analysed for the content of phosphate. The values in parentheses are (a) Ca^{2+} /P ratio or (b) Mg^{2+} /P ratio found in the precipitates.

cations had an additive effect on each other. In the precipitates, the sum of the two cations amounted to 1.23–1.25 in molar ratio to phosphate.

It is possible to explain the phenomena described above as follows. There are two types of binding sites for the bivalent metal cations in the protein, the phosphate groups of phosphoserine residues and the pairs of univalent anions (carboxyl groups) of the adjacent protein molecules. To the phosphate groups Ca^{2+} or Mg^{2+} is able to bind without serious competition with Na^+ , and the binding potencies of the two bivalent cations are of similar order. The binding of Ca^{2+} or Mg^{2+} to phosphate groups alone does not result in the precipitation of the protein, but the binding greatly decreases the electrostatic repulsive force among protein molecules and enables the other type of binding to occur. On the other hand, to bind the pairs of carboxyl groups of adjacent protein molecules, the bivalent cations have to compete with Na^+ , and in the competition Ca^{2+} is much more potent than Mg^{2+} . Further, the binding potency itself of Ca^{2+} is far greater than that of Mg^{2+} . As the result of the intermolecular cross-links via bivalent cations, precipitation occurs.

The postulate seems to be supported by the analytical results of CM-fraction and by the values in Table 3. Table 1 shows that 100mg of the ash-free CM-fraction contained 246 μmol of phosphate and 132 μmol of the residues with carboxyl groups, that is, the sum of aspartic acid, glutamic acid and *S*-carboxymethylcysteine minus amide. Consequently, 100mg of protein is able to bind a total of 312 μg -atoms of the bivalent metal cations, and the ratio of this total to phosphate is 1.27, being very close to the values found in the precipitates as Ca^{2+}/P or Mg^{2+}/P (Fig. 4) and as $(\text{Ca}^{2+} + \text{Mg}^{2+})/\text{P}$

(Table 3). In Table 3, two columns of values are given to relate the ratios of the added two bivalent cations to those found in the precipitates. The results of a plain comparison of the ratios of added two cations with those found in the precipitates (column B/A) are to indicate that when the amount of added Mg^{2+} increases relative to that of Ca^{2+} , Mg^{2+} gradually loses its binding potency. On the other hand, the values in column C/A, which were calculated on the basis of the above-mentioned postulate, are close to one another, and they may indicate that Ca^{2+} is slightly more potent than Mg^{2+} in binding to the phosphate groups, and that the relative amounts of the two bivalent cations bound to the phosphate groups depend on the ratios of the two cations added. The latter values seem to be more reasonable than the former.

The results and interpretation described above were those obtained from the experiments where CM-fraction was precipitated from its relatively dilute solution (0.25%). When more-concentrated solutions were added with Ca^{2+} or Mg^{2+} , the precipitation occurred with smaller amounts of the cations in molar ratio to phosphate in the protein. This was not marked with Ca^{2+} , but was remarkable with Mg^{2+} . Complete precipitation of CM-fraction from its 0.5 and 1% solutions required Ca^{2+} at 1.5 and 1.25 times the amount of phosphate, respectively, whereas the amounts of Mg^{2+} needed for 0.35, 0.5 and 1% solutions were five-, four- and two-fold, respectively. Since the required amounts of the cations were still more than 1.25 in molar ratio to phosphate and all the precipitates were readily soluble in 0.2M-sodium chloride, the results may be regarded as essentially the same as those obtained with 0.25% solution. The distance between a pair of carboxyl groups to be cross-

Table 3. *Precipitation of CM-fraction by the addition of both Ca^{2+} and Mg^{2+}*

The experimental procedure was same as in the legend to Fig. 4, except that both Ca^{2+} and Mg^{2+} were added without the addition of 100 μmol of NaCl. The $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratio bound to phosphate was calculated under an assumption that all of Mg^{2+} found in the precipitates was bound to phosphate and none of this cation linked pairs of univalent anions in the protein, whereas Ca^{2+} was bound to phosphate and also linked pairs of univalent anions (carboxyl groups) of adjacent protein molecules competing with pre-existing Na^+ .

Amount of the added cations (molar ratio to P of CM-fraction)		Precipitation (%)	Ratios found in ppt.			Mg ²⁺ /Ca ²⁺ ratio				
Mg ²⁺	Ca ²⁺		Mg ²⁺ /P	Ca ²⁺ /P	(Mg ²⁺ + Ca ²⁺)/P	(A)	(B)	(C)	B/A	C/A
						Added to soln.	Found in ppt.	Bound to phosphate		
1.0	1.5	100	0.34	0.90	1.24	0.67	0.38	0.52	0.57	0.78
1.0	1.0	79	0.44	0.79	1.23	1.0	0.56	0.79	0.56	0.79
1.5	1.0	100	0.53	0.71	1.24	1.5	0.75	1.13	0.50	0.75
4.0	1.0	100	0.76	0.48	1.24	4.0	1.58	3.17	0.40	0.79
7.0	1.0	100	0.84	0.41	1.25	7.0	2.04	5.25	0.29	0.75

EXPLANATION OF PLATE I

A photomicrograph of the network structure formed by the re-oxidation of SH-fraction. A 1 ml sample of a 0.25% solution of SH-fraction (pH 8.6) was frozen in a slanted test tube for 36 h. The network formed was suspended in water, and a thin piece was taken out. It was mounted in water for the phase-contrast microscopy.

linked by the bivalent cation should have been decreased by increasing the concentration of protein, which might have affected the binding potency more favourably for Mg^{2+} than for Ca^{2+} since the former is far smaller than the latter.

Some other bivalent metal cations, which have greater atomic weight than Ca^{2+} , were also tested for efficiency in precipitating CM-fraction from its 0.25% solution. The necessary minimum amount to cause complete precipitation was 1.25 times the amount of phosphate with Cu^{2+} , Zn^{2+} and Pb^{2+} , 1.5 times with Mn^{2+} and twice with Ba^{2+} , indicating the absence of strict relationship between the necessary amount and atomic weight. It was found, however, that the precipitation of CM-fraction with these heavier cations was not appreciably affected by the presence of a large excess of Na^+ and the precipitates were insoluble in 0.2M-sodium chloride. This fact seems to suggest that a bivalent metal cation larger than Ca^{2+} does not have to compete with Na^+ in cross-linking pairs of carboxyl groups of different molecules. Besides the concentration of the protein, the size of the cation would also be an important factor in connexion with the distance of the anions to be cross-linked.

Re-oxidation of SH-fraction to form the inter-molecular disulphide linkage. An aqueous solution (pH 4.5–10) of newly prepared SH-fraction formed a gel when it was exposed to air for a certain period. At first, the gel was transparent, but before long a network of fine fibres developed throughout the gel. The fibres became thicker and, when the process was complete, the network occupied the whole volume of the original solution and was easily seen with the naked eye. A photomicrograph of the network structure is shown in Plate 1. Freezing of the solution in a slanted test-tube was found to be the most suitable condition for complete network formation. The completed structure contained all the protein that was originally present in the solution, and gentle washing of the structure with water did not dissolve an appreciable amount. The network structure occupied a certain domain and its volume altered with changes in the metal cations present. The gel or developing network dissolved almost immediately after the addition of a slight excess of dithiothreitol, but dissolution of the completed structure by the reducing reagent required the presence of 8M-urea, suggesting that the development of the network structure involved the formation of hydrogen bonds in addition to the re-oxidation of cysteinyl residues. The completed structure was insoluble at room temperature in 10M-sodium hydroxide and in 8M-urea but was soluble in 0.1M-sodium hydroxide and in 0.15M-sodium sulphide.

A typical experiment will be described as an example. SH-fraction (20mg) was dissolved in

1.2ml of the solvent composed of urea (8M), EDTA (30mM), dithiothreitol (10mM) and tris-HCl buffer (0.2M, pH 8.6), and the solution was dialysed in 8/32 Visking cellophane tubing against 500ml of 10mM-tris-HCl buffer, pH 8.6, with stirring at 3°C. During dialysis for 24h the buffer was renewed twice. The content of the tubing (2.5ml) was transferred into a test-tube, and the tube was placed aslant in a freezer (–20°C). At this step, the solution was freely movable as a liquid, but next morning when the frozen sample was thawed, the liquid nature was found to be lost partially. After further freezing for 24h, the gel formation was complete: there was no freely movable liquid in the test-tube and the tube could be held upside down without worry of losing its content. A fine network was observable in the gel through a magnifying glass. The gel was again frozen for 24h, and the formation of the network structure was then complete. When the sample after the dialysis was frozen in the presence of sodium chloride (0.2M), the process was retarded for three days, but when the sample was frozen at pH 4.5 or diluted sample (0.25–0.5% solutions) were frozen at pH 8.6, it took only 24–36h for completion. When the sample was kept at 3°C, the network structure was incomplete in the amount of fibres and in their cross-links even after 8 days, but placing it in the freezer overnight brought the process to completion.

The network structure that was formed as described above had a volume of about 2.8ml when it was gently washed with water to pH 7.0 and centrifuged at 1500g for 10min. The network shrank to 0.8ml when it was equilibrated with 1M-sodium chloride and centrifuged in the same conditions. Washing the shrunken structure with water, however, restored the volume to 2.6ml. The addition of Ca^{2+} let the network shrink more than did the equilibration with 1M-sodium chloride. By increasing the amount of Ca^{2+} , it was found that the minimum volume (about 0.6ml) was obtainable when the cation was added in more than about 1.5 molar ratio to the phosphate in the protein. The volume was also restored to near the original one (to about 2.4ml) by repeated washing with 25mM-EDTA (trisodium salt). During these experiments, it was observed that whenever the network shrank or the shrunken one swelled markedly, a considerable amount of the protein went into solution. For example, by equilibration with 1M-sodium chloride about 7% of the protein was lost in solution, and rapid swelling of the network by depletion of Ca^{2+} with EDTA freed 15% of protein. In other experiments, it was found that the volume of the network structure did not change appreciably at pH values between 2.5 and 9.0, but shrank markedly at below pH 1.8 (e.g. from 2.4ml to 0.3ml) without dissolution of the protein. Washing the shrunken structure

with water to pH 5 allowed it to regain its original volume.

DISCUSSION

Results of the present investigation revealed that the egg-shell of *G. mitratus* contained two fractions of structural protein, designated SH-fraction and insoluble residue, in an approximate ratio of 5:1. The major fraction, SH-fraction or its *S*-carboxymethyl derivative, CM-fraction, contained serine and phosphate as the main constituents, and *O*-phosphoserine was identified in a partial acid hydrolysate of the fraction. CM-fraction was a fibrous protein of medium molecular weight, and it behaved as a typical polyanion owing to the high content of phosphate. The procedure of dissolving the scleroprotein fraction readily suggested that the native form of the major protein had a chemical structure stabilized by two types of intermolecular linkage, the disulphide linkage and a kind of linkage via bivalent metal cations. The importance of the two linkages was further emphasized by observations on the re-oxidation of SH-fraction, precipitability of the major protein fraction with bivalent metal cations, and the actual presence of calcium and magnesium in the scleroprotein fraction. The intermolecular disulphide linkage alone may result in the formation of a network structure such as those observed *in vitro*, but the structure may not be strong enough to keep the integrity of the egg-shell because of the low content of cystinyl residues. Thus, besides the hydrogen bonds, bivalent metal cations, particularly calcium, reinforce the structure by binding pairs of carboxyl groups of adjacent protein molecules. The stabilized structure, however, must be changeable according to the relative amounts and concentrations of metal cations. In this connexion, McFarlane (1968) observed a noteworthy phenomenon: a solution of 0.5M-sodium chloride or potassium chloride collapsed the newly laid egg of *A. domesticus* and the effect was prevented by the presence of 5–10mM-calcium chloride and, to a lesser extent, by 10mM-magnesium chloride.

Lack of a suitable solvent for the insoluble residue did not allow us more detailed studies on this minor fraction. It was, however, distinguishable from the main fraction in chemical composition and in solubility. As to the relationship between the two protein fractions, it is possible that the two fractions form two different morphological parts of the egg-shell. Furneaux, James & Potter (1969) found that the egg-shell or chorion of *A. domesticus* is made of two morphologically distinct layers, the outer layer 6.5–7.0 μ m thick and the inner layer 0.6–1.0 μ m thick. In addition, the acidic nature of the outer layer was suggested by an observation that it readily took up basic dyes but not acid dyes

(McFarlane, 1966). If this is also the case in the egg-shell of *G. mitratus*, the major protein, a polyanion, may form the outer layer and the minor one the inner layer.

The structure of silkworm egg-shells (Kawasaki *et al.* 1971) was also stabilized by the disulphide linkages of the major proteins, but their cystine contents (2.9–3.5mol % of the total amino acids) were far greater than that of the major protein fraction of the *G. mitratus* egg-shell (0.9mol %). Further, the silkworm egg-shells studied did not contain appreciable amounts of phosphorus. The egg-shell of *Bombyx mori* (family Bombycidae) was made of three structural proteins, proteins I, II and III, in the approximate proportions of 76:23:1, whereas the shells of five species of family Saturniidae contained protein I as the major protein and protein III in various amounts (2–15%) but lacked protein II that was unique in the abundant content of cystine (14.7mol %). Unlike proteins I, proteins III differed from one another in amino acid composition and were insoluble in thiol-urea solutions like the insoluble residue of the present study. Consequently, although the egg-shell of *G. mitratus* is similar to those of silkworms in the importance of the disulphide linkage of the major protein fraction and in some properties of the minor one, a marked difference exists among these shells as to the presence and importance of a large amount of phosphoserine and accompanying metal cations. This difference must be a reflexion of the different protective function of the shells and may have important physiological implications. At present, we feel that more information about other insect egg-shells are necessary to understand these things.

As a structural protein, the major protein fraction of *G. mitratus* egg-shell is unique in its being a phosphoprotein with high content of serine (29.8mol %) and phosphorus (7.6% of ash-free CM-fraction). Phosvitin, the main phosphoprotein of avian yolk, resembles this structural protein in some respects: phosvitin contained a large amount of serine (about 50mol %) and phosphorus (9.7%) (Mecham & Olcott, 1949; Lewis, Snell, Hirschmann & Fraenkel-Conrat, 1950); it was precipitated by the addition of magnesium sulphate and the precipitate was soluble in 10% sodium chloride (Joubert & Cook, 1958); and it was shown to be a fibrous protein (frictional ratio, 1.98–2.09) (Joubert & Cook, 1958). There are certainly differences between the two phosphoproteins in molecular parameters and in amino acid composition. However the absence of cystinyl residue in phosvitin (Mecham & Olcott, 1949) seems to be one of the basic differences that make one phosphoprotein a structural protein and the other probably a protein of storage function. Detailed studies on other basic differences would be worthwhile.

Further, we would like to mention the structural proteins of the developing enamel of various mammalian teeth. Although the proteins have not yet been well characterized, they were found to contain significant quantities of organic phosphorus (17–36 atoms of P/1000 amino acid residues), and *O*-phosphoserine was identified in partial acid hydrolysates of all the samples from 14 species studied (Glimcher, Friberg & Levine, 1964; Levine, Glimcher & Krane, 1967; Levine, Seyer, Huddleston & Glimcher, 1967). Seyer & Glimcher (1969) isolated two *O*-phosphoserine-containing tripeptides from a partial hydrolysate of the matrix of embryonic bovine enamel and characterized them. A phosphoprotein, thus, could be the structural protein of the calcified tissue as well. When a pure enamel protein is obtained, a comparison of its properties with those of the major protein of the cricket egg-shell, which is not calcified, will provide useful information as to its physiological meanings, particularly as to the possible role of the *O*-phosphoseryl residue in initiating calcification.

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