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The Isolation and Composition of Two Phosphoproteins from Hen's Egg

By R. C. CLARK
The National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria, South Africa

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1. Phosvitin extracted from domestic hen's-egg yolk was resolved on Sephadex G-100 into two phosphoprotein components. 2. The major component has a molecular weight of about 3.4×10^4 and alanine as an *N*-terminal residue. Glucosamine is present, but tyrosine is virtually absent. 3. The minor component has a molecular weight of about 2.8×10^4 and lysine as an *N*-terminal residue. Missing residues are glucosamine, methionine and leucine. Lysine, histidine, threonine, glycine, phenylalanine and tyrosine contents differ significantly from those of the major component. 4. Sephadex G-100 also removes small amounts of an impurity with a much higher molecular weight.

The findings of several previous workers have indicated that hen's-egg-yolk phosvitin is not a homogeneous protein. Fractionation into components of differing composition has been achieved by Connelly & Taborsky (1961) on DEAE-cellulose, by Belitz (1963) on DEAE-Sephadex, and by Mok, Grant & Taborsky (1966) using countercurrent distribution. This last procedure resolved the material into components of different molecular weights (Taborsky & Mok, 1967). Also reported is the use of Sephadex G-200 in a short column (Ho, Magnuson, Wilson, Magnuson & Kurland, 1969), which only separated a phosphorus-free contamination from phosvitin.

MATERIALS AND METHODS

Phosvitin. Extraction from hen's eggs was according to the method reported by Joubert & Cook (1958*a*,*b*) with the modification that the sodium acetate buffer dialysis stage was carried out at pH4.5 and *I*0.1 (0.1M-sodium acetate, adjusted to pH4.5 with acetic acid) in which 0.01M-EDTA was present. Salt was removed from the final product by dialysis at 5°C against flowing water.

Enzymes. Carboxypeptidase A and B (both di-isopropyl phosphorofluoridate-treated) as well as bacterial alkaline phosphatase (*Escherichia coli*) were purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Phosphorylated amino acids. *O*-Phospho-L-serine and *O*-phospho-DL-threonine were supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A. Purity was confirmed by analysis of nitrogen.

Fractionation on Sephadex G-100. Jacketed columns (150cm long × 3.8cm diam.) maintained at 5°C were filled with Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden), particle size 40–120μm, after equilibration in sodium acetate buffer, pH4.5 and *I*0.3 (i.e. 0.3M-sodium acetate adjusted to pH4.5 with acetic acid). Elution with buffer of the same composition was carried out in the upward mode at a pumping rate of about 45ml/h. Effluent was monitored by measuring the extinction at 280nm, the spectrophotometer output being connected to a time-base chart recorder. Smaller-scale fractionations, where indicated, made use of columns 150cm long × 1.9cm diam., with elution at a pump rate of about 20ml/h. Protein recovery from eluate fractions was carried out by freeze-drying after dialysis against flowing water at 5°C.

Analyses. Nitrogen was determined by the method of McKenzie & Wallace (1954) and phosphorus by the method of Morrison (1964). Glucosamine and amino acid analysis was carried out by using a 4h run on a Beckman model 120 Amino Acid Analyser fitted with a model 125 automatic integrator, after hydrolysis of the sample (approx. 1mg) in constant-boiling HCl (0.5ml) for 24h at 110°C in sealed evacuated ampoules. Tyrosine losses were minimized by the addition of phenol (10–20μl of aq. 5% solution of freshly sublimed material) according to the method of Sanger & Thompson (1963). By using the above-described conditions and quoting mean result, S.D. and number of experiments performed, it was found that a *O*-phospho-L-serine standard was dephosphorylated virtually completely, with a serine recovery of 72.3± 0.8% (3). *O*-Phospho-DL-threonine afforded 26.7± 0.4% (4) unhydrolysed starting material and 51.6± 0.9% (4) threonine. These means have been utilized in correcting for serine destruction, original phosphothreonine content and the contribution to threonine made by the hydrolysis of phosphothreonine. Serine was assumed, for the purpose of these calculations, to occur only as the phosphate ester in the protein. Unphosphorylated threonine in phosvitin was assumed to be degraded to an extent of 3.8%, a value derived in these laboratories from the analysis of phosphorus-free proteins. *N*-Terminal amino acid analysis by the 1-fluoro-3,4-dinitrobenzene procedure was carried out on a Technicon Automated DNP-amino acid chromatograph as described by Kestner, Muntwyler, Griffin & Abrams (1963), but by using a shortened elution

gradient prepared in a nine-chambered Technicon Varigrad Apparatus with 200ml of *n*-hexane in chambers 1–6, containing 2%, 2%, 2%, 8%, 10% and 2% (v/v) 2-methylbutan-2-ol respectively, and 170ml, 170ml and 172ml of freshly distilled butan-2-one in chambers 7–9. Peak identities were verified by collection as they emerged from the colorimeter unit, and after removal of solvent in a rotary evaporator they were chromatographed on Merck silica gel t.l.c. plates with standard DNP-amino acid samples as reference. The three different developing solvent systems were the same as those used by Belitz (1966) for similar investigations. *N*-Terminal amino acids were also identified by using DNS-chloride (Gray & Hartley, 1963; Gray, 1967). The hydrazinolytic *C*-terminal amino acid procedure was that of Braun & Schroeder (1967). No analyses were carried out for carbohydrates, although these are known to be present in phosvitin (Allerton & Perlmann, 1965; Tunman & Silberzahn, 1962).

Molecular weights. The short-column procedure (method III; Van Holde & Baldwin, 1958) was carried out on a Beckman Spinco model E Analytical Ultracentrifuge. Where specified, the meniscus-depletion equilibrium method (Yphantis, 1963) was also utilized. Buffer was sodium acetate, pH4.5 and *I*0.3, in all cases.

Electrophoresis. Tiselius moving-boundary electrophoresis was carried out at 1°C with a Beckman model H instrument and at 0°C with a Perkin–Elmer model 238 Electrophoresis Apparatus. Schlieren optics were used in both instances for photographic records.

RESULTS

Extraction. Yields of water-dialysed freeze-dried phosvitin ranged between 0.3 and 0.4% of the weight of white-free egg yolks extracted. Analysis data for this material are given in Table 1 together with values published previously by other workers.

Sephadex G-100 fractionation. Passage of phosvitin through a single 150cm-long Sephadex column on either analytical (200mg on 1.9cm diam.) or preparative (1.5g on 3.8cm diam.) scale gave an elution pattern similar to that shown in Fig. 1. The inclusion of 10mM-EDTA in the eluting buffer was not found to bring about significant alteration of this pattern. The initial peak, fraction S_A, which was variable in height with different batches of starting material, accounted for 5% or less of the total weight fractionated. High extinction readings arise from a turbidity in this fraction. The dialysed freeze-dried product isolated from fraction S_A did not readily redissolve in aqueous buffers, whereas protein from the remainder of the chromatogram gave clear solutions with no difficulty. The phosphorus content of fraction S_A was found to be about 5% and *N*-terminal amino acid analysis by the fluorodinitrobenzene procedure gave several DNP-amino acids in low yield.

The remaining material eluted was cut to give fractions S_B, S_C and S_D, as indicated in Fig. 1. Passage of these fractions through analytical-scale

Table 1. *Composition of whole phosvitin*

Source	N (%, w/w)	P (%, w/w)	Amino acid content (mol of residues/10^4g of dry protein)																				
			Ser	Thr	Arg	Lys	His	Asp	Glu	Gly	Ala	Val	Leu	Ile	Pro	Phe	Tyr	Trp	Cys	Met	GlcN
Tunman & Silberzahn (1962)																					0.5
Belitz (1963)	11.35	10.0	31.0		3.3	5.2	2.8	3.6	3.7	1.5	2.3	0.8	0.6	0.5	0.8	0.3	0.1				
Mecham & Olcott (1949)	11.90	9.7	31.0	Trace	2.8	4.0	3.1	3.2	2.3	2.1		Trace	0.8	Trace	0.9	0.4	<0.1	0.3	0	0.3	
Lewis, Snell & Hirschmann (1950)			31.5	1.2	2.8	4.1	3.1	3.3	2.3	2.1	1.7	0.9	0.8	0.4	0.8	0.3				0.2	
Allerton & Perlmann (1965)			35.5	1.4	3.2	4.7	2.9	3.9	3.7	1.5	2.2	0.9	0.7	0.5	0.9	0.5	0.2	0.2	0	0.2	0.9
Taborsky & Allende (1962)		10.0	37.2	1.2	3.1	4.5	3.0	3.6	3.2	1.6	2.0	0.6	0.7	0.4	0.9	0.7	0.7	0.3	0	0	
This work	12.24	9.4	34.3	1.4	3.3	4.7	2.8	4.0	4.0	1.6	2.3	1.1	0.8	0.6	1.0	0.6	0.3		0	0.2	

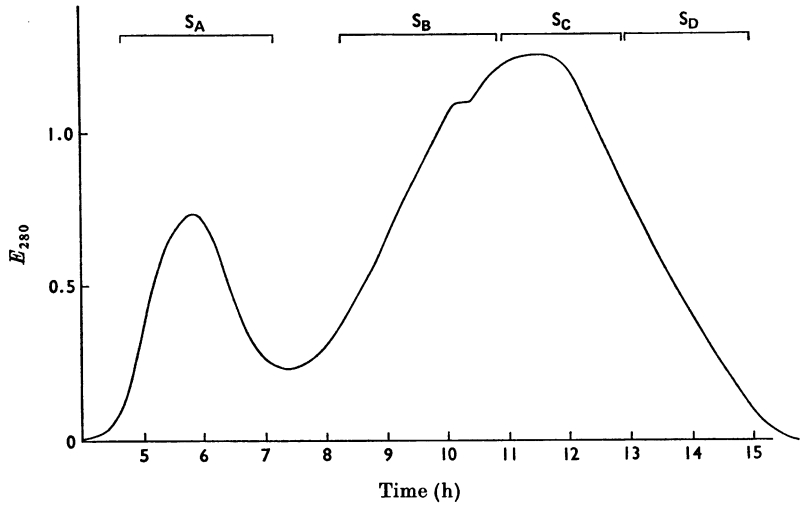

Fig. 1. Elution diagram of phosvitin on Sephadex G-100. Conditions: column length, 150cm; diam., 3.8cm; buffer, 0.3M-sodium acetate, pH4.5.

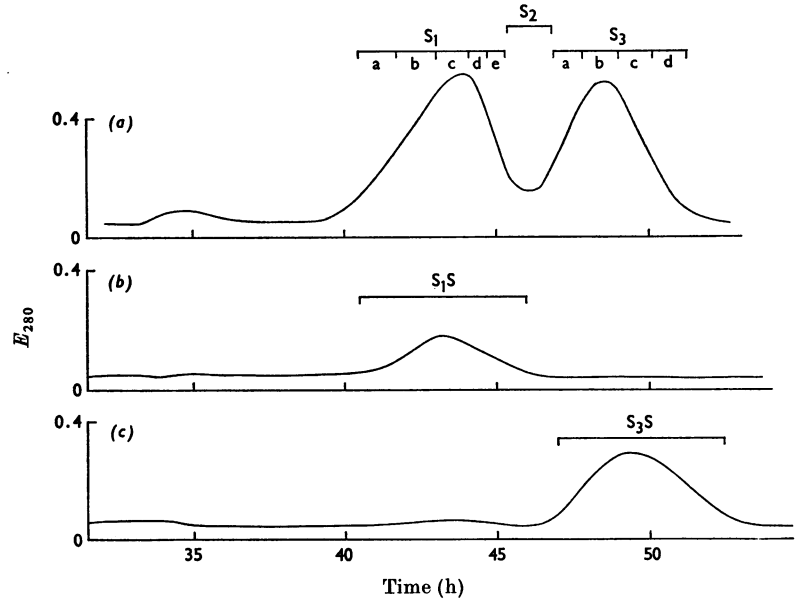

Fig. 2. Elution diagrams on Sephadex G-100. Conditions: column length, 450cm; diam., 3.8cm; buffer, 0.3M-sodium acetate, pH4.5. (*a*) Phosvitin; (*b*) fraction S_1; (*c*) fraction S_2.

150cm-long Sephadex columns gave asymmetrical peaks that differed in amino acid composition from one another. Serine and phosphate content, on the other hand, differed to a much smaller degree. Electrophoretic mobilities at 0°C of ascending and descending boundaries were found to be: fraction S_B, 10.26 and 9.98; fraction S_C, 9.99 and 9.50; fraction S_D, 9.99 and $9.19\times10^{-5}cm^2V^{-1}s^{-1}$. Schlieren patterns of the boundaries of fractions S_B and S_D were notably asymmetrical.

Passage of phosvitin at conventional loadings (1.5–3.0g for a column diameter of 3.8cm) through three 150cm lengths of Sephadex G-100 connected in series gave a type of elution pattern similar to that

Table 2. *Composition of Sephadex fractionation products of phosvitin*

Fraction	Recovery of crude weight (%, w/w)	P (%, w/w)	Amino acid content (mol of residues/10^4 g of dry protein)																
			Ser	Thr*	Arg	Lys	His	Asp	Glu	Gly	Ala	Val	Leu	Ile	Pro	Phe	Tyr	Met	PThr	GlcN
S_1	50–55	9.3	38.4	0.53	3.44	4.61	3.78	4.23	3.47	1.60	2.20	0.83	0.85	0.44	0.90	0.29	0.04	0.27	0.98	0.96
S_3	15–20	9.3	38.1	1.64	3.65	6.00	2.27	3.85	4.81	1.70	2.30	0.74	0.30	0.30	0.62	0.71	0.34	0.09	0.82	0.54
S_1S	40–45	9.5	32.1	0.59	2.93	4.01	3.18	3.45	2.89	1.24	1.75	0.72	0.82	0.49	0.84	0.20	0.07	0.25	0.69	0.84
S_3S	10–15	9.2	33.4	2.20	3.72	6.50	1.37	4.16	6.42	1.91	2.69	1.00	0.02	0.27	1.05	1.18	0.64	0	0.42	0
S_{1a}	9		33.0	0.58	2.91	3.63	2.85	3.82	3.08	1.43	2.31	0.88	0.96	0.51	0.73	0.31	0.13	0.26	0.28	0.30
S_{1b}	16		34.0	0.53	2.92	4.17	3.24	3.80	2.97	1.38	2.21	0.78	0.82	0.49	0.90	0.30	0.12	0.29	0.99	0.94
S_1	14		37.2	0.55	3.12	4.35	3.37	3.97	3.26	1.51	2.34	0.89	0.82	0.54	0.93	0.31	0.12	0.21	1.16	1.09
S_{1d}	9		37.6	0.50	2.98	3.98	3.01	3.89	2.71	1.40	2.15	0.80	0.81	0.50	0.88	0.31	0.07	0.24	1.09	1.04
S_{1c}	7		36.5	0.57	3.03	4.34	3.35	3.92	3.24	1.61	2.49	0.93	0.94	0.57	0.98	0.33	0.08	0.24	1.07	1.04
S_{3a}	6		33.5	1.64	3.47	5.76	1.71	4.10	5.53	1.90	2.61	0.79	0.25	0.37	0.60	0.98	0.43	0.03	0.78	0.21
S_{3b}	7		36.1	2.29	3.89	6.80	1.66	4.41	6.34	2.10	2.92	0.95	0.09	0.28	0.85	1.23	0.58	0.04	0.63	0.04
S_{3c}	6		34.3	1.97	3.51	6.07	1.40	4.18	6.17	1.96	2.77	0.92	0.09	0.29	0.94	1.19	0.59	0.03	0.79	0.04
S_{3d}	4		33.2	1.89	3.69	6.42	1.45	4.09	6.06	1.90	2.67	0.85	0.09	0.25	0.99	1.13	0.52	0.07	0.79	0.03

* Excluding phosphothreonine (PThr).

Table 3. N-*Terminal amino acid analysis results of phosvitin and Sephadex fractions (fluorodinitrobenzene procedure)*

	Amino acid content (mol of residues/10^4 g of dry protein)	
	Lysine	Alanine
Phosvitin	0.14	0.24
S_1	0.05	0.39
S_3	0.41	0.05

shown in Fig. 1. However, if the loading was decreased to 0.5–0.8g, separation of the kind shown in Fig. 2(*a*) could be achieved. Cuts to give fractions S_1, S_2 and S_3 were made as indicated. In one instance there were smaller subdivisions made to give fractions S_{1a}, S_{1b}...S_{3d}. Refractionation of fractions S_1 and S_3 gave elution patterns shown in Figs. 2(*b*) and 2(*c*) respectively. Head and tail portions of each peak were rejected and the recovered fractions were designated S_1S and S_3S. Compositions and yields of the various fractions are given in Table 2. Amounts of fraction S_2 were 15–20% of the original weight of phosvitin, and on refractionation yielded further amounts of fractions S_1 and S_3.

Small amounts of material were isolatable from the minor peak or peaks eluted near the fronts of the patterns in Fig. 2. Properties were similar to those described for fractions S_A from the short-column experiments. No turbidity of the eluate was noticeable at the light column loadings used.

End-group analysis. Results are given in Table 3. The DNP-amino acid chromatograms showed only small amounts of material corresponding to DNP-serine (about 0.15mol of residues or less/10^5g).

Qualitative *N*-terminal amino acid detection by using DNS-chloride confirmed the identity of alanine and lysine as end groups of fractions S_1 and S_3 respectively.

Hydrazinolysis of phosvitin as well as Sephadex fractionation products gave free amino acid recoveries of 0.1mol of residues or less/10^4g for each of several amino acids. Carboxypeptidase A or B, either in the presence or absence of bacterial alkaline phosphatase (*E. coli*), did not liberate free amino acids in significant amounts.

Molecular weights. Table 4 gives values of molecular weights measured in a variety of media. Partial specific volume for phosvitin and the fractions is taken as 0.545 (Joubert & Cook, 1958*a*), and densities of guanidine hydrochloride and urea solutions are calculated on the basis of the empirical equations of Kawahara & Tanford (1966).

Moving-boundary-electrophoresis patterns of phosvitin, fraction S_1S and fraction S_3S. Table 5 lists mobility data. The shapes of the schlieren patterns of both ascending and descending boundaries of

Table 4. *Weight-average molecular weights by ultracentrifugation*

Material	Method*	Buffer†	$10^{-4}M_W$
Whole phosvitin	MDE	A	3.21
			3.13
Whole phosvitin	MDE	B	3.17
Whole phosvitin	MDE	C	2.83
Fraction S_1	SC	A	3.44
Fraction S_3	SC	A	2.85
Fraction S_1	MDE	B	3.20
			2.99
			3.07

* MDE, Meniscus-depletion equilibrium; SC, short-column technique.

† A, Sodium acetate buffer, pH 4.5 and I 0.3; B, sodium acetate buffer, pH 4.5 and I 0.3, + 6M-urea+mercaptoethanol in approx. 100-fold stoicheiometric excess over the protein molecular weight; C, sodium acetate buffer, pH 4.5 and I 0.3, +6M-guanidine hydrochloride.

Table 5. *Electrophoretic mobilities of phosvitin and Sephadex fraction in* 0.05M-*sodium acetate*–0.05M-*sodium chloride buffer*, pH 4.5 *at* 1°*C*

	$10^5 \times$ Mobility ($cm^2 V^{-1} s^{-1}$)	
	Ascending	Descending
Phosvitin	10.95	9.77
S_1S	10.86	9.85
S_3S	10.34	9.63

fractions S_1S and S_3S reflect a very small degree of cross-contamination and/or the presence of minor amounts of high-molecular-weight aggregates. Characteristic of phosvitin and its fractions is the large difference in the degree of spreading of the ascending and descending boundaries. The former is very sharp, whereas the latter is very much more diffuse.

DISCUSSION

The analytical results in Table 1 reveal no radical differences between my preparation of phosvitin and the materials studied by previous workers. Variations in serine content are mainly from differences in the correction applied for *O*-phosphoserine destruction during protein hydrolysis.

The leading shoulder on the main peak eluting from 150cm-long Sephadex columns (Fig. 1) indicates protein heterogeneity, and this is substantiated by the asymmetrical elution curves obtained when fractions S_B, S_C and S_D are passed through Sephadex again. The progressive differences in amino acid composition, *N*-terminal amino acid residue content, electrophoretic mobility and molecular weight of fractions S_B, S_C and S_D show that proteins of substantially different chemical and physical properties are present. Serine and phosphate contents, on the other hand, being more constant, suggest that, although there is more than one component present, the material is all phosphoprotein. The EDTA experiment presents evidence that metal ion complexes do not play a significant role in the type of fractionation obtained with Sephadex. The minor fraction S_A, because of low phosphate content, low yield, inhomogeneity as evidenced by *N*-terminal amino acid analysis and intractable solubility, was disregarded. Its origin could well be from denaturation and aggregate formation of phosvitin with itself or with other egg proteins that had not been completely removed by the extraction procedure.

Effective resolution of two phosphoproteins is possible provided that the Sephadex column length is sufficiently great and sample loading small. Aggregate formation, giving peaks ahead of the main phosphoprotein bands, is also lessened by the lower concentration of protein on the column. Fractions S_1S and S_3S show marked differences in amino acid composition, notably that glucosamine, methionine and leucine are exclusive to fraction S_1S and that tyrosine content is so low that it is likely to be only a contamination. Glutamic acid, threonine, lysine and tyrosine are in much higher proportions in fractions S_3S, whereas the histidine content is lower. It is evident from these results that fraction S_3S does not merely consist of a portion of the molecule in fraction S_1S. Amino acid analysis data of fractions S_{1a}–S_{1e} and S_{3a}–S_{3d} show that cross-contamination of the two peaks is relatively small and that each peak is otherwise homogeneous. Refractionation to give fractions S_1S and S_3S involves rejection of the head and tail portions of each peak, where most of the cross-contamination is present.

Alanine and lysine as well as serine have been reported as *N*-terminal residues by previous workers (Mok, Martin & Common, 1961; Neelin & Cook, 1960; Belitz, 1966). Assigning alanine to the

major component in fraction S_1S and lysine to the minor component in fraction S_3S concurs with the findings of the first two groups of workers. Belitz (1966) proposed, on the other hand, that phosvitin is a tripolypeptide with two alanines and one serine as *N*-terminal residues. The inaccuracy involved in the correction for serine destruction when hydrolysing phosvitin and DNP-phosvitin is of such magnitude that a difference analysis of one serine in approximately 100 residues cannot be performed reliably. Amounts of alanine listed in Table 4 are lower than published values [0.5mol of residues/10^4g (Neelin & Cook, 1960); 0.6mol of residues/10^4g (Belitz, 1966)] and do not account for the even higher values Taborsky (1963) obtained for α-amino acid content as measured by a selective Van Slyke analysis (1.1–1.2mol of residues/10^4g). However, values in Table 4 are equivalent to 1.3mol of alanine residues/3.4×10^4g and 1.1mol of lysine residues/2.8×10^4g, which approximate to unity for the molecular weights of fractions S_1S and S_3S as measured by ultracentrifugation. The result of the hydrazinolysis experiments favour the identity of the *C*-terminal group or groups as being either basic or amide amino acids, since these are not isolatable by the technique used. The usual commercial carboxypeptidases and phosphatases are ineffective in attack of this highly acidic substrate, and are thus not amenable as tools for sequence investigation here. Also relevant to this is our experience that manually executed Edman degradation of either whole phosvitin or fraction S_1S, based on conditions described by Edman & Begg (1967), failed. A likely cause of difficulty was the phenylthiourea derivative, which forms an insoluble glass in the system required for its conversion into the thiazolinone.

Molecular-weight measurements show that that of fraction S_1S is slightly greater than that of fraction S_3S. Intermolecular disulphide bridging or non-covalent association of polypeptide chains appears to be absent since mercaptoethanol and lyotropic agents do not cause significantly large changes in measured molecular weight. Cystine or cysteic acid is not found in acid hydrolysates, but, as pointed out by Allerton & Perlmann (1965), these can be destroyed by hydrolysis conditions if present in only very small amounts. Yuan (1967) reports on the assay of thiol in phosvitin, finding up to 0.45 equiv./3×10^4g. No criteria as regards the purity of his phosvitin have been given.

The larger molecule present in fraction S_1S must bear a higher net charge at pH4.5 compared with fraction S_3S, since electrophoretic mobility is slightly greater.

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