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Solubilization of hemoglobin S by other hemoglobins

(sickle cell hemoglobin/hemoglobin mixtures/copolymerization)

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ABSTRACT **The polymerization of mixtures of Hb S with hemoglobins A, A_2, and F has been investigated by analysis of the proportions of S and non-S hemoglobin both in the supernate and in the pellet after centrifugation. In all cases the non-S hemoglobin was incorporated into the polymer even in the absence of hybrids in the order A > A_2 > F. The solubility of Hb S is substantially increased by the other hemoglobins, especially by Hb F, which would account for its antisickling effect. It appears that the excluded volume effect of the other hemoglobin on Hb S is largely counterbalanced by the solubilizing effect arising from the interaction between the two hemoglobins in solution. The ability of hybrid hemoglobins to gel was demonstrated directly with tetramers in which $\alpha\beta^S$ dimers were covalently linked to $\alpha\beta^A$, $\alpha\delta^{A_2}$, and $\alpha\gamma^F$ dimers.**

The so-called "sickle-sparing" effect of other hemoglobins on hemoglobin S (Hb S) was described more than 20 years ago by Singer and Singer (1) and by Allison (2), who found that some hemoglobins such as Hb A and Hb C greatly decrease the concentration of Hb S necessary for gelling, whereas Hb F had a much smaller effect. It was assumed by these authors and in much of the subsequent work with other hemoglobins (3–6) that the "sickle-sparing" hemoglobins act by substituting for Hb S in the polymer, i.e., by copolymerizing with it. By contrast, it was concluded that Hb F and, more recently, Hb A_2 (7–9) do not copolymerize with Hb S.

However, it has now been realized that another factor plays a major role in the gelling of Hb S in the presence of other hemoglobins (10–12). In these very concentrated and nonideal solutions the presence of any other hemoglobin [and, indeed, any other protein (13, 14)] causes an increase in the activity coefficient of Hb S by an excluded volume effect. This, by itself, must lead to a decrease in the *concentration* of Hb S necessary for gelation.

It is clear, then, that another hemoglobin can affect Hb S polymerization in at least three ways: (*i*) by increasing the activity coefficient, (*ii*) by copolymerizing, and (*iii*) by forming hybrids of the type $\alpha_2^A\beta^A\beta^S$, which can in turn act by either or both of the first two mechanisms.

The magnitude of the first effect can be calculated from the values of the activity coefficient as a function of hemoglobin concentration derived from sedimentation and osmotic pressure data (12). Hybrid formation can be prevented by mixing the hemoglobins in the absence of oxygen. However, experiments to determine the extent of copolymerization have led to conflicting results, especially for Hb F. Measurement of the concentration of Hb F in the pellet after centrifugation of the gel led Bertles *et al.* (15) to the conclusion that Hb F does not copolymerize with Hb S, in agreement with Behe and Englander (16), who analyzed the supernate. Goldberg *et al.* (17), on the other hand, found evidence of copolymerization with Hb F under both hybridizing and nonhybridizing conditions. Finally, from an analysis of the total solubility of the gels formed from hemoglobin mixtures as well as theoretical considerations, Sunshine *et al.* (18) postulated that only deoxy-Hb S molecules polymerize in mixtures with Hb F.

The purpose of the work reported here was therefore to investigate the participation of Hb F and Hb A_2 in the polymerization of Hb S by direct analysis, not only of the supernate, but also of the gel. In addition, hemoglobins in which an $\alpha\beta^S$ dimer was covalently crosslinked to either an $\alpha\gamma$ or an $\alpha\delta$ dimer were used to test the ability of these mixed tetramers to polymerize.

MATERIALS AND METHODS

The hemoglobins were isolated from hemolysates, prepared as described (19) from normal blood, sickle trait blood, and cord blood. After dialysis against 0.1 M glycine at pH 7.6, the hemolysates were fractionated on DEAE-cellulose, Whatman DE52 (20), under the conditions shown in Table 1. Sectional columns (Kontes Glass) were used and the desired hemoglobin was removed from the appropriate segment without elution of the column (21).

Crosslinked (XL) hemoglobins were prepared by using the reagent 2-nor-2-formylpyridoxal 5′-phosphate as described (22, 23). For the preparation of crosslinked mixed tetramers, the reaction was performed on equimolar amounts of Hb S and the other hemoglobin that had been mixed under oxygen to allow for hybridization. After the coupling reaction, which takes place under anaerobic conditions, the hemoglobins were converted to the cyanmet form by oxidation with 1.2 equivalents of potassium ferricyanide in the presence of 2.0 equivalents of potassium cyanide. Ferro- and ferricyanide were removed by passage through a Sephadex G-25 column in 0.1 M potassium phosphate buffer, pH 7.3/0.01 M cyanide. After dialysis against 0.1 M glycine at pH 7.6 containing 0.005% KCN, the crosslinked tetramers SSXL, ASXL, and A_2SXL were isolated by chromatography on DEAE-cellulose as shown in Table 1. In the case of F_ISXL it was necessary to isolate the crosslinked hybrid in two steps: the crosslinked tetramers were first separated from the uncrosslinked ones on a column of Sephadex G-100 in 1 M $MgCl_2$ (24). The F_ISXL was then isolated by chromatography of the crosslinked fraction on DEAE-cellulose, using the same gradient as for ASXL, except that after 24 hr the solution in the reservoir was replaced by 0.2 M glycine/0.06 M NaCl.

After elution from the DEAE-cellulose the hemoglobins were concentrated to about 1 mM by pressure filtration through Amicon PM10 membranes and dialyzed against 0.1 M phosphate buffer, pH 7.3, containing 0.1 mM EDTA in the case of oxyhemoglobin and 0.01 M CN^- for cyanmethemoglobin. They were then further concentrated to 35–40 g/dl by ultrafiltration through 1-cm Visking dialysis tubing suspended in a large evacuated separatory funnel at 5°C. Total hemoglobin concentration was determined as cyanmethemoglobin (25).

For mixtures in which hybridization between Hb S and the other hemoglobin was meant to reach equilibrium, the hemo-

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Table 1. Conditions for the preparative separation of hemoglobins on DEAE-cellulose

Hemoglobins	Sample applied	Gradient, in 0.2 M glycine at pH 7.6: Constant-volume mixer	Gradient, in 0.2 M glycine at pH 7.6: Reservoir	Components in order of mobility
A, A_2, S	Sickle trait blood hemolysate, 10–12 μmol	0.005 M NaCl	0.02 M NaCl	A_2, S, A
F, F_I	Cord blood hemolysate, 9 μmol	0.02 M NaCl	0.04 M NaCl	F, F_I
SSXL	Crosslinked reaction mixture, 6 μmol	0.005 M NaCl	0.02 M NaCl	S, SSXL
ASXL	Crosslinked reaction mixture, 6 μmol	0.01 M NaCl	0.03 M NaCl	S, SSXL + A, ASXL, AAXL
A_2SXL	Crosslinked reaction mixture, 6 μmol	0.005 M NaCl	0.02 M NaCl	A_2, S, A_2A_2XL, A_2SXL, SSXL

See ref. 20. Columns were 4 × 26 cm and were developed at 90–100 ml/hr at 5°C.

globins were first mixed in various proportions in air. Then 200-μl samples were layered over a drop of FC43 fluorocarbon (Spinco) in a 3.4-mm NMR tube (26). The tube was filled up with mineral oil and then 10 μl of 1 M sodium dithionite in deoxygenated 0.1 M phosphate buffer, pH 7.3, was introduced under the mineral oil. After thorough mixing with a barbell-shaped magnetic stirring bar, the tubes were incubated for 2 hr at 30°C and then centrifuged for 2 hr at the same temperature in a 50.1 swinging-bucket rotor in a Spinco L 5-50 centrifuge at 190,000 × *g*.

The mixtures without hybrids were prepared from cyanmethemoglobins essentially as described by Goldberg *et al.* (17). The non-S hemoglobin was introduced between the nonaqueous layers in the centrifuge tube followed by the addition of 1.2 equivalents of 1 M deoxygenated dithionite solution. Incubation for 15 min at room temperature was sufficient for complete reduction. The tube was then cooled on ice, the cyanmethemoglobin S was added, and the solutions were mixed thoroughly. After removal of an aliquot for determination of the relative proportions of the two hemoglobins, a second portion of dithionite was added to reduce the Hb S, bringing the total dithionite to 2 equivalents per total heme. The tubes were incubated and centrifuged as described above.

After centrifugation, the supernate and the gel were analyzed separately. The total hemoglobin concentration (c_{sat}) was first determined on an aliquot of the supernate. The remaining supernate was removed, diluted 1:2–1:3 with 0.01 M phosphate buffer, pH 7.3, containing 0.01 M CN^- and catalase at 5 μg/ml, followed by passage through a 8 × 60 mm Sephadex G-25 column in the same buffer to remove dithionite and its oxidation products. In the case of mixtures of Hb S with Hb A and Hb A_2, the proportions were determined by discontinuous polyacrylamide gel electrophoresis with 7.5% acrylamide gels in Tris/glycine buffers (27). The gels were scanned at 280, 531, or 540 nm. Calibration with known mixtures gave close agreement with the expected results. The proportions of Hb F and Hb S were obtained by complete amino acid analysis of the globin prepared from the mixtures by the method of Teale (28). Because there is no isoleucine in Hb S, whereas Hb F has 8 isoleucines per tetramer (29), the Ile/Leu or Ile/Phe ratio provides an accurate measure of the Hb F content of these mixtures.

After removal of the supernate, the gels were washed with phosphate buffer, removed from the centrifuge tube with about 5 vol of the cyanide and catalase-containing phosphate buffer, dispersed by stirring, and left overnight at 5°C to dissolve. After passage through Sephadex G-25, the solutions were analzed by the same methods as those used for the supernates.

RESULTS AND DISCUSSION

We have investigated the possible incorporation of hemoglobins A, A_2, and F into Hb S polymers in three different ways: (*i*) the gelling behavior of covalently crosslinked hybrid tetramers, (*ii*) the non-S hemoglobin content of the pellets from centrifuged mixtures, and (*iii*) the activity of Hb S in the supernatant of gelled mixtures.

(*i*) *Gelling of crosslinked hybrid tetramers.* The question whether $\alpha\gamma$ or $\alpha\delta$ dimers can be incorporated into a Hb S polymer is answered very directly by the observation that tetramers in which these dimers are covalently linked to $\alpha\beta^S$ dimers are capable of gelling, albeit with a higher c_{sat} (Table 2). It should be emphasized that in these gels and their supernates only a single species of molecule can exist—i.e., the hybrid tetramer. Therefore, hybridization and changes in activity coefficients that complicate the study of mixtures are completely avoided in these uniform populations of nondissociable tetramers. The lower hemoglobin concentration in the gels of Hb A_2SXL and Hb F_ISXL, despite their higher c_{sat} (18), suggests that a "looser" polymer is formed by these hybrids than by either Hb ASXL or Hb SSXL (Table 2).

Two series of experiments on mixtures of Hb S with Hb A, Hb A_2, and Hb F were carried out, one in which the mixture had been hybridized to equilibrium before deoxygenation and the other in which hybridization was prevented. In each case the proportions of S and non-S hemoglobin were analyzed separately in the pellet and the supernate after centrifugation.

(*ii*) *Analysis of the pellet.* The proportion of non-S hemoglobin in the centrifuged gels is shown in Tables 3 and 4. Because the centrifuged gels contain "trapped" supernate, the values in the last columns of Tables 3 and 4 were corrected, using the estimate of Sunshine *et al.* (18) that the volume fraction of polymer in the gel is 0.65 and its hemoglobin concentration is 69 g/dl, the remainder being trapped supernate with a hemoglobin concentration equal to c_{sat}. It is clear that all three non-S hemoglobins are incorporated into the polymer both as hybrids and as foreign tetramers in the order A > A_2 > F.

The incorporations of hemoglobins A, A_2 and F into the polymer are compared in Figs. 1 and 2. In each case the proportion of the non-S hemoglobin in the polymer is greater when hybrid is present than when it is not (Fig. 1), but the polymer

Table 2. Polymerization of crosslinked hybrid hemoglobins

Hemoglobin	Conc., g/dl: Supernate (c_{sat})	Conc., g/dl: Gel
S	15.6	48
SSXL	13.5	48
ASXL	21.8	48.5
A*fn*2SXL	32.8	44
F_ISXL	32.8	44

The cyanmethemoglobins were reduced with 2 equivalents of dithionite, incubated, and centrifuged at 30°C as described in the text. In each case the c_{sat} was found to be independent of the initial hemoglobin concentration.

Table 3. Distribution of hemoglobins after centrifugation: Hybridized mixtures

Hemoglobins	Total hemoglobin conc., g/dl		Non-S Hb / Total Hb × 100			
	Initial	Supernate	Initial	Supernate	Gel	Polymer
A + S	36.0	28.7	61.1	78.7	51.2	45.0
	35.3	26.4	50.0	71.2	43.0	37.2
	36.0	24.0	40.0	51.6	38.1	35.6
	34.0	20.5	33.0	46.2	30.5	28.0
	33.3	19.0	23.0	34.8	22.6	20.8
	34.3	16.8	13.0	28.0	16.0	14.5
F + S	36.0	30.3	57.3	64.6	40.0	34.2
	36.0	28.9	52.8	62.2	38.5	33.1
	35.9	30.5	44.1	65.1	36.8	29.9
	31.6	26.2	38.0	47.8	17.0	10.7
	32.0	24.4	29.9	42.3	13.5	8.0
	32.5	23.7	19.0	37.4	11.0	6.1
A_2 + S	31.0	26.2	51.0	61.7	36.1	30.8
	34.0	30.0	50.0	54.5	34.3	30.0
	36.0	31.5	50.0	52.0	35.6	32.0
	36.0	29.2	43.4	50.0	30.5	26.0
	31.6	24.6	42.2	57.2	30.0	24.8
	34.7	28.2	38.0	46.8	27.0	23.0
	32.5	24.0	31.3	51.7	24.2	19.0
	33.6	27.2	30.0	43.0	22.1	18.0
	32.8	25.4	26.0	36.7	21.5	18.0
	33.2	23.4	25.2	41.1	16.8	12.3
	31.8	23.7	22.5	30.5	14.5	12.0
	34.0	22.0	14.4	31.2	10.9	7.4

Polymer data were calculated as explained in the legend to Fig. 1.

can evidently incorporate up to 30% of the foreign hemoglobins in the form of unhybridized tetramers without any β^S chains at all (Fig. 1, filled symbols, and Fig. 2). Furthermore, in contrast to Hb A, hemoglobins A_2 and F copolymerize only when their proportion in the mixture exceeds a minimum value, which in the case of Hb F is about 15–20%. The difference between the three hemoglobins is maximal at low concentration of the non-S hemoglobin in the supernate (Fig. 2).

(*iii*) ***Analysis of the supernate.*** The concentration of Hb S in the supernate provides an independent test for copolymerization, because the *activity* of Hb S (i.e., the concentration corrected for the excluded volume effect of the other hemoglobin) must remain constant and independent of the composition of the mixture if no incorporation of the other hemoglobin takes place. This analysis was restricted here to mixtures without hybrids and the results are shown in Table 4. Several conclusions can be drawn from these data:

(*a*) The activity of Hb S in the supernate of the mixtures is not constant, and is considerably higher than the activity of pure Hb S in equilibrium with its polymer, so that Hb S becomes more soluble in the presence of the other hemoglobins. Thus, for example, the experimentally determined Hb S concentration in the supernate of a 48% Hb F mixture (Table 4) is 10.1 g/dl, whereas Sunshine *et al.* (18) calculated the Hb S concentration to be only 2.1 g/dl for a similar mixture on the assumption that no copolymerization takes place.

(*b*) The solubilizing effect is greatest for Hb F and least for Hb A, which parallels the known antisickling effect of these hemoglobins.

(*c*) Interaction of the other hemoglobin with Hb S in solution would therefore explain the inhibition of polymerization of Hb S observed in these mixtures. It seems clear that the excluded volume effect, which lowers the solubility of Hb S in mixtures, must be substantially counteracted by interaction between Hb S and the other protein.

This leads to an alternative interpretation of the cause for the difference in the "sickle sparing" effects of different hemoglobins. In contrast to the previous assumption that Hb F is not "sickle sparing" because it does not interact with Hb S, it must now be concluded that, on the contrary, hemoglobins F and A_2 solubilize Hb S by interacting with it in solution. At a sufficiently high concentration they then give rise to copolymers with an increased solubility.

The problem of the interaction of other hemoglobins with

Table 4. Distribution of hemoglobins after centrifugation: Mixtures without hybrids

Hemoglobins	Supernate				Non-S Hb / Total Hb × 100		
	c_{total}, g/dl	c_S, g/dl	Activity coefficient	Activity of Hb S, g/dl	Initial	Gel	Polymer
A + S	28.5	9.4	19	179	58.9	35.8	28.9
	27.7	9.0	17	153	48.2	34.7	27.6
	26.0	10.6	13	138	41.6	31.5	25.8
	23.0	10.9	8.5	93	37.8	28.5	24.2
	21.9	12.8	7.5	96	26.1	20.6	17.0
A_2 + S	29.6	7.5	22	165	58.9	34.1	24.7
	28.6	10.8	19	205	47.0	28.8	21.4
	28.1	14.4	18	259	35.3	21.4	15.4
	25.3	15.2	12	182	22.4	14.5	9.5
	24.3	16.9	10	169	17.0	8.1	3.9
F + S	31.4	8.4	32	270	54.4	35.9	26.7
	30.8	10.1	28	283	48.2	28.2	18.8
	30.5	12.3	26	319	40.6	21.8	12.7
	25.8	12.4	13	161	29.3	12.2	4.2
	24.3	15.3	10	153	18.0	6.6	0.8
S	15.0	15.0	3.4	50			

Activity coefficients were taken from ref. 12. Polymer data were calculated as explained in the legend to Fig. 1. Data in boxes are discussed in the text.

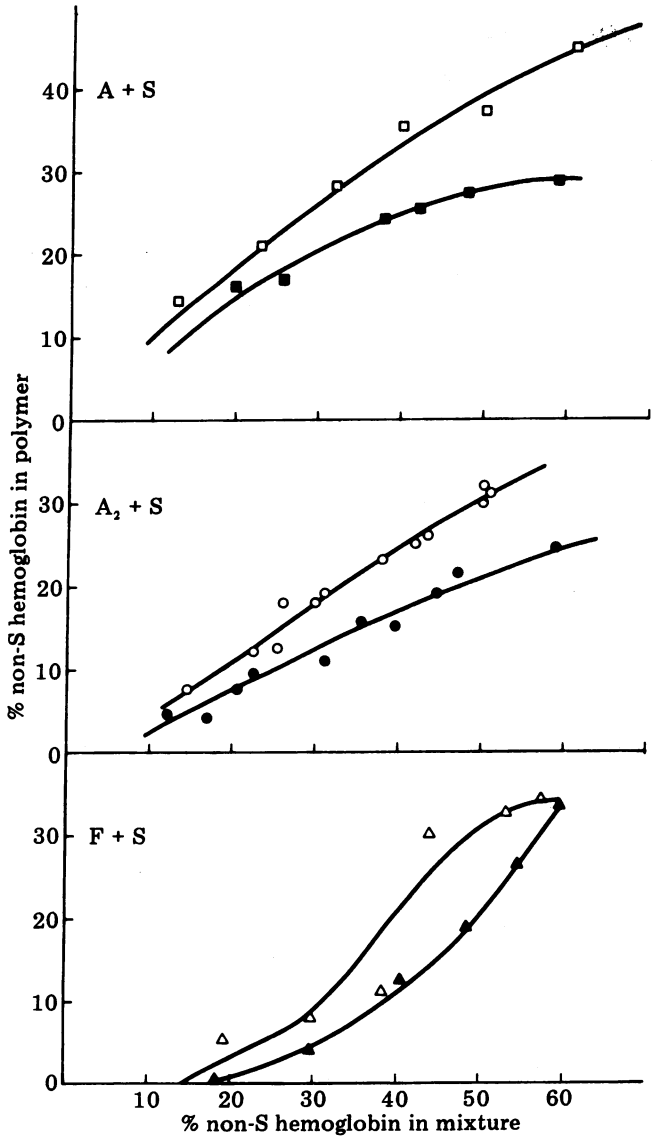

FIG. 1. Incorporation of hemoglobins A, A_2, and F into the Hb S polymer. Filled symbols, mixtures without hybrids; open symbols, mixtures hybridized to equilibrium. The proportion of non-S hemoglobin in the polymer was calculated from the measured proportion in the centrifuged gel by correcting for "trapped" supernate as explained in the text.

Hb S in its polymerization has been approached in two different ways. Only a few workers have measured the distribution of the foreign hemoglobin by direct analysis of the supernatant, gel, or both after centrifugation. In this way Goldberg *et al.* (17) found that Hb F is incorporated, whereas Bertles *et al.* (15) and Behe and Englander (16) reached the opposite conclusion. Because the latter investigators only used solutions containing no more than 20% Hb F, whereas Goldberg *et al.* confined their experiments to mixtures containing at least 45% Hb F, our data (Fig. 1), which show that incorporation of Hb F begins only at about 20%, readily resolve this discrepancy.

On the other hand, a large number of workers have attempted to infer the incorporation of other hemoglobins into the polymer from measurements of the *total* hemoglobin concentration necessary for gelation of mixtures (1–6) and, more recently, from the *total* hemoglobin concentration remaining in the supernate after centrifugation of gelled mixtures (8, 18). In order to interpret these results in terms of incorporation into a polymer, a satisfactory model is necessary. The common assumption has been that the foreign tetramers merely replace Hb S tetramers (with various efficiencies) in a Hb S polymer of otherwise unchanged structure and therefore unchanged solubility. On this basis many workers have concluded that Hb F does not copolymerize, in contrast to the results presented here, which suggest that substitution by a non-S hemoglobin can give rise to a polymer that has a different structure and an increased solubility. This hypothesis is supported by our previous finding that a tetramer composed of β^S chains and mutant α chains ($\alpha^{47\ Asp \rightarrow His}$) forms gels that have a much greater solubility and a lower hemoglobin concentration than those of Hb S (21). Moreover, recent electron microscopy studies in collaboration with R. Crepeau and S. Edelstein have shown that this double mutant hemoglobin forms fibers with very specific structural alterations, in which certain pairs of strands are missing and others are added (unpublished). When a single substitution in the α chains can lead to such profound changes in the structure of the polymer, it is not surprising that multiple substitutions with Hb F or Hb A_2 have decisive effects on the structure and solubility of the copolymer.

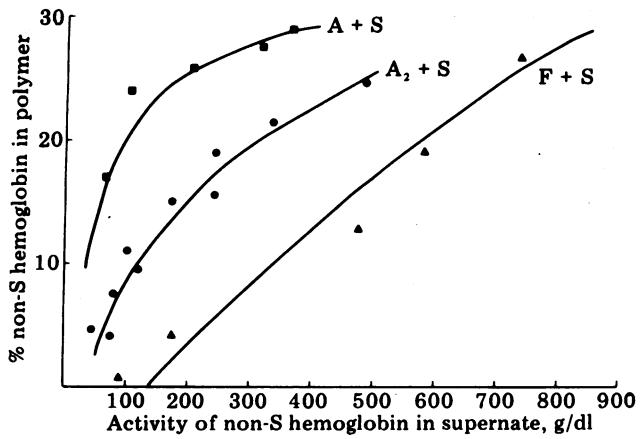

FIG. 2. Activity of non-S hemoglobin in the supernate and incorporation of non-S hemoglobin into the polymer. The activity of non-S hemoglobin in the supernate was calculated from the measured proportion of non-S hemoglobin, the total hemoglobin concentration of the supernate, and the corresponding activity coefficient (12).

While the structure of the copolymers of Hb S with other hemoglobins remains to be elucidated, the analogy with the double mutant hemoglobin suggests a possible reason for the observation (Fig. 1) that, in contrast to Hb A, the incorporation of Hb F appears to be cooperative. This behavior would be expected if Hb F molecules could build into the polymer only in pairs or even sets of pairs to form a looser, more soluble, structure.

The formation of a "softer" polymer by incorporation of a different hemoglobin might also explain some surprising observations—e.g., why relatively low levels of carbamoylation have led to unexpectedly large antisickling effects (30).

Note Added in Proof. Since this manuscript was submitted we have performed analogous experiments on mixtures of Hb S and human serum albumin. With 40% and 51% albumin in the initial mixture, the activity of Hb S in the supernate was 48 and 82 g/dl and the albumin content of the polymer was 6.3% and 5.2%. These results show that, in contrast to the hemoglobins (Table 4), serum albumin does not solubilize Hb S and its effect can be accounted for by its excluded volume alone. The small amount of albumin found in the polymer is probably due to some sedimentation of the albumin itself in non-sector-shaped centrifuge tubes (16). The details of these experiments, which were done with S-carboxamidomethylated serum albumin and using yeast instead of dithionite for deoxygenation in order to avoid polymerization of the albumin by disulfide exchange, will be described in a future publication.

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