Hemoglobin Interaction: Modification of Solid Phase Composition in the Sickling Phenomenon

Abstract. *Direct analyses of solid phase formed by deoxygenating solutions of sickle-cell hemoglobin (Hb S) in the presence of certain other hemoglobin species show that hemoglobins A and C can participate in the filamentous fine structure characteristic of the sickling phenomenon. In contrast, fetal hemoglobin (Hb F) is nearly completely excluded.*

Close ordering of deoxygenated molecules of sickle-cell hemoglobin (Hb S) into linear aggregates is generally accepted as the basic process underlying distortion of erythrocytes in sickle-cell anemia (Hb SS disease) into bizarre silhouettes of sickles or scythes (*1–4*). This change in phase toward a state of greater order, whether within cells (*5*) or in cell-free solution (*3, 6*), manifests itself as an increase in viscosity of the suspension or solution. Other hemoglobins can partially replace Hb S in solution without diminishing the viscosity response to deoxygenation. In this respect the abnormal Hb C is more efficient than Hb A (the major normal hemoglobin of adults), whereas human fetal hemoglobin (Hb F) acts as an inert diluent (*6, 7*). Presumably modification of the sickling phenomenon (that is, promotion or inhibition of solid-phase formation) by hemoglobins other than Hb S reflects a physical relationship among the hemoglobin species involved: the term "interaction" has been applied to this relationship, but mechanisms have remained unclear. We report here results of direct analyses of the solid phase which demonstrate that the apparently negligible interaction of Hb F with Hb S during sickling represents rejection of Hb F during solid-phase formation. In contrast, either Hb C or Hb A can join with Hb S to form a filamentous solid phase indistinguishable by electron microscopy from solid phase composed only of Hb S.

Blood was drawn into anticoagulant (ethylenediaminetetraacetate, 2 mg per milliliter of blood) from normal humans (who are homozygous for Hb A) and from patients with proven Hb SS disease, Hb SC disease, and Hb AS disease (sickle-cell trait). Solutions of cell-free hemoglobin, all at a concentration of approximately 30 g/100 ml, were prepared according to Singer and Singer (*7*) with minor modifications. Appropriate mixtures of hemoglobin solutions, none containing more than 2 percent methemoglobin, were then deoxygenated at room temperature by exposure to humidified atmospheres of either 95 percent N_2 and 5 percent CO_2 or 100 percent CO_2 until increasing viscosity nearly halted flow. Varying the conditions of deoxygenation had no apparent effect on subsequent determinations. The preparations were transferred anaerobically to centrifuge tubes, and the tubes were sealed with thin layers of mineral oil and centrifuged at 40,000 rev/min (170,000*g*) in a Spinco SW 50 L bucket rotor for 1 hour at 20°C. The solid phase sedimented into a smooth-surfaced pack which subsequently melted slowly on exposure to O_2. Proportional contributions of individual hemoglobins to liquid supernatant phases and to their corresponding packed solid phases were obtained by conventional techniques of chromatography (*8*) and electrophoresis (*9*).

Transmission electron microscopy (Fig. 1) of cell-free lysate composed of Hb S and Hb F, sampled during deoxygenation of the lysate, revealed bundles of parallel filaments, each filament having the same transverse dimension (approximately 170 Å) as filaments found in sections of deoxygenated Hb SS erythrocytes (*10, 11*), or dilute solutions of Hb S (*12*), regardless of whether deoxygenation was carried out in an atmosphere of 95 percent N_2 and 5 percent CO_2 or of 100 percent CO_2. Single filaments occurred rarely, a fact conceivably signifying that lateral stabilizing forces between hemoglobin filaments contribute to filament growth. The appearance of these filaments—as clusters of parallel rods—resembles descriptions of liquid crystals of the nematic type. Similarities of sickling to liquid crystal formation were recognized by earlier workers (*3, 4*) who noted the birefringence of deoxygenated Hb S in cells and in solution. Tranverse dimensions of filaments be-

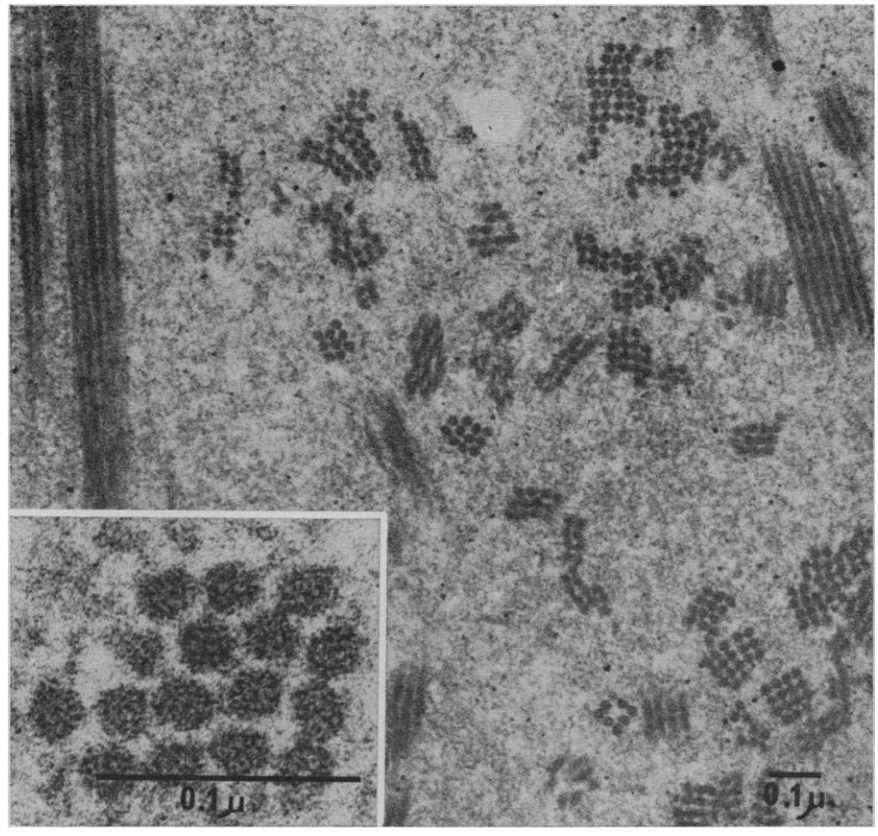

Fig. 1. Transmission electron micrograph of cell-free lysate, from Hb SS erythrocytes, sampled during deoxygenation. Samples were fixed first in cold 1 percent glutaraldehyde in 0.1*M* phosphate buffer (*p*H 7.4) for over 18 hours, and then in 1 percent OsO_4, and prepared for electron microscopy as described (*11*). Filaments lie parallel in bundles. No internal details (inset) are apparent (× 69,000; inset × 380,000).

Table 1. Change in composition of hemoglobin mixtures during phase-change from liquid to solid produced by deoxygenation. Numerals in brackets identify individual experiments. Hb F was determined by column chromatography on carboxymethyl Sephadex (*8*) to a precision of ± 0.2 percent (1 S.D.). As Hb F and Hb A_I subfractions elute together from CM Sephadex, Hb F in the presence of Hb A (experiment 5) was determined by alkali denaturation (*17*) to a precision of ± 0.7 percent (1 S.D.). Hemoglobins S, C, and A were determined by optical-density scanning (420 nm) of electrophoretic patterns on polyacrylamide (*9*) to a precision of ± 2 percent (1 S.D.), followed by appropriate subtractions of Hb F values. This calculation gives Hb A as Hb A_0, free of Hb A_I subfractions. Contributions by the minor hemoglobin A_2 have been neglected.

Hb S	Hb C	Hb S	Hb A_0	Hb F	Hb S	Hb F
Composition of solution before deoxygenation (%)						
[1] 45.9	50.8	[3] 47.7	43.5		[6] 94.1	5.9
[2] 44.2	54.0	[4] 46.7	42.1		[7] 91.5	8.5
		[5] 72.6	13.4	14.0	[8] 88.6	11.4
					[9] 80.3	19.7
Composition of solid phase produced by deoxygenation (%)						
[1] 64.7	35.3	[3] 68.2	29.4		[6] 98.7	1.3
[2] 62.0	36.6	[4] 71.4	26.7		[7] 98.9	1.1
		[5] 90.4	7.5	2.1	[8] 100.0	0.0
					[9] 97.7	2.3
Change in composition (%)						
[1] + 41.0	− 30.5	[3] + 43.0	− 32.4		[6] + 4.9	− 78.0
[2] + 40.3	− 32.2	[4] + 52.9	− 36.6		[7] + 8.1	− 87.1
		[5] + 24.5	− 44.0	−85.0	[8] + 12.9	−100.0
					[9] + 21.7	− 88.3

fore and after ultracentrifugation were identical.

Electron microscope sections of the liquid phase from centrifuged preparations were structureless: all solid-phase material was found in the sedimented pack. However, trapping of some liquid phase within packed solid phases was apparent both from observations of solid-phase consistency and from microscopy. Attempts to wash liquid phase out of solid phase were unsuccessful. We therefore determined extent of trapping through the use of either human serum albumin labeled with ^{131}I or human serum globulin labeled with ^{99m}Tc (*13, 14*). Added in trace amounts to hemoglobin solutions before deoxygenation, either of these labeled proteins serves as a marker for liquid phase, and the compositional analysis of packed solid phase can be accordingly adjusted to yield at least a close approximation of true solid. This technique was used to determine changes in composition during solid-state formation in various mixtures of Hb S with Hb C, Hb A, and Hb F (Table 1). Hemoglobins C and A were rejected from solid phase to a similar extent (experiments 1 through 4), and it is noteworthy that each comprised a significant proportion of solid phase. In contrast, rejection of Hb F was marked (experiments 6 through 9), regardless of its concentration in solution before deoxygenation. Experiment 5 provides a direct comparison of the behavior of Hb F and Hb A initially present at the same concentration; rejection of Hb F from solid phase widely exceeded that of Hb A. These observations now explain the well-known lack of interaction of Hb F with Hb S during the sickling phenomenon. The reason for this demonstrated exclusion of Hb F ($\alpha_2\gamma_2$) from solid phase presumably lies in the effect of γ chains on the structural conformation of deoxygenated Hb F molecules: because of configurational differences in regions of intermolecular contact, they cannot bind to (interact with) deoxygenated molecules of Hb S ($\alpha_2\beta^S{}_2$).

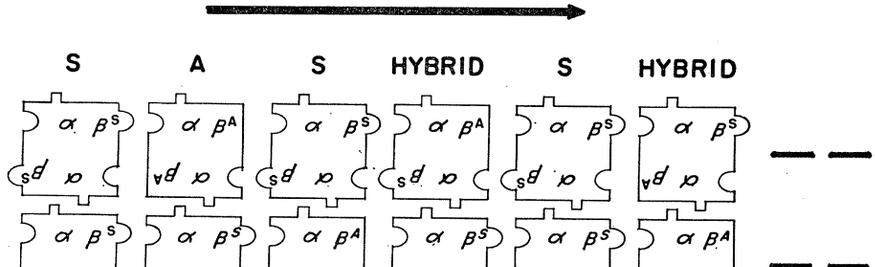

Fig. 2. Two-dimensional scheme of possible modes of interaction of Hb A (or Hb C) with Hb S in filamentous solid state composed predominantly of deoxygenated Hb S. Details are modified according to this text from previous proposals by Pauling *et al.* (*1*), Allison (*6*), Murayama (*16*), and Perutz and Lehmann (*15*). If β^S chains are primarily responsible for the complementary intermolecular fit between deoxygenated hemoglobin molecules, then attractions are generally strongest (and therefore filament formation would be initiated) when Hb S molecules interact. As filament growth proceeds (direction of arrow), molecules containing $\alpha\beta^A$ dimers participate, possibly alternating with Hb S molecules to give the proportions shown in Table 1. If as suggested (*16*) several strands entwine to form microscopically visible filaments (Fig. 1), then minor lateral forces would help stabilize single strands containing Hb A or, more likely, AS hybrids. Filaments would presumably terminate where intermolecular attractive forces are weakest—for example, where local concentrations of Hb S fall below critical levels. Therefore filament length should be (and is) greater in the absence of Hb A (or Hb C).

Transverse dimensions (140 to 175 Å) of individual filaments composing solid phase from each hemoglobin mixture, observed in electron micrographs of thin sections, were identical to those of filaments shown in Fig. 1. Although determinations of the mean length of filaments in sections are not significant, for filaments necessarily terminate at cut surfaces, approximate *maximum* length was greater in solid phase from mixtures of Hb S and Hb F (2.6 μm) than from mixtures of Hb S and Hb A (0.8 μm) or mixtures of Hb S and Hb C (0.4 μm).

Complementary fit between certain surface regions on deoxygenated Hb S molecules, as originally proposed by Pauling *et al.* (*1*), can account for filamentous solid phase composed of linear aggregates of Hb S molecules only. The scheme suggested by Perutz and Lehmann (*15*) depicts Hb AS hybrid molecules as possible terminators of these linear aggregates. Our observations that Hb A (or Hb C) can form a substantial proportion of solid phase (Table 1), and that fine structural characteristics remain identical to those of solid phase composed of Hb S alone, strongly suggest that Hb A molecules or Hb AS hybrids, though linking weakly to Hb S, take part in the linear array. If Murayama's model (*16*) has validity,

linear aggregates (strands or "monofilaments") entwine helically as cables: hence lateral forces between monofilaments would stabilize weak linkages within them. Figure 2 presents these concepts in schematic form.

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13. Human serum albumin and human serum globulin, two proteins grossly dissimilar in composition and configuration from human hemoglobin and therefore unlikely to enter solid phase, hypothetically should serve as markers of trapped liquid phase. We examined this possibility as follows. Either ^{131}I-albumin or ^{99m}Tc-globulin were added to various mixtures of Hb S with Hb C, Hb A, and Hb F. After deoxygenation and centrifugation of these mixtures, volumes and hemoglobin composition of each resulting liquid phase and packed solid phase were measured, and radioactivity in each phase was assayed in a well-type scintillation counter to within 3 percent probable error. Calculations of total hemoglobin content of true solid phase, based on the assumption that all radioactivity in packed solid represented trapped liquid, yielded a value of 580 mg/ml ± 71 (1 S.D.) ($n = 14$). The hemoglobin content of horse methemoglobin crystals, determined by Perutz by flotation on organic solvents immiscible with water, is approximately 640 mg/ml (*14*). The near agreement of these two figures serves to validate our assumption that these two nonhemoglobin proteins are excluded from solid phase and therefore can be used to provide a close estimate of the amount of liquid phase trapped in centrifuged solid phase. The slightly greater hemoglobin concentration of horse hemoglobin crystals may reflect a tighter packing of protein molecules in crystal lattices than in liquid crystals.
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Pion Cancer Therapy: Positron Activity as an Indicator of Depth-Dose

Abstract. *Negative pions are useful for radiation therapy of localized carcinoma because of their large end-of-range energy deposition upon capture in nuclei and the potentially accurate spatial localization of the radiation dose. Accurate determination of the depth of penetration of the pion beam in vivo can be accomplished by counting the back-to-back annihilation gamma rays resulting from positron activity induced by the stopped pions.*

The possibility of employing negative pions in cancer therapy was suggested as far back as 1961 (*1*). However, this application has not been practical owing to the lack of pion beams of suitable intensity. Recent advances in accelerator technology have made possible the construction of "meson factories" which will produce intense pion fluxes. Three such facilities are planned or under construction today: one in Switzerland, one in Canada, and one in the United States—the Los Alamos Meson Physics Facility for which a clinical facility has been proposed (*2*). Interest in this facility has caused a renewed interest in biomedical applications of negative pions.

One problem associated with any radiation therapy is the development of techniques to concentrate the dose in a prescribed volume. Even if the absorption properties of the radiation are known exactly, an accurate calculation of the dose in vivo is limited by individual variations in body geometry and composition. The use of pions in therapy holds promise of such precise localization that the usual uncertainty in depth-dose is significant. Furthermore, the complexity of pion capture permits new methods whereby the uncertainty of the dose may be reduced. As the pions are captured, many processes contribute to the highly localized dose, including a small amount of short-lived positron activity. The ensuing pairs of back-to-back annihilation gamma rays can be detected to pinpoint the dose position. We report here initial measurements of positron activity from negative pions stopping in water.

When a negative pion is captured by a nucleus, its rest mass (140 Mev) appears as energy which tends to fragment the nucleus ("star" production). Part of this energy goes toward overcoming nuclear binding, and the remainder appears as kinetic energy of the fragments. From calculations of the yield of various light elements from the capture of π^- by ^{16}O (*3*), we have estimated that about 1 percent of the "star" products will be positron unstable (primarily ^{11}C and ^{13}N).

With a 100-Mev negative pion beam (*4*) an experiment was performed to determine the positron yield from water as a function of depth. The data were obtained by irradiating 1.5-cm thick, gelatin-filled plastic petri dishes with a fixed pion flux and by counting the resulting activity for a fixed time with an NAI detector. Slabs of polyethylene were used to degrade the pion beam. The pion flux was measured by counting coincident pulses from two thin plastic scintillators placed in front of the degrader.

The resulting yield of positrons (corrected for activity due to the petri dish) as a function of depth in water is presented in Fig. 1. The range of 100-Mev pions in water is just over 25 cm. In the end-of-range peak, a plot of decay of activity shows that the primary components are ^{11}C and ^{13}N. These two products probably result from decomposition of the ^{16}O nucleus by capture:

$$^{16}\mathrm{O} + \pi^- \rightarrow {}^{13}\mathrm{N} + 3\mathrm{n} \qquad (1)$$

and

$$^{16}\mathrm{O} + \pi^- \rightarrow {}^{11}\mathrm{C} + 4\mathrm{n} + \mathrm{p} \qquad (2)$$

A decay curve of the entrance activity in addition to the ^{11}C and ^{13}N activity also shows a component due to ^{15}O which can only result from the reaction

$$^{16}\mathrm{O} + \pi^- \rightarrow {}^{15}\mathrm{O} + \pi^- + \mathrm{n} \qquad (3)$$

Part of the ^{11}C activity may be due to the gelatin by means of the reaction

$$^{12}\mathrm{C} + \pi^- \rightarrow {}^{11}\mathrm{C} + \pi^- + \mathrm{n} \qquad (4)$$