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Molecular Basis for the Anti-Sickling Activity of Aromatic Amino Acids and Related Compounds: A Proton Nuclear Magnetic Resonance Investigation†

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ABSTRACT: High-resolution proton nuclear magnetic resonance spectroscopy and relaxation techniques have been used to investigate the interactions of sickle cell hemoglobin (Hb S) and human normal adult hemoglobin (Hb A) with *p*-bromobenzyl alcohol, L-phenylalanine, L-tryptophan, and L-valine. With the exception of valine, all these compounds inhibit the polymerization of deoxy-Hb S [Noguchi, C. T., & Schechter, A. N. (1978) *Biochemistry 17*, 5455]. Using transferred nuclear Overhauser effects among the proton resonances of the compound of interest and the corresponding longitudinal relaxation rates (T_1^{-1}), we have shown that the binding of each of the compounds investigated to deoxy-Hb S is comparable to that to deoxy-Hb A. Intermolecular transferred nuclear Overhauser effects have been observed between proton resonances of the anti-sickling compounds and specific protons situated in the heme pockets of Hb. On the basis of these results, we suggest that one binding site, common to all compounds with anti-sickling activity, is at or near the heme pockets in the α and β chains of both deoxy-Hb S and deoxy-Hb A. The proton T_1^{-1} values of the histidyl residues situated over the surface of the hemoglobin molecule indicate that a second binding site is located at or near the β6 position, containing the mutation in Hb S (β6Glu → Val). The binding of the compounds investigated to the latter site induces conformational changes in the amino-terminal domains of the β chains. Our present NMR results support the following two mechanisms for the anti-sickling activity: (i) the allosteric mechanism, in which alterations in the local conformation(s) at intermolecular contact site(s) between Hb S molecules in the polymer occur as a result of binding of an anti-sickling compound to adjacent regions on the Hb S molecule; (ii) the competitive mechanism, in which the binding of an anti-sickling compound blocks one or more of the intermolecular contact sites between Hb S molecules in the polymer.

Sickle cell hemoglobin (Hb S)[1] is a mutant hemoglobin (β6Glu → Val) occurring in individuals with sickle cell anemia or sickle cell trait. In the deoxygenated form, under physiological conditions, Hb S molecules can polymerize into long fibers, which can then distort and rigidify the red blood cells. The polymerization of Hb S is the main molecular process responsible for the clinical manifestations in patients with sickle cell anemia. A large variety of compounds have been found to inhibit the polymerization of deoxy-Hb S either by reacting covalently to modify the Hb S molecule or by noncovalent interactions [for recent reviews, see Noguchi & Schechter (1985) and Schechter et al. (1985)]. Among the noncovalent agents, inhibition of polymerization has been demonstrated for aromatic amino acids (L-phenylalanine, L-tryptophan, and L-tyrosine) (Noguchi & Schechter, 1978), alkylureas (Elbaum et al., 1974), phenyl derivatives (Behe & Englander, 1979; Ross & Subramanian, 1977, 1978), benzyl esters of aromatic and hydrophobic amino acids (Gorecki et al., 1980a), and benzyloxy and phenoxy acids (Abraham et al., 1984). In addition, phenylalanine-containing di- and tripeptides and various oligopeptides have been shown to be able to inhibit Hb S polymerization (Noguchi & Schechter, 1978; Gorecki et al., 1980b; Franklin et al., 1983, Votano & Rich, 1985).

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[1] Abbreviations: Hb S, sickle cell hemoglobin; Hb A, human normal adult hemoglobin; Phe, L-phenylalanine; Trp, L-tryptophan; Tyr, L-tyrosine; His, L-histidine; CFA, 2-(*p*-chlorophenoxy)-2-methylpropionic acid; p-BrBzlOH, *p*-bromobenzyl alcohol; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; T_1^{-1}, longitudinal relaxation rate; NOE, nuclear Overhauser effect; TRNOE, transferred nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid.

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The molecular mechanisms by which these noncovalent anti-sickling agents inhibit the polymerization of Hb S have been under active investigation during the last decade. Quantitative relationships have been established between various chemical modifications of known agents and their potency to raise Hb S solubility and to lengthen the delay times in the polymerization process (Ross & Subramanian, 1977, 1978; Gorecki et al., 1980). The X-ray diffraction method has been recently used to characterize the stereochemistry of the binding sites of weak anti-sickling compounds to Hb (Abraham et al., 1983). For aromatic amino acids and for an anti-sickling dipeptide containing tyrosine, the X-ray diffraction studies failed to identify specific binding sites to human normal adult hemoglobin (Hb A) in either deoxy or CO form. A unique binding site for *p*-bromobenzyl alcohol (p-BrBzlOH) to HbCO A has been located by the X-ray diffraction method in the cleft between helices A and E, near the site of α14Trp. Alternative binding sites have been found for 2-(*p*-chlorophenoxy)-2-methylpropionic acid (CFA) in deoxy-Hb A in the internal cavity between the two α chains (Abraham et al., 1983). These X-ray diffraction studies have provided some information on the molecular mechanisms for anti-sickling activity and on the design of new anti-sickling drugs. Nevertheless, they are limited by the necessity of using Hb A instead of Hb S and by the experimental conditions necessary for growing Hb crystals [namely, 2.05–2.25 M phosphate buffer or 1.8–2.3 M $(NH_4)_2SO_4$ and, in certain cases, the addition of toluene]. Under these experimental conditions, the binding of the compounds investigated to Hb S, and thus their anti-sickling activity, can be greatly altered. One way to overcome these limitations of the X-ray diffraction method is to apply suitable spectroscopic methods to study the binding modes of potential anti-sickling agents to Hb in solution. Proton nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful experimental methods presently available to study the conformations of the Hb S and Hb A molecules in solution and the molecular events taking place during the polymerization of Hb S (Ho & Russu, 1978, 1981). In our laboratory, we have initiated an investigation by means of NMR spectroscopy of the surface conformations of Hb A and Hb S (Ho & Russu, 1978; Russu & Ho, 1980, 1982a) and of the specific molecular interactions between noncovalent anti-sickling agents and Hb S, aiming at characterizing the common features of the molecular mechanism(s) of action of these compounds.

In this work, we report our recent ^{1}H NMR findings on the binding modes of three anti-sickling compounds, L-phenylalanine (Phe), L-tryptophan (Trp), and p-BrBzlOH, and one amino acid with no anti-sickling activity, L-valine (Val) (Figure 1), to Hb A and Hb S. These compounds have been chosen as the first stage of our NMR investigation of potential anti-sickling agents because they represent simple model systems for this study and because their anti-sickling activity is well documented (Noguchi & Schechter, 1978; Behe & Englander, 1979; Abraham et al., 1983).

Materials and Methods

Materials. Hb A and Hb S were purified by the standard procedures used in our laboratory (Lindstrom & Ho, 1972; Russu & Ho, 1982a). The Hb solutions (3.0 mM on a dimer basis) in 0.05 M phosphate buffer plus 0.2 mM ethylenediaminetetraacetic acid (EDTA) in D_2O at pH 6.9 were exchanged 6 times with the corresponding solution of the anti-sickling compound in the same buffer by using ultrafiltration membrane cones (Centriflo, Amicon Corp.). The final concentrations of the anti-sickling compounds were 24 mM for Phe, Trp, and Val and 10 mM for p-BrBzlOH. The deoxygenation of the Hb samples was carried out as described previously (Lindstrom & Ho, 1972).

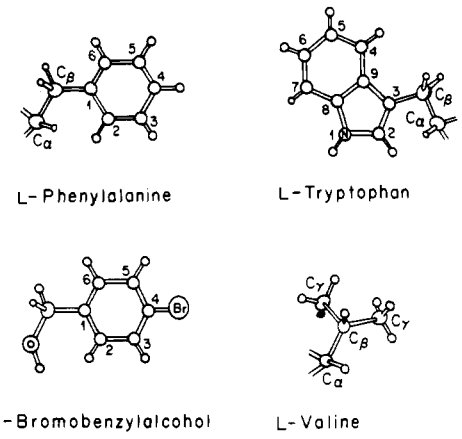

FIGURE 1: Anti-sickling compounds investigated in this work.

Methods. All ^{1}H NMR experiments were carried out on a Bruker WH-300 NMR spectrometer operating at 300 MHz and at 29 °C. The transferred nuclear Overhauser effect (TRNOE) measurements were carried out by using the following pulse sequence: $(t_1-t_2-\text{PW}-\text{AT}-t_3)_n$. The selective irradiation was applied during the time interval t_1; t_2 was a short delay before the acquisition pulse PW (t_2 = 2 ms); AT was the time required for data digitalization; t_3 was the relaxation delay, chosen as 5 times the longest T_1 in the spectrum. For the TRNOE experiments among proton resonances of the anti-sickling compounds, the t_1 delay was chosen such that the steady-state TRNOEs were observed. The t_1 values in the TRNOE measurements between proton resonances of Hb and of the anti-sickling compounds are given in the text. The data were collected in the interleaf mode as differences between the equilbrium spectrum (eight scans) and the spectrum with selective irradiation (eight scans). The total number of scans n for each experiment was 1600. In the experiments where only a single resonance was irradiated, the equilibrium spectrum was obtained by switching the irradiation frequency to a value remote from any proton resonance of Hb. In the experiments where more than one resonance was irradiated, the irradiation frequency was switched between the desired values at a rate adjusted according to the T_1 values of the irradiated resonances such that complete saturation was achieved during the first fifth of the time interval t_1. In these experiments, the equilibrium spectra were obtained by reducing the power of the irradiation pulse to less than 8×10^{-8} W. With this setting, no significant changes from the equilibrium intensities were observed.

The T_1^{-1} measurements were carried out by the nonselective inversion recovery method with the following pulse sequence: $[\pi-t-(\pi/2)-\text{AT}-t_2]_n$. The $\pi/2$ pulse was measured for each Hb sample and was found to be 5.5 μs. The total number of scans n for each experiment was 200. The relaxation delay t_2 was chosen $\sim$5 times the longest T_1 in the spectrum. The T_1^{-1} values were calculated by fitting the intensity of the corresponding resonance as a function of the time interval t to a simple exponential with a nonlinear least-squares computer program. The intensities of the His C2 proton resonance were measured as described previously (Russu & Ho, 1982b).

The ^{1}H chemical shifts are expressed as ppm with respect to HDO, which is 4.73 ppm downfield from the methyl protons of a water-soluble standard, the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The positive sign indicates that the observed resonance is downfield from that

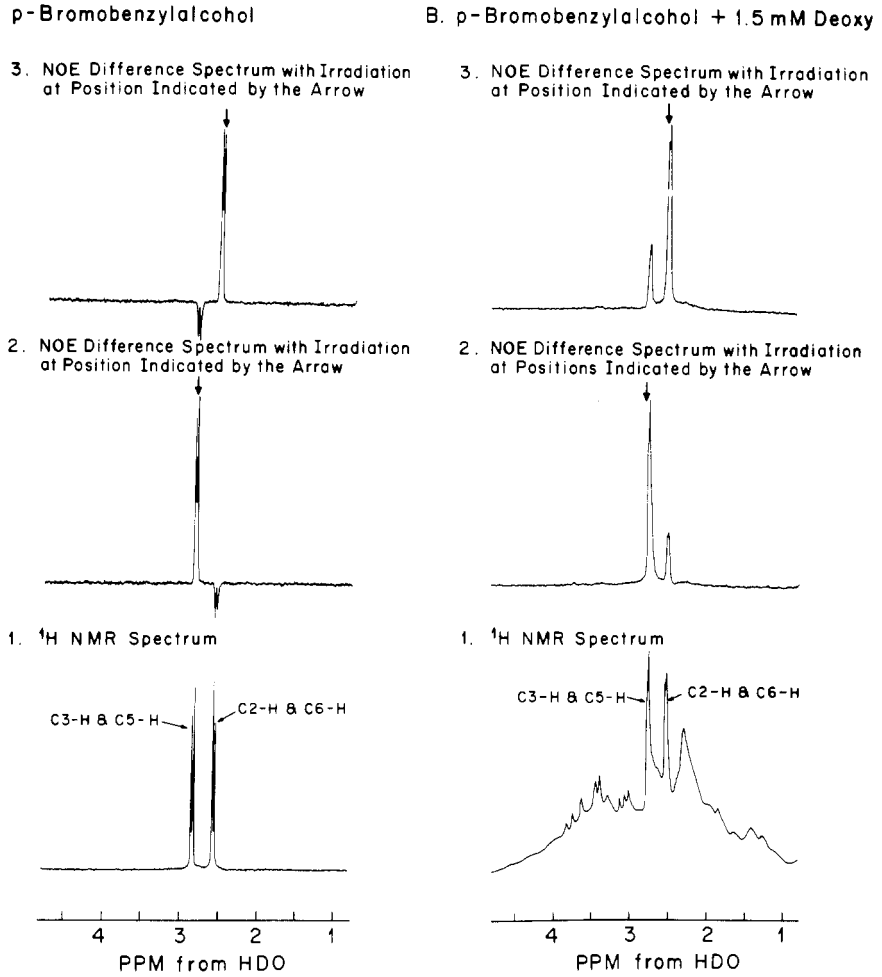

FIGURE 2: The 300-MHz ^{1}H NMR spectrum and TRNOE difference spectra of 10 mM p-BrBzlOH in 0.1 M phosphate buffer + 0.2 mM EDTA, at pH 6.9 and at 29 °C, in the absence and in the presence of deoxy-Hb S (3.0 mM on a dimer basis). The assignment of the aromatic proton resonances of p-BrBzlOH is shown. The NOE on the methylene protons of p-BrBzlOH is difficult to observe due to the position of this ^{1}H resonance close to that of water protons (−0.6 ppm upfield from HDO).

of HDO, and the negative sign indicates that the observed resonance is upfield from that of HDO.

Results

Transferred Nuclear Overhauser Effects among Resonances of the Anti-Sickling Compounds. In the presence of deoxy-Hb A or deoxy-Hb S, the ^{1}H NMR resonances of the anti-sickling compounds investigated occur at the same spectral positions as the corresponding resonances in pure solutions of the compounds. Hence, these resonances should originate from the fractions of the anti-sickling compounds that are free. The corresponding resonances from the compound molecules bound to Hb are very likely broadened or shifted, and thus, they are not resolved in the ^{1}H NMR spectra.

The TRNOE measurements among proton resonances of the anti-sickling compounds were carried out by irradiating one resonance or a set of resonances of a given compound and measuring the changes in the intensities of the remaining resonances at the steady-state values (Figure 2). The results obtained in deoxy-Hb S solutions are summarized in Table I, where, for each of the anti-sickling compounds investigated, they are compared with the NOEs observed in pure solutions. In deoxy-Hb A, the TRNOE values have been found to be, within experimental errors, the same as those in deoxy-Hb S. Control experiments have been carried out in order to ensure that the TRNOEs observed here do not originate from the simultaneous irradiation of the Hb resonances overlapping the proton resonances of the anti-sickling compounds. In these experiments, the irradiation frequency was placed 30–50 Hz away from proton resonances of the anti-sickling compound such that only the intensity of the envelope of broad Hb proton resonances was affected. No changes in the intensities of the proton resonances of the anti-sickling compounds have been observed, indicating that the TRNOEs reflect the exchange of magnetization between the free and the bound molecules of the anti-sickling compound.

Longitudinal Relaxation Rates of Protons of the Anti-Sickling Compounds. We have measured the proton T_1^{-1} values for each of the anti-sickling compounds investigated in pure solutions and in the presence of deoxy-Hb S or deoxy-Hb A. For each of the compounds investigated, the T_1^{-1} values in deoxy-Hb S solutions have been found to be the same as the corresponding values in deoxy-Hb A solutions. The results are summarized in Table II, where the T_1^{-1} values for deoxy-Hb A and deoxy-Hb S solutions are shown in a single column.

Transferred Nuclear Overhauser Effects between Proton Resonances of Hb and Proton Resonances of the Anti-Sickling Compounds. In deoxy-Hb, several hyperfine-shifted proton resonances occur over the spectral region 6–20 ppm downfield from HDO. These resonances originate from protons of the porphyrin structures and/or amino acid residues situated in the heme cavities of the α and the β chains. Each of the hyperfine-shifted resonances of deoxy-Hb A has been assigned by this laboratory to the α and the β chains of the molecule, as summarized in Figure 3 (Lindstrom & Ho, 1972; Takahashi

Table I: TRNOEs among Proton Resonances of the Anti-Sickling Compounds in Deoxy-Hb S Solutions in 0.05 M Phosphate Buffer plus 0.2 mM EDTA at pH 6.9 and at 29 °C and Corresponding NOEs in Pure Solutions of the Anti-Sickling Compounds

	p-Bromobenzyl Alcohol irradiated protons			
	C2-H and C6-H		C3-H and C5-H	
obsd protons	p-BrBzlOH	p-BrBzlOH + Hb S (3:1)	p-BrBzlOH	p-BrBzlOH + Hb S (3:1)
C2-H and C6-H			~17.0	~−25.0
C3-H and C5-H	~21.0	~−27.0		

	L-Phenylalanine irradiated protons					
	aromatic H		Cβ-H		Cα-H	
obsd protons	Phe	Phe + Hb S (8:1)	Phe	Phe + Hb S (8:1)	Phe	Phe + Hb S (8:1)
C2-H and C6-H			11.2	−7.9	2.3	−9.2
C3-H, C4-H, C5-H			−0.9	−7.9	0.0	−9.2
Cβ-H	5.3	0.0			0.0	−3.1
Cα-H	8.1	0.0	27.5	−12.9		

	L-Tryptophan irradiated protons							
	C4-H		C7-H		Cβ-H		Cα-H	
obsd protons	Trp	Trp + Hb S (8:1)	Trp	Trp + Hb S (8:1)	Trp	Trp + Hb S (8:1)	Trp	Trp + Hb S (8:1)
C4-H			~−2.0	~−3.8	11.0	−12.0	8.0	3.0
C7-H	~−1.0	~−5.2			0.0	0.0	0.0	−5.0
C2-H	~−2.0	~−1.8	~−3.1	~−4.0	19.0	−6.0	6.0	−1.0
C6-H	~−3.1	~−6.0	~16.0	~−15.0	0.0	−1.5	0.0	−4.0
C5-H	~18.2	~−13.0	~−3.0	~−6.8	−2.0	−2.0	0.0	−4.0
Cβ-H	3.0	−4.1	0.0	0.0			5.0	−7.0
Cα-H	8.0	−3.8	0.0	0.0	15.0	−26.0		

	L-Valine irradiated protons					
	Cγ-H		Cβ-H		Cα-H	
obsd protons	Val	Val + Hb S (8:1)	Val	Val + Hb S (8:1)	Val	Val + Hb S (8:1)
Cγ-H			3.1	−4.3	0.2	−0.5
Cβ-H	8.9	0.0			0.8	0.0
Cα-H	4.8	0.0	7.1	−3.5		

Table II: T_1^{-1} Values of Anti-Sickling Compounds in the Absence and Presence of Deoxy-Hb A or Deoxy-Hb S in 0.05 M Phosphate plus 0.2 mM EDTA at pH 6.9 and 29 °C

		T_1^{-1} (s^{-1})	
compd	resonance	compd alone	compd + 1.5 mM Hb
p-BrBzlOH (10 mM)	C2-H and C6-H	0.10	1.11
	C3-H and C5-H	0.12	1.10
Phe (24 mM)	aromatic H	0.32	0.59
	Cα-H	0.35	0.65
	Cβ-H	1.00	1.28
Trp (24 mM)	C4-H, C5-H, C6-H	0.40	0.72
	C7-H	0.20	0.72
	C2-H	0.20	1.02
	Cα-H	0.45	0.85
	Cβ-H	1.25	1.46
Val (24 mM)	Cα-H	0.26	0.32
	Cβ-H	0.37	0.65
	Cγ-H	0.75	1.75

Table III: TRNOEs between Anti-Sickling Compounds and Hb A or Hb S

	irradiation	
compd	ferrous hyperfine-shifted resonances of α and β chains in deoxy-Hb S or A	ring current shifted resonances of αE11- and βE11Val in HbCO S or A
p-BrBzlOH	present	absent
Phe	present	present
Trp	present	present
Val	absent	absent

et al., 1980). In this work, we have investigated the TRNOEs resulting from the irradiation of the β-chain hyperfine-shifted resonance at 17.8 ppm and/or the α-chain hyperfine-shifted resonance at 12.1 ppm. An illustration of the NOEs observed for a deoxy-Hb S solution in the presence of Phe is given in Figure 3. Upon irradiation of the α- and the β-chain hyperfine-shifted resonances, decreases in the intensity of the aromatic proton resonances of Phe are observed for the shortest time of irradiation (namely, 0.1 s, Figure 3). For longer irradiation times (such as 0.3 s), the intensity of the Phe Cα-H proton resonance is also decreased, but no changes in the intensity of the Phe Cβ-H proton resonances are observed. Similar results have been obtained upon the separate irradiation of the α- or the β-chain hyperfine-shifted resonances as shown in Figure 4. In this case, the TRNOE from the β-chain hyperfine-shifted resonance occurs at longer irradiation times than the corresponding NOE from the α-chain hyperfine-shifted resonance. The results of the TRNOE measurements in deoxy-Hb A and deoxy-Hb S solutions, in the presence of all the compounds investigated, are summarized in Table III.

An alternative marker for the heme pockets of the Hb molecule is provided by the resonance at −6.5 ppm from HDO in the ^{1}H NMR spectra of HbCO (Figure 5), which has been assigned by this laboratory to the γ_2-methyl group protons of the distal αE11- and βE11-valinyl residues (Lindstrom et al., 1972; Dalvit & Ho, 1985). We have carried out TRNOE experiments with irradiation of the αE11- and βE11-Val resonances at −6.5 ppm. The results are illustrated in Figure 5 for a HbCO S solution in the presence of Phe. Negative

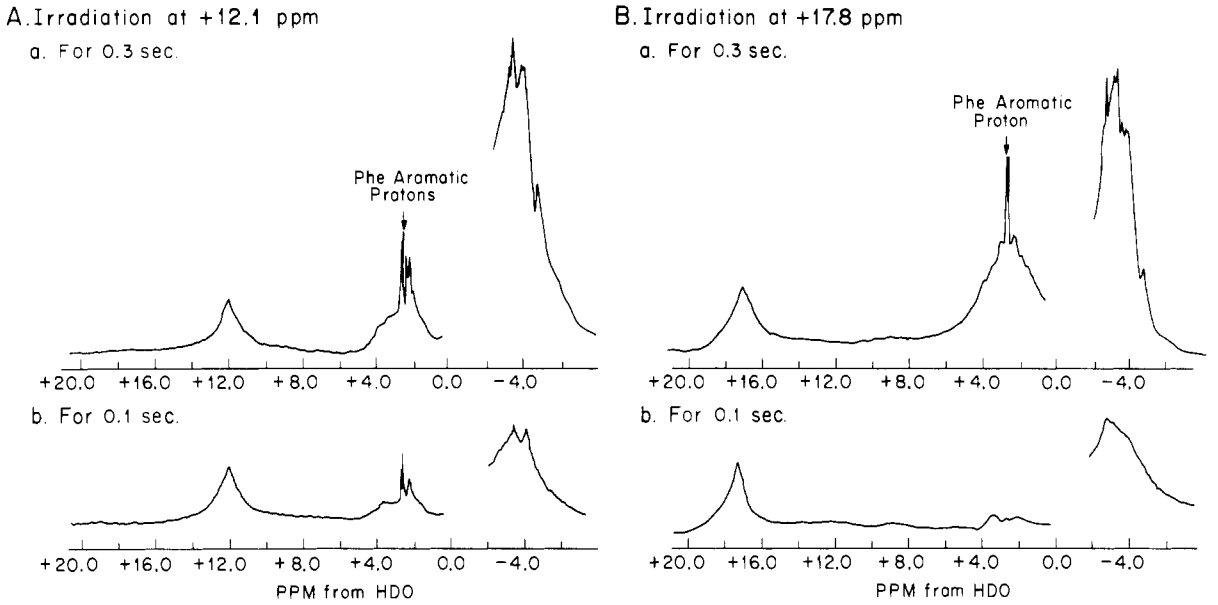

FIGURE 3: Difference spectra for the TRNOEs on free Phe resonances upon irradiation of the ferrous hyperfine-shifted resonances of the β (17.8 ppm) and the α (12.1 ppm) chains of deoxy-Hb S: Hb concentration 3.0 mM (on a dimer basis); total Phe concentration 24 mM in 0.05 M phosphate buffer + 0.2 mM EDTA at pH 6.9 and at 29 °C.

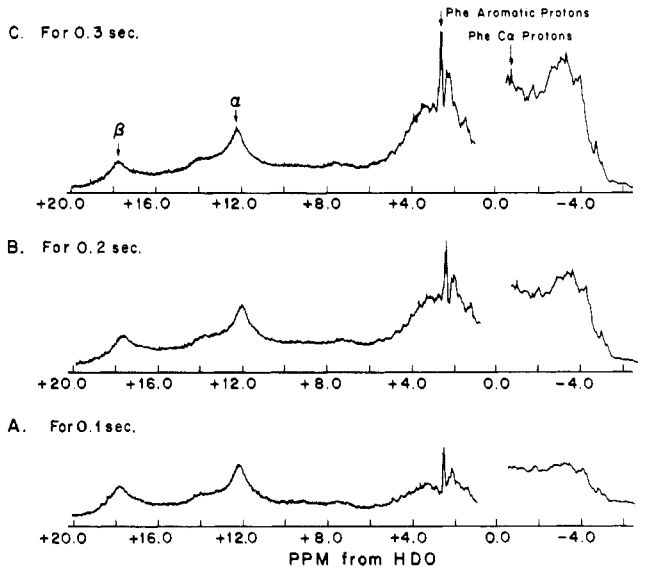

FIGURE 4: Difference spectra for the TRNOEs on free Phe resonances upon separate irradiation of the ferrous hyperfine-shifted resonance of the β chains (17.8 ppm) and ferrous hyperfine-shifted resonance of the α chains (12.1 ppm) of deoxy-Hb S: Hb concentration 3.0 mM (on a dimer basis); total Phe concentration 24 mM in 0.05 mM phosphate buffer + 0.2 mM EDTA at pH 6.5 and at 29 °C.

NOEs are observed for the remaining protons of the αE11- and βE11-Val residues (namely, Cα-H, Cβ-H, and γ_1-CH_3), for specific protons of the α- and β-porphyrins (such as the methyl groups at C1 and C8 positions and the meso protons δ), and for the aromatic proton resonances of Phe. Similar effects have been observed for the aromatic protons of Trp in HbCO A and HbCO S solutions, but the effects are absent in the HbCO solutions containing p-BrBzlOH or Val (Table III).

In order to assess the specificity of the observed TRNOEs, we have carried out two control experiments. In one experiment, the irradiation frequency was placed in the aliphatic proton resonance region, at various spectral positions between −2.5 and −4.2 ppm from HDO. No changes in the intensities of the proton resonances of the anti-sickling compounds were observed, indicating that the TRNOEs summarized in Table III are specific to protons situated in the heme pockets of the Hb A and Hb S molecules. A second experiment was aimed at comparing the time scale of the observed TRNOEs to that of the spin-diffusion process within the Hb molecule. We have measured the time development of the spin-diffusion process in deoxy-Hb and HbCO. The integrated intensities of the aromatic and the aliphatic proton resonances of Hb were followed as a function of the irradiation time of the heme

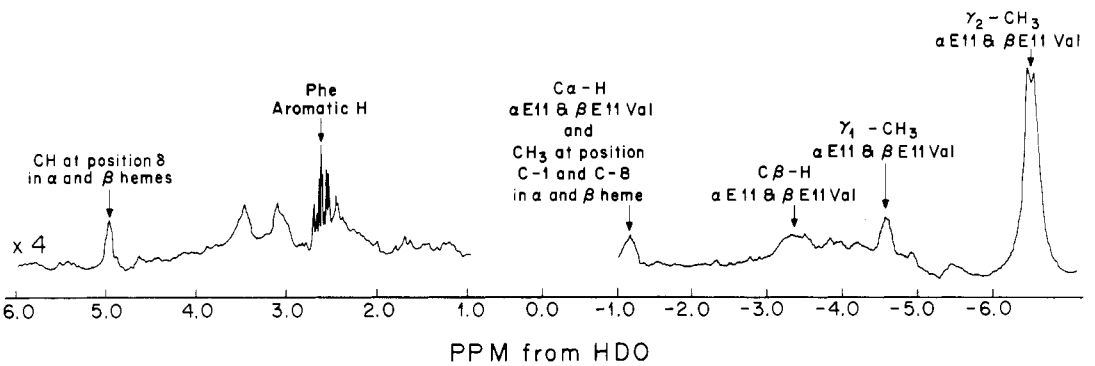

FIGURE 5: Difference spectrum for the TRNOEs on free Phe proton resonances upon irradiation of the γ_2-CH_3 resonances of the αE11- and βE11-Val of HbCO S: Hb concentration 3.0 mM (on a dimer basis); total Phe concentration 24.0 mM + 0.2 mM EDTA at pH 6.9 and at 29 °C. The irradiation time was 0.1 s.

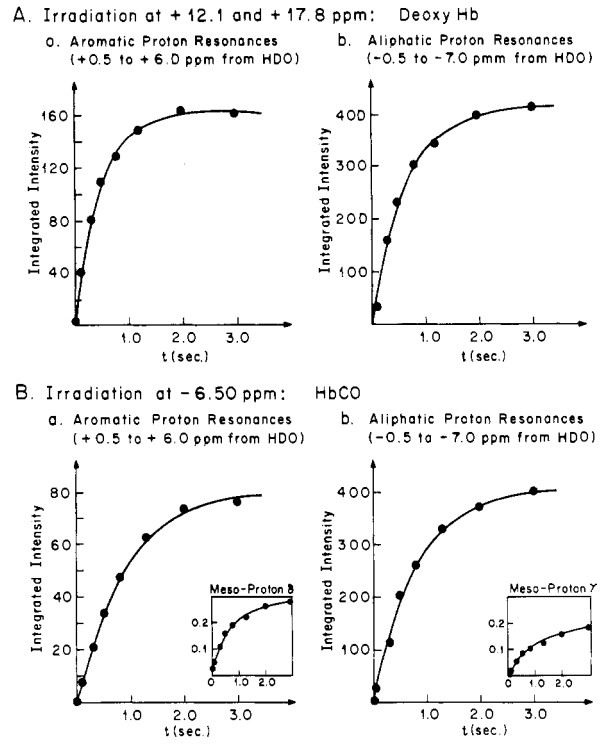

FIGURE 6: Time dependence of the intramolecular NOEs and of spin-diffusion in 3 mM Hb solutions in 0.05 M phosphate buffer in D_2O + 0.2 mM EDTA. (A) Irradiation of the β-chain (17.8 ppm) and α-chain (12.1 ppm) ferrous hyperfine-shifted resonances in deoxy-Hb. (B) Irradiation of the γ_2-CH_3 resonances of αE11- and βE11-Val in HbCO.

proton resonance, and the experimental data were fitted to a simple exponential equation. The results are shown in Figure 6. The following time constants for the spin-diffusion process have been found: 0.59 ± 0.04 and 0.46 ± 0.03 s for the aliphatic and the aromatic proton resonances, respectively, in deoxy-Hb (A or S); 0.78 ± 0.04 and 0.88 ± 0.04 s for the aliphatic and the aromatic proton resonances, respectively, in deoxy-Hb (A or S). The steady-state intensities in the spin-diffusion process have been found to correspond to 31.6 and 51.9% of the equilibrium-integrated intensity of the aromatic proton resonances in HbCO and deoxy-Hb, respectively, and to 22.9 and 13.9% of the equilibrium-integrated intensity of the aliphatic proton resonances in HbCO and deoxy-Hb, respectively. The insert in Figure 6B compares the time development of the spin-diffusion process within the Hb molecule to the NOE buildup between two specific pairs of Hb protons, namely, the NOEs on mesoprotons δ and γ of the porphyrin upon irradiation of the methyl proton resonances of E11-Val residues in α and β chains. The time constants corresponding to the buildup of these NOEs are 0.7 ± 0.1 s for the mesoproton δ and 0.9 ± 0.1 s for the mesoproton γ.

Longitudinal Relaxation Rate Measurements of the C2 Protons of Surface His Residues in Hb A and Hb S. The C2 proton resonances of the 22 surface His residues (11 His residues/$\alpha\beta$ dimer) of deoxy-Hb A and deoxy-Hb S that can be individually observed in the aromatic proton resonance region of the ^{1}H NMR spectra (1.0–5.0 ppm downfield from HDO) are labeled 1–10 in Figure 7 as in our previous publications (Russu et al., 1982; Russu & Ho, 1982a). The currently available assignments of these resonances to specific His residues of Hb are also shown.

We have measured the T_1^{-1} values for the 22 His C2 protons of deoxy-Hb S and deoxy-Hb A in the presence of all the anti-sickling compounds investigated. The results indicate that,

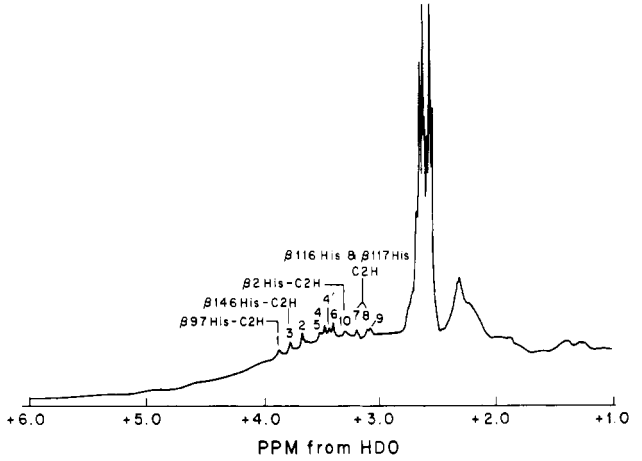

FIGURE 7: The 300-MHz aromatic proton resonances of 3 mM deoxy-Hb S (on a dimer basis) in 0.05 M phosphate buffer in D_2O + 0.2 mM EDTA in the presence of 24 mM Phe at pH 6.9 and at 29 °C. The surface His C2 proton resonances are labeled 1–10, and the presently available assignments to specific His residues are shown [from Russu et al. (1982)].

Table IV: Effects of Anti-Sickling Compounds on the T_1^{-1} of C2 Protons of β2His of Deoxy-Hb A and Deoxy-Hb S in 0.05 M Phosphate plus 0.2 mM EDTA at pH 6.9 and 29 °C

	T_1^{-1} (s^{-1})	
sample	Hb A	Hb S
Hb	1.54	1.85
Hb + Phe (1:8)	1.06	3.45
Hb + p-BrBzlOH (1:3)	2.56	3.34
Hb + Trp (1:8)	2.13	4.00
Hb + Val (1:8)	2.38	1.37

with only one exception, the T_1^{-1} values of all the 22 His C2 protons are not affected by the presence of the anti-sickling compounds, in either deoxy-Hb A or deoxy-Hb S. The only change in the T_1^{-1} value was observed for the β2His C2 protons.[2] The results are summarized in Table IV. The chemical shifts of the 22 His C2 proton resonances of deoxy-Hb A and deoxy-Hb S were found to be unaffected by the presence of the anti-sickling compounds investigated except for a slight upfield shift of ~0.1 ppm for the resonances labeled 4 and 5 in the presence of Trp and p-BrBzlOH.

DISCUSSION

Longitudinal Relaxation Rates and Transferred Nuclear Overhauser Effects among Resonances of the Anti-Sickling Compounds. The T_1^{-1} values of the protons of the anti-sickling compounds are increased by the same amount in the presence of deoxy-Hb S or deoxy-Hb A (Table II). These enhancements are a direct indication that the anti-sickling compounds investigated bind, to a comparable extent, to both deoxy-Hb A and deoxy-Hb S.

Further evidence for the similar binding of the anti-sickling compounds to deoxy-Hb S and deoxy-Hb A is provided by the

[2] During the course of these measurements, we have observed that the T_1^{-1} values of the β2His C2 protons in deoxy-Hb solutions in phosphate buffer are very sensitive to the presence of small amounts of metal ion impurities in the samples. This effect results from the following facts: (i) the large affinity of the inorganic phosphate ions for metal ions; (ii) the specific binding of inorganic phosphate to the β2His residues of deoxy-Hb (I. M. Russu, A. K.-L. C. Lin, and C. Ho, unpublished results). To eliminate this effect, we have added 0.2 mM EDTA to our samples. This EDTA concentration was found to reduce the line width of the ^{31}P NMR resonance of inorganic phosphate ions from 40 to 0.36 Hz and to increase its T_1 value from 1.0 to 16.4 s. These results indicate that, in the presence of 0.2 mM EDTA, the inorganic phosphate ions are free of metal ions and, thus, the paramagnetic effect of these metal ions upon the T_1^{-1} values of the β2His C2 protons should be largely eliminated.

TRNOE results shown in Table I. All of the positive NOEs among protons of an anti-sickling compound change, in the Hb solutions, to negative values. These effects originate from the chemical exchange between molecules bound to Hb (a situation in which the proton–proton NOEs are negative with an extreme value of −1) and free molecules characterized by very short correlation times (a situation in which proton–proton NOEs are positive with a maximum value of $^1/_2$). The small negative NOEs observed for pure solutions of the anti-sickling compounds (Table I) result from the presence in the molecule of more than two spins (Noggle & Schirmer, 1971). In these cases, the TRNOEs produce further decreases in the observed NOEs. The TRNOEs are, within experimental error, the same in deoxy-Hb S and in deoxy-Hb A.

The observation of negative TRNOEs for the free molecules of anti-sickling compounds implies that the chemical exchange rates between the free and the bound states (k_1, k_{-1}) are intermediate or fast on the spin–lattice relaxation scale of the free ligand [i.e., k_1, $k_{-1} \geq 10\delta_F$, or k_1, $k_{-1} \gg 10^3\delta_f$, where δ_f is the spin–lattice relaxation of a given proton in the free state (Clore & Gronenborn, 1982)]. The T_1^{-1} values of the anti-sickling compounds in the free state range from 0.1 to 1.2 s^{-1} (Table II). Accordingly, for a negative TRNOE to be observed, the lifetimes of the ligand molecules in the free and the bound states, τ_f and τ_b, respectively, should be τ_f, $\tau_b \leq 0.1$ to 1 s. This condition imposes a lower limit for the rates of the on/off binding reaction of the anti-sickling compounds studied. An upper limit for these rates can be inferred from the fact that the chemical exchange between free and bound states is slow on the chemical shift scale (for example, for a minimum chemical shift difference between the proton resonances of free and bound ligand of 0.5 ppm at 300 MHz, τ_b, $\tau_f > 10^{-3}$ s). Hence, we suggest that the most probable ranges for the lifetimes of the anti-sickling compounds investigated in the bound and the free states are from 1 to 0.01 s. This estimation may prove useful for relating the kinetics of the binding process of the anti-sickling compounds to the kinetics of the deoxy-Hb S polymerization.

Transferred Nuclear Overhauser Effects between Proton Resonances of Hb and Proton Resonances of the Anti-Sickling Compounds: Binding Sites of the Anti-Sickling Compounds Investigated to Hb. We have observed intermolecular TRNOEs between the free Phe, Trp, and p-BrBzlOH resonances and the ferrous hyperfine-shifted resonances of the α and β chains in deoxy-Hb S and deoxy-Hb A. In HbCO solutions, similar TRNOEs have been detected between the aromatic resonances of Phe and Trp and the γ_2-methyl protons of the αE11- and βE11-Val residues (Table III).

The intermolecular TRNOEs observed in the present work reflect the following two processes: (i) the transfer of magnetization from the saturated heme protons to the protons of ligand molecules bound to Hb via proton–proton cross-relaxation within the Hb–ligand complex; (ii) the transfer of magnetization between the bound and the free ligand molecules via a chemical exchange process. The transfer of magnetization via cross-relaxation within the Hb–ligand complex results, at shorter irradiation times, in specific first-order NOEs between the irradiated heme protons and the protons situated in their close proximity (Dalvit & Ho, 1985). At longer irradiation times, the transfers of magnetization by cross-relaxation develop into a generalized intramolecular spin-diffusion effect. The intermolecular TRNOEs observed here occur for irradiation times as short as 0.1 s (Figures 3–5). The extent of spin-diffusion within the Hb molecule during these irradiation times is 12–15% from the attainable steady-state values (Figure 6). This finding indicates that within the time ranges of our present experiments the intramolecular transfers of magnetization are dominated by first- and second-order cross-relaxation transitions between protons in close proximity. Therefore, we can conclude that the ligand molecules in the bound state must be situated in close proximity to the heme pockets of Hb A and Hb S. The transfer of magnetization between bound and free ligand molecules occurs via a chemical exchange process. The TRNOEs among proton resonances of the anti-sickling compounds have indicated that the rate constants for this process exceed the relaxation rates of protons of anti-sickling compounds in the free state. Consequently, the transfer of saturation from the bound to the free ligand resonances should be very effective (Forsen & Hoffman, 1963), as observed experimentally.

In conclusion, the intermolecular TRNOEs observed in this work indicate that one binding site for Phe, Trp, and p-BrBzlOH to deoxy-Hb A and deoxy-Hb S is at or near the heme pockets of the α and β chains in the Hb molecule (Table III). A similar binding site is also present in HbCO A and HbCO S for Phe and Trp but appears to be absent for p-BrBzlOH (Table II). The exact location and the detailed stereochemistry of these binding sites are not yet known. However, the X-ray diffraction studies of deoxy-Hb S crystals indicate that, in the double-strand structure, one lateral contact region consists of a hydrophobic pocket at the corner between the E and F helices of the β chains (Wishner et al., 1975, 1976). The EF corner is situated just outside the heme cavity, and its hydrophobic character is conferred by the groups on the exterior edge of the porphyrin and by a cluster of amino acid residues such as β85Phe, β88Leu, and β91Leu. These hydrophobic pockets in the β-chain hemes have been proposed by Ross & Subramanian (1978) as a main site of intermolecular interaction involved in anti-sickling activity. The binding sites identified in this NMR work as being at or near the heme pockets provide support to this model. Further support has been provided by Johnson & Zeidan (1983), who found that the binding site of Phe to HbCO A and HbCO S is located within 7–13 Å from the unpaired electron of the spin-label at the β93 position.

The binding of an anti-sickling compound at or near the heme pockets raises an important question about the functional integrity of the Hb molecule under these conditions. We have found that the oxygen dissociation curves of Hb A and Hb S solutions in the presence of all the anti-sickling compounds investigated are identical with those in the absence of the anti-sickling compounds [corresponding $p_{1/2} = 15.0 \pm 0.5$ mmHg in 0.05 M phosphate buffer at pH 6.9 and at 29 °C (results not shown)]. These results suggest that the local stereochemistry of the binding sites of the anti-sickling compounds near the heme pockets is such that the bound ligand molecules do not interfere with the molecular mechanism or pathways for the oxygen binding. Thus, under these experimental conditions, the functional integrity of Hb A and Hb S appears to be preserved.

Longitudinal Relaxation Rates of the Surface His C2 Protons of Deoxy-Hb S and Deoxy-Hb A. Among the 22 His residues situated over the surface of the Hb molecule, the T_1^{-1} value of a single His residue, namely, β2His, is affected by the presence of the anti-sickling compounds studied (Table IV). The extent of this effect is specific to deoxy-Hb S or deoxy-Hb A and to each of the compounds investigated. In deoxy-Hb S, the compounds with anti-sickling activity induce an enhancement in the T_1^{-1} values of the β2His C2 protons. On the other hand, valine, a compound that does not have

significant anti-sickling activity, induces a decrease in the T_1^{-1} value of the β2His C2 protons.

The longitudinal relaxation of the β2His C2 protons is mainly determined by their dipolar interactions with protons of adjacent methyl and methylene groups, namely, those of β1Val, β3Leu, β4Thr, and β132Lys (Russu & Ho, 1982b). In the absence of the anti-sickling compounds investigated, the T_1^{-1} value of the β2His C2 protons in deoxy-Hb S is larger than the corresponding value in deoxy-Hb A [Table IV and Russu & Ho (1980)]. This difference clearly reflects the existence of a different conformation for the polypeptide chain around the β6 mutation site in deoxy-Hb S even in the absence of the anti-sickling compounds. The T_1^{-1} values obtained in the present work for the β2His C2 protons (Table IV) indicate that all the anti-sickling compounds investigated perturb the relative distances of the β2His C2 protons to the protons of neighboring groups. This finding suggests that all the compounds investigated interact with the deoxy-Hb molecule (both Hb S and Hb A) at a site within the amino-terminal regions of the β chains. As a result of this interaction, the conformational differences between deoxy-Hb S and deoxy-Hb A at these regions are enhanced (Table IV). Our present suggestion on the existence of an anti-sickling binding site within the amino-terminal regions of the β chains is in excellent agreement with the recent finding that the binding of a spin-labeled Phe analogue to Hb S broadens the C2 proton resonances of β2His and β146His (Lu et al., 1984). One possible site for the interaction between anti-sickling compounds and the amino-terminal regions of the β chains may consist of the β-terminal hydrophobic pocket of Hb. This pocket is formed by the A and H helices and the amino-terminal fragment, NA (residues β1 to β4), which closes it like an arm. Its hydrophobic character is conferred by the following residues: β3(NA3)Leu, β11(A8)Val, β133(H11)Val, β130(H8)Tyr, β126(H7)Ala, and β78(EF2)Leu. Inspection of the molecular model of the deoxy-Hb molecule indicates that the binding of an anti-sickling compound into this pocket would certainly perturb the local conformations at the β6 mutation sites. Such a conformational perturbation could play an important role in inhibiting the polymerization process since one of the β6 sites of the Hb S molecule provides a key lateral contact for the fiber formation (Wishner et al., 1975, 1976). This molecular model awaits further experimental verification. At the present stage, our ^{1}H NMR results indicate that the anti-sickling compounds investigated interact with the Hb molecule at a site close to the β2His and to the β6Glu (or Val) residues. This suggests that one molecular mechanism involved in the anti-sickling effect of the compounds investigated consists of well-defined alterations in the local conformation around intermolecular contact sites induced by the specific binding of an anti-sickling compound.

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Registry No. Hb S, 9035-22-7; *p*-$BrC_6H_4CH_2OH$, 873-75-6; L-phenylalanine, 63-91-2; L-tryptophan, 73-22-3; L-valine, 72-18-4.

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