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Spin Equilibria in Human Methemoglobin: Effects of Bezafibrate and Inositol Hexaphosphate As Measured by Susceptometry and Visible Spectroscopy

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ABSTRACT: The effects of inositol hexaphosphate (IHP) and a second allosteric effector, bezafibrate, on the spin-state equilibria of the mixed-spin derivatives of ferric human hemoglobin A are examined. Changes in spin-state equilibrium are monitored by measuring absorption spectra in the visible region (460–700 nm) as well as by direct measurements of magnetic susceptibility by means of a superconducting fluxmeter. The addition of IHP at pH 6.5 results in a measurable shift in the spin equilibria of these derivatives toward higher spin. However, the addition of bezafibrate in the presence of IHP results in still larger shifts toward the high-spin form. The changes in the free energies of the spin-state equilibria resulting from the combination of these two effectors are similar in magnitude to that which results from the R-state to T-state transition in carp hemoglobin.

The origin or mechanism of the ligand-linked changes in the ligand affinity of hemoglobin remains a topic of exploration and controversy. Perutz (1972) postulated a linkage between the ligand-induced movement of the proximal histidine residue with respect to the heme plane and the conformational equilibrium of the protein. Within the framework of the two-state model of Monod, Wyman, and Changeux (1965), the movement of this histidine toward the heme plane is resisted in the low-affinity T state while occurring with greater facility in the high-affinity R state of the protein. This of course results in a thermodynamic linked function (Wyman, 1964) between ligand binding and protein conformation.

Tests of this hypothesis have given mixed results. Rousseau et al. (1984) examined the resonance Raman spectra of the heme regions of ligand-saturated R and T states of hemoglobin and found no evidence for differences in bond energies to account for their differences in ligand affinity. On the other hand, Nagai and Kitagawa (1980) demonstrated a clear difference in the stretching frequencies of the iron–proximal histidine bond in deoxygenated high- and low-affinity states of hemoglobin. Studies on a wide variety of hemoglobins have demonstrated a correlation between this iron–histidine stretching frequency and ligand affinity (Friedman, 1985). However, the actual changes in bond energy indicated by these frequency shifts are small compared to the associated changes in the free energy of ligand binding (Ondrias et al., 1982).

An alternative basis for an affinity change could be a steric resistance to the positioning of the ligand at the heme iron on the distal side of the heme. However, in examining the binding of the family of isonitriles to hemoglobin, Mims et al. (1983) and Lin et al. (1988) did not find the relationship between ligand size and affinity difference that would be expected if distal, steric effects contributed to the affinity differences between R and T states.

A relationship exists between the strength of the bond to the dissociable ligand, the position of the iron atom with respect to the heme plane, and the energy splitting of the d orbitals of the iron atoms. All ligands that bind to the reduced, ferrous form of hemoglobin are strong-field ligands and result in a fully low spin electronic distribution of the iron atoms (Philo et al., 1984; Sawicki et al., 1984; Cerdonio et al., 1985). However, ligands that bind to the ferric form of hemoglobin vary enormously in field strength. They result in a full range of energy splittings, and many are characterized by measurable equilibria between high-spin and low-spin states (Hoard, 1975). Perutz's model suggests that the T state should favor and be favored by the high-spin state of the iron while the R state should favor low-spin forms.

As demonstrated by Tan and Noble (1973) liganded derivatives of carp hemoglobin can be reversibly converted from the R to the T quaternary state with ease by the addition of inositol hexaphosphate (IHP) and adjustment of the pH. By use of this model system a relationship between protein conformation and spin-state equilibrium was demonstrated by Perutz et al. (1978) and Messana et al. (1978) and subsequently quantitated at room temperature and shown to exhibit an average linkage free energy of approximately 700 cal/mol of ligand (Noble et al., 1987; Henry et al., 1985).

Demonstration of a quantitatively similar linkage relationship in human hemoglobin has proven to be more difficult. Ligand-saturated ferrous derivatives of human hemoglobin cannot be converted to their T states by adjustment of pH and addition of organic phosphate the way that carp hemoglobin can. However, Perutz et al. (1974) obtained a variety of

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evidence suggesting that ferric hemoglobin derivatives which are predominantly of high spin can be converted to their T states by the addition of IHP at about pH 6.5. The fact that this did not occur for low-spin derivatives established a qualitative linkage between the electronic configuration of the iron atom and the structural state of the protein. However, quantitation of this relationship by direct measurement of magnetic susceptibility by Philo and Dreyer (1985) demonstrated that IHP addition is linked to the spin equilibrium of the high-spin forms of human hemoglobin by a free energy which is at most half that observed with carp hemoglobin and for some derivatives virtually zero. Thus, the generality of this linkage relationship was brought into question.

In examining critically the evidence that IHP converts high-spin derivatives of human hemoglobin into their T states, one is struck by the persuasiveness of the evidence for a conformational change. However, the evidence that the IHP-induced state is truly the quaternary T state associated with deoxygenation is lacking, as is the evidence that the conformational transition is complete. This is especially true in light of recent evidence of the inadequacy of the two-state model and the need to invoke the occurrence of intermediate functional states in the process of sequentially binding four ligands to the hemoglobin tetramer (Ackers & Smith, 1986; DiCera et al., 1987).

Perutz and Poyart (1983) reported that the compound bezafibrate (BZF) binds preferentially to the deoxygenated hemoglobin molecule and thereby lowers the ligand affinity. Perutz et al. (1986) subsequently showed it to bind in the central cavity of the tetramer without interfering with the organic phosphate binding site. This explained the observation of Perutz and Poyart that the effects of IHP and bezafibrate on oxygen affinity are synergistic. The question then became, can the two effectors in combination produce larger changes in the structures and spin equilibria of derivatives of human ferric hemoglobin than are caused by IHP alone?

We have explored the effects of these two allosteric effectors on spectral and magnetic properties of several derivatives of human ferric hemoglobin.

Experimental Procedures

Magnetic susceptibilities were measured with a superconducting fluxmeter as previously described (Cerdonio et al., 1981; Vitale et al., 1982; Noble et al., 1987). The gram diamagnetic susceptibility, χ_g, of protein was taken to be -0.587×10^{-6} mL/g as measured previously by us (Cerdonio et al., 1985).

This apparatus yields measurements with an overall accuracy of 0.03% of the susceptibility of water at room temperature. At the heme concentration used in this study, this results in an error in the estimate of the paramagnetic contribution to the overall susceptibility of between 0.5 and 1%. The principle error in the determination of molar paramagnetic susceptibility lies in the measurement of protein concentration. This was measured in triplicate for each sample with an estimated standard error of 2%. In the experiments reported here the effects of additives on the paramagnetic susceptibility of any one hemoglobin derivative were measured with a single stock hemoglobin solution. In addition, the experiments were designed so that small volumes of reagents were added to a much larger volume of hemoglobin. As a result, the variations in hemoglobin concentration within such a set of measurements was considerably less than the 2% uncertainty in the estimate of the absolute concentration and are in fact small relative to the error of the estimate of the volume paramagnetic susceptibility.

Visible spectra were recorded with an OLIS conversion of a Cary Model 14 spectrophotometer connected on-line to an OLIS 3820Z data acquisition and instrument control system (OLIS Inc., Rt. 2, Jefferson GA 30549). For the spectral studies the final hemoglobin concentration was 0.65 mM in heme equivalents. Spectra were recorded from 700 to 460 nm at 20 °C by using a 2 mm path length cuvette. All measurements at a single pH for a particular derivative were carried out by making identical additions of water or stock solutions of allosteric effectors to aliquots of the same stock hemoglobin solution, thus minimizing dilution errors.

Ferric human adult hemoglobin (HbA) was prepared as described by DiIorio (1981). For susceptibility measurements buffer was prepared at high enough concentration that the addition of 0.1 volume or less to deionized HbA yielded the desired final buffer chloride concentration. Buffer was prepared by adding the basic form of Bis-Tris (pH 7.0 and below) or Tris (above pH 7.0) to an amount of HCl equal to the total chloride ion needed. In all experimental samples the final chloride ion concentration was 100 mM. All ligand concentrations were 100 mM except formate, which was 1 M.

Inositol hexaphosphate (IHP) was obtained as the sodium salt. The pH of this stock solution was adjusted by means of the acid form of Amberlite IR-120 to avoid the addition of extraneous anions to the sample. The amount of IHP added to each sample was 1 mM in excess of the concentration of hemoglobin tetramers.

Bezafibrate (BZF) [2-[4-[2-(*p*-chlorobenzamido)ethyl]-phenoxy]-2-methylpropionic acid] was obtained from Boehringer-Mannheim Corp. On the basis of a molecular weight of 361 g, a 250 mM stock solution was prepared by adding 0.181 g to 1.0 mL of H_2O. With vigorous stirring, 1 N NaOH was added until the BZF was dissolved. This solution was then carefully back-titrated with 0.2 N HCl to a final pH between 9.2 and 9.7. The volume was then adjusted to 2 mL. The final concentration of BZF in all experimental samples was 5 mM.

Results

Unfortunately, bezafibrate absorbs UV radiation, and its presence precludes the precise determination of the spectrum of the hemoglobin molecule in the region of the aromatic amino acids. For this reason the spectral studies reported here are limited to the visible region of the heme spectrum from 460 to 700 nm.

In Figure 1 are presented the spectra of the nitrite derivative of human hemoglobin at pH 6.5 in Bis-Tris buffer along with the spectra obtained when bezafibrate, IHP, and a combination of these two effectors were added to the buffer. Both of the effectors alter the spectrum of this hemoglobin derivative, the effect of bezafibrate being the smaller. However, in combination these effectors cause a much larger spectral change than either alone. If one compares the spectra of high- and low-spin derivatives of hemoglobin, it is clear that the spectral band near 630 nm is indicative of the high-spin forms, while those between 540 and about 570 nm are indicative of the low-spin forms. Choosing the wavelengths of maximum absorbance change, we have computed the overall spectral change by the function

$$\Delta\Delta OD = (OD_{HS} - OD_{LS})_{effectors} - (OD_{HS} - OD_{LS})_0$$

As shown in Figure 2, for the nitrite derivatives this becomes

$$\Delta\Delta OD = (OD_{635nm} - OD_{549nm})_{effectors} - (OD_{635nm} - OD_{549nm})_0$$

where the subscript zero refers to the absence of the effectors. By use of this function the pH dependencies of these effects

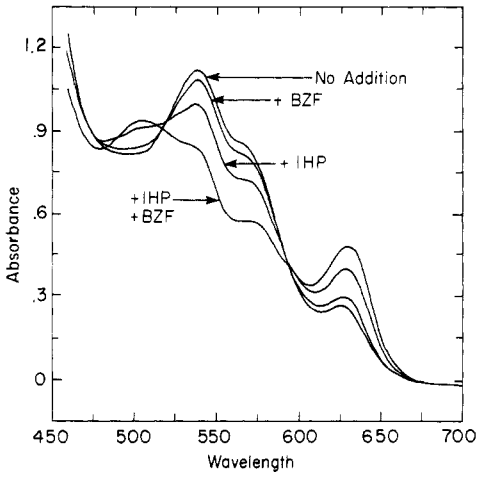

FIGURE 1: Absorption spectra of the nitrite derivative of human ferric hemoglobin at pH 6.5 in the absence of effectors, in the presence of 5 mM BZF, in the presence of 1 mM IHP, and in the presence of 1 mM IHP plus 5 mM BZF. The hemoglobin concentration was 0.65 mM in heme equivalents, and spectra were recorded at 20 °C by using a 2 mm path length cuvette.

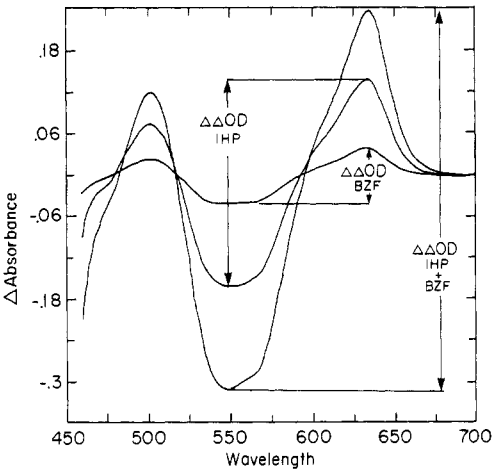

FIGURE 2: Difference absorption spectra obtained from the data in Figure 1 by subtracting the spectrum of the nitrite derivative of human hemoglobin in the absence of effectors from spectra obtained in their presence. The graphical origins of the ΔΔOD (ΔΔ absorbance) terms in Figure 3 and Table I are depicted in this figure.

were determined as shown in Figure 3, again for the nitrite derivative. Above pH 8 no significant effects are seen, and at pH 7.5 they remain small. The effects of IHP and the combination of IHP plus bezafibrate are greatest around pH 6.5. In contrast, the smaller effect of bezafibrate alone continues to increase as the pH is lowered.

Philo and Dreyer (1985) reported that the nitrite derivative of human hemoglobin was unstable in their hands. To control for the possibility of time-dependent changes, spectra of solutions of this derivative were examined as a function of time. This was done at two hemoglobin concentrations, 6.2 mM in heme equivalents, to simulate the condition of the susceptibility measurements, and 0.62 mM, the concentration used for the rest of these spectroscopic studies. At the high hemoglobin concentration no spectral changes were observed at 20 °C over a period of 1.5 h. This is a much longer time than is required for a measurement of magnetic susceptibility. In contrast, at the lower hemoglobin concentration spectral changes were observed that were progressive over a period of 2 h. However, in the first 30 min, the time required for a full set of spectral measurements at a single pH, these time-dependent changes were small compared to the spectral effects of IHP and/or

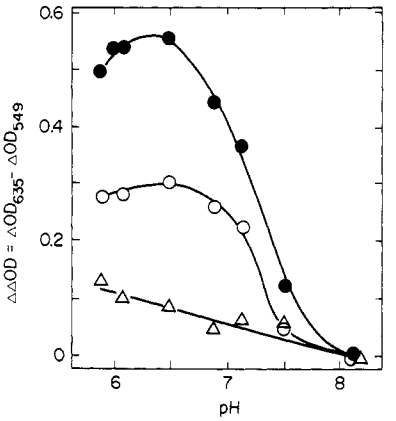

FIGURE 3: pH dependencies of the values of ΔΔOD (ΔΔ absorbance) for the nitrite derivative of human ferric hemoglobin that result from the addition of 5 mM BZF (△), 1 mM IHP (○), or their combination (●). For this derivative the maximum value of ΔΔ absorbance resulting from the addition of BZF plus IHP occurs at pH 6.5.

Table I: Maximum ΔΔOD Changes for Five Ferric HbA Derivatives in the Absence and Presence of BZF and/or IHP

			ΔΔOD		
derivative	pH	nm–nm	+BZF	+IHP	+BZF + IHP
NO_2^-	6.5	635–549	0.085	0.300	0.550
OCN^-	6.6	642–571	0.065	0.160	0.220
SCN^-	6.9	650–569	0.070	0.170	0.180
$HCOO^-$	6.5	636–562	0.055	0.110	0.160
N_3^-	6.45	635–542	0.035	0.060	0.100

BZF. Nevertheless, the order of measurement of the spectral changes associated with these allosteric effectors was varied and found not to alter the final result.

Choosing the pH of maximum effect of the combination of bezafibrate and IHP for each derivative, we have calculated the values of ΔΔOD for several derivatives of ferric HbA, and they are compared in Table I. The first derivative in the table is nitrite ferric HbA, the data for which have already been presented. The other derivatives, in order of the maximum ΔΔOD resulting from the combination of IHP and bezafibrate, are cyanate, thiocyanate, formate, and azide. With the exception of the thiocyanate, these derivatives exhibit a significantly greater spectral change with the combination of bezafibrate and IHP than with IHP alone.

The greater effects of an IHP–bezafibrate mixture than of IHP alone on the spectral properties of several derivatives of ferric HbA having been established, the effects of these effectors on the magnetic properties of the heme-iron atoms were determined. In Table II are reported the values of μ^2, the square of the average magnetic moment of the iron atoms in Bohr magnetons, for four derivatives of ferric HbA in buffer alone, in buffer plus 1 mM IHP, and in buffer plus 1 mM IHP and 5 mM BZF. These results parallel quite closely those obtained from the visible spectra. IHP and bezafibrate in combination have a much greater effect on the spin equilibria of three of these ferric derivatives of HbA than does IHP alone. As before, the exception to this pattern is the thiocyanate derivative, on which bezafibrate has no significant effect when IHP is present.

As in our previous work on the hemoglobin of the carp, we have calculated the changes in the standard free energies, $\Delta\Delta G°$, of the spin equilibria associated with the observed changes in magnetic moment. To do this, we have taken the square of the magnetic moment for the fully high spin state, μ^2_{HS}, to be 35 (Bohr magnetons)^2. This involves no assumptions. However, in the low-spin state the orbital magnetic

Table II: Effects of IHP and Bezafibrate on μ^2 and the Free Energy of the Spin-State Equilibrium of Derivatives of Human Ferric Hemoglobin

derivative	pH	addition	μ^2 (Bohr magnetons)^2	$\Delta\mu^2$ (Bohr magnetons)^2	$\Delta\Delta G°$ (cal/mol)
NO_2	6.5		14.3 ± 0.6		
NO_2	6.5	IHP	21.4 ± 0.8	7.1 ± 0.5	530 ± 40
NO_2	6.5	IHP and BZF	24.9 ± 0.9	10.6 ± 0.6	810 ± 70
SCN^-	6.5		28.0 ± 1.2		
SCN^-	6.5	IHP	31.7 ± 1.3	3.7 ± 1.0	520 ± 200
SCN^-	6.5	IHP and BZF	31.5 ± 1.1	3.5 ± 0.9	480 ± 170
OCN^-	6.38–6.53		28.6 ± 1.1		
OCN^-	6.38–6.53	IHP	31.0 ± 1.2	2.4 ± 0.8	330 ± 150
OCN^-	6.38–6.53	IHP and BZF	32.6 ± 1.3	4.0 ± 0.8	660 ± 250
$HCOO^-$	6.5		29.1 ± 1.1		
$HCOO^-$	6.5	IHP	30.9 ± 1.3	1.8 ± 0.9	250 ± 150
$HCOO^-$	6.5	IHP and BZF	32.5 ± 1.3	3.4 ± 0.8	580 ± 250

moments do not generally cancel and can contribute significantly to the total magnetic moment of the system. Nevertheless, we have taken the spin-only value for the square of the magnetic moment of this state, μ^2_{LS} = (3 Bohr magnetons)^2. For most derivatives of ferric hemoglobin this orbital contribution appears to be small, and for many it is not known. Since all of the derivatives we have examined in this particular study are of relatively high spin, this approximation introduces only a small error, but it does mean that the values of $\Delta\Delta G°$ reported here represent lower limits.

By use of these limiting values of μ^2, $\Delta\Delta G°$ was computed according to

$$\Delta\Delta G° = RT \ln \left(\frac{\mu^2_E - \mu^2_{LS}}{\mu^2_{HS} - \mu^2_E} \right) \left(\frac{\mu^2_{HS} - \mu^2_0}{\mu^2_0 - \mu^2_{LS}} \right)$$

where μ^2_0 is the experimental value of μ^2 in the absence of allosteric effectors and μ^2_E is the value in the presence of effectors. These values of $\Delta\Delta G°$ appear in Table II. The largest value of $\Delta\Delta G°$, also associated with the smallest error, is obtained with the nitrite derivative, the derivative with the lowest spin of those examined by magnetic susceptibility in this study. Here the combined effects of IHP and bezafibrate change the standard free energy of the spin-state transition by over 800 cal/mol.

Discussion

There is an excellent correspondence between the effects of IHP and bezafibrate on the paramagnetic susceptibilities of ferric derivatives of human HbA and the effects of these allosteric effectors on the visible spectra of these derivatives. A qualitative agreement can be discerned by examining the data presented in Tables I and II. However, as shown in Figure 4, $\Delta\Delta$OD as measured in Figure 2 appears to offer a nearly linear measure of $\Delta\mu^2$, the change in paramagnetic susceptibility that results from the addition of IHP and bezafibrate to these materials. $\Delta\mu^2$ is interpreted to result from an interconversion between two heme populations, those with high spin and those with low spin. If $\Delta\Delta$OD can be interpreted in the same way, then one would expect a family of isosbestic spectra for each derivative examined. In Figures 1 and 2 it is seen that this is very nearly the case for the nitrite derivative. However, the isosbestic points for these spectra are not perfect, nor are they generally found to be perfect for the other ferric derivatives. This may indicate that these allosteric effectors also perturb the spectra of the high- and low-spin forms.

A similar linear relationship between $\Delta\mu^2$ and changes in the intensities of spin marker lines in the resonance Raman spectra of these derivatives is presented in the accompanying paper. Taken together these results suggest that there are no significant errors other than those already recognized in the procedures used for the physical measurements or the analysis of the resulting data.

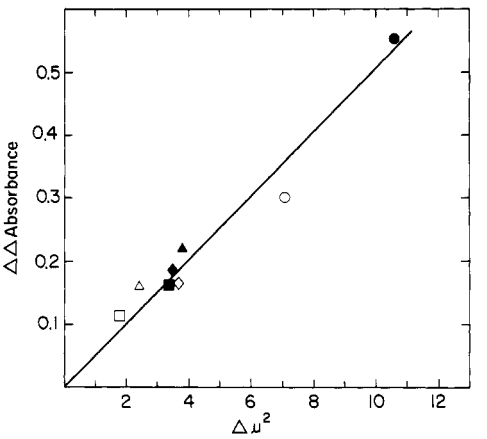

FIGURE 4: Values of $\Delta\Delta$ absorbance from Table I are plotted as a function of $\Delta\mu^2$ from Table II for derivatives and experimental conditions for which both parameters are determined. Open symbols indicate the effect of addition of 1 mM IHP and solid symbols that of the addition of 1 mM IHP plus 5 mM BZF to the nitrite (○, ●), thiocyanate (◇, ◆), cyanate (△, ▲), and formate (□, ■) derivatives of ferric human hemoglobin. The linear relationship between these two parameters is well within the error limits for $\Delta\mu^2$ listed in Table II.

It is clear from these data that the addition of IHP to mixed-spin derivatives of ferric HbA results in an increase in the amounts of the high-spin components of their spin equilibria. This is reflected in their spectral properties as well as by an increase in their magnetic susceptibilities. However, the addition of bezafibrate in the presence of IHP causes a further shift in the spin equilibria toward high spin. This is consistent with the suggestion that IHP alone may be unable to convert these derivatives entirely to the low-affinity, T quaternary states. The exception to this pattern is the thiocyanate derivative. With this derivative IHP by itself appears to produce the complete change in spin equilibrium, the addition of bezafibrate causing little or no additional effect. It appears that this derivative is shifted to its T state more easily than the others. This was also observed to be the case for the thiocyanate derivative of carp Hb (Perutz et al., 1978).

If one averages the changes in the free energies of the spin-state equilibria produced by the combination of IHP and bezafibrate (see Table II), one obtains a value of 635 cal/mol of heme. This compares well with the value of 720 cal/mol, which is the average change in this free energy produced by the R- to T-state transition in carp hemoglobin reported by Noble et al. (1987). Therefore, the effect of quaternary state on the spin equilibria first observed with carp hemoglobin appears to be a more general relationship than was concluded by Philo and Dreyer (1985). It is important to note that we cannot state that even the combination of IHP and BZF results in a complete transition to the T state of these ferric derivatives, only that it produces larger effects than IHP alone. Still

larger effects may be possible by using more potent allosteric effectors such as compound LR16 described by Lalezari et al. (1988).

This thermodynamic linkage is consistent with the model, first proposed by Perutz (1972), that the quaternary state of the protein is thermodynamically coupled to the state of the iron porphyrin system via their mutual interaction with the proximal histidine residue. As previously noted (Noble et al., 1987), these data offer no direct insight into the actual mechanism by which the relative free energies of the high- and low-spin electronic states are affected by the structural state of the protein. However, they clearly reinforce other studies, particularly on the deoxygenated hemoglobins, that have established a modulation of the iron–proximal histidine bond by the structure of the protein (Friedman, 1985).

Acknowledgments

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Registry No. BZF, 41859-67-0; IHP, 83-86-3; MetHbA, 12646-21-8; Fe, 7439-89-6; nitromethemoglobin A, 74665-89-7; thiocyanatomethemoglobin A, 72175-40-7; cyanatomethemoglobin A, 39340-60-8; formatomethemoglobin A, 96150-56-0; azidomethemoglobin A, 9072-23-5; heme, 14875-96-8.

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