The Origin of the Adams-Schuster Difference Spectrum of Oxyhemoglobin*

Francis C. Knowles, Melisenda J. McDonald+
and Quentin H. Gibson

Department of Biochemistry, Molecular and Cell Biology

Wing Hall, Cornell University, Ithaca, N.Y. 14853

Received July 23,1975

ABSTRACT

The characteristic difference spectrum reported by Adams and Schuster (Biochem. Biophys. Res. Commun. 1974, 58, 525) on the addition of inositol hexaphosphate to oxyhemoglobin is similar to the difference spectrum between (i) isolated α- and β-chains, (ii) α- and β-semihemoglobins, (iii) addition of inorganic phosphate to oxyhemoglobin, (v) change in temperature of a solution of oxyhemoglobin, (v) change in pH of carp carboxyhemoglobin and (vi) addition of inositol hexaphosphate to α-semihemoglobin. The spectrum may also be generated by differentiation of the spectra of oxyhemoglobin and carboxyhemoglobin, implying that the common feature of the results reported above is a shift in the position of the absorption bands. This shift may arise from several causes and so its interpretation is uncertain.

The absorption spectra of individual hemoglobin derivatives are generally believed to be constant for any one species such as, say, cyanide methemoglobin, and to be little influenced by the environment. The first exception was deoxyhemoglobin where Gibson (1) showed that deoxyhemoglobin prepared by flash photolysis of carboxyhemoglobin had a transient spectrum in the Soret region with the peak shifted slightly to the red and reduced in intensity as compared with normal deoxyhemoglobin. The same altered spectrum has since been found to occur constantly in deoxyhemoglobins with high ligand affinity and is often interpreted as indicating a deoxyhemoglobin in the oxy conformation or R-state.

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* Supported by U.S.P.H.S. Grant GM 14276 and NSF Grant BMS 74-08233.

+ Recipient of National Institutes of Health Postdoctoral Individual Research Fellowship 1-F22-AM-00531-01.

Very recently, Adams and Schuster (2) have described a difference spectrum obtained from oxyhemoglobin on the addition of inositol hexaphosphate[1] and have suggested that it may be due to a shift in the R-T equilibrium of the liganded hemoglobin. In the experiments reported here the same or closely related spectra have been obtained as the difference between (i) α- and β-oxy chains, (ii) α- and β-carboxysemihemoglobins, (iii) α-carboxysemihemoglobin ± IHP, (iv) oxyhemoglobin A ± inorganic phosphate, (v) carp carboxyhemoglobin ± IHP, (vi) carp carboxyhemoglobin at various pH values, (vii) oxyhemoglobin A at various temperatures and, (viii) by differentiation of the spectra of oxy- and carboxy-hemoglobins. In some of these cases an R-T transition almost certainly occurs, in others it quite certainly does not. The success of procedure (viii) shows that the Adams and Schuster spectrum derives from a shift in the position of the absorption bands, and it is plausible that such a shift might have more than one chemical cause.

Examples of some of the individual results follow. (i). The difference spectrum between α- and β-oxyhemoglobin A chains, prepared by the method of Geraci et al. (3), was obtained with a computer controlled digital spectrophotometer constructed in the laboratory. The instrument recorded the absolute absorption spectrum of each solution, which was introduced in turn into a cuvette which remained fixed in place throughout the experiment. The difference spectra were generated arithmethically from the raw data (160 points per spectrum) and were stored on magnetic tape. The wavelength reproducibility of the spectrophotometer as judged by the differences between successive spectra of oxyhemoglobin A was better than 0.1Å. The limit appeared to be set by the effects of temperature changes in the room on the monochromator. The difference spectrum for chains together with an Adams and Schuster spectrum obtained by adding 2 mM IHP to hemoglobin A, and a synthetic spectrum obtained by numerical differentiation (procedure viii) are shown in Figure 1. Note that the total excursion

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[1] IHP, Inositol hexaphosphate.

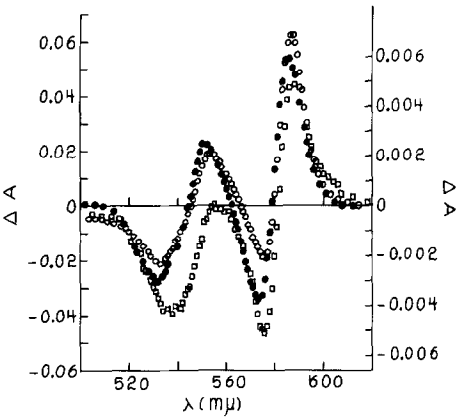

Figure 1. Chain and IHP Difference Spectra in the Visible Region. The difference spectrum between the oxy derivatives of chain preparations (○) 40 μM in heme at pH 7.0 (0.050 M potassium phosphate) and 25° (oxy β_{SH}-chains minus oxy α_{SH}-chains, 10 mm light path). The difference spectrum between 262 μM oxyhemoglobin A at pH 7.0 (0.050 M tris, 0.100 M NaCl) and 20° ± 2 mM IHP (□) for a 1.0 mm light path (plotted on expanded scale). A derivative spectrum of oxyhemoglobin A (●).

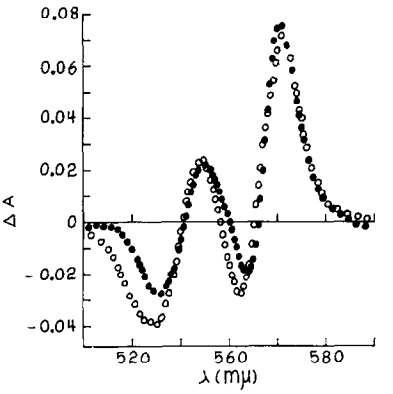

Figure 2. Difference and Derivative Spectra of Carboxysemihemoglobins in the Visible Region. The difference spectrum between the carboxy-derivatives of semihemoglobins (●) 40 μM in heme at pH 7.0 (0.050 M potassium phosphate) and 25° (β-carboxysemihemoglobin minus α-carboxysemihemoglobin, 10 mm light path). The derivative spectrum of β-carboxysemihemoglobin (○).

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is only ±0.005 for the Adams and Schuster spectrum. With this in mind, the agreement of the three spectra is considered striking.

Similar relations subsist for carboxy derivatives, items (ii) and (iii).

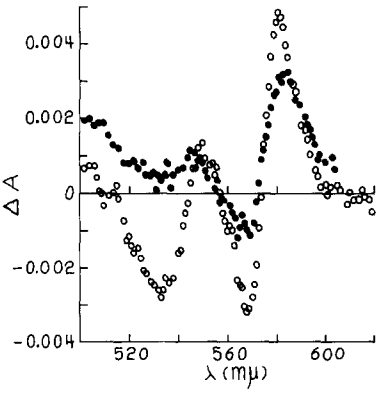

Figure 3. IHP Difference Spectra for Carboxysemihemoglobins in the Visible Region. The difference spectra between α-carboxysemihemoglobin ± 500 μM IHP (○) and β-carboxysemihemoglobin ±500 μM IHP (●) preparations 40 μM in heme at pH 7.0 (0.050 M potassium phosphate) and 25° for a 10.0 mm light path.

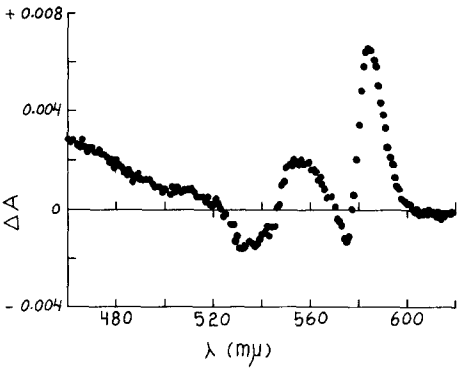

Figure 4. The difference spectrum of 415 μM oxyhemoglobin A ± 0.080 M potassium phosphate at pH 7.0 (0.050 M bis-tris) and 20° for a 1.0 mm light path.

Semihemoglobins, derivatives with heme on either α- or β-chains, were prepared by a modification of the method of Cassoly (4,5). An Adams and Schuster spectrum was obtained as the difference between α- and β-semihemoglobins and upon addition of IHP to these derivatives. It is interesting to note that IHP has much more effect on the spectrum of α-semihemoglobin than on that of β-semihemoglobin. This is paralled by the effects of IHP on the kinetics of ligand binding which are generally in agreement with the idea that IHP promotes

a shift from R to T behavior even in these greatly modified compounds, and which is much greater for α-semihemoglobin. The experimental and synthetic spectra are presented in Figures 2 and 3.

(iv). The Adams and Schuster spectrum is again obtained upon the addition of inorganic phosphate to oxyhemoglobin, though the effect is quite small at 20°. In principle, an apparent dissociation constant for phosphate may be calculated from the absorbance changes, but it is necessary to assume that there is only one way in which phosphate can bind to produce the spectral change. As inorganic phosphate is not a strong effector it seems somewhat improbable that the T-form of oxyhemoglobin becomes substantially populated in this case. An example of the data is given in Figure 4.

(v) and (vi). Functional studies of carp hemoglobin (6-9) have suggested that this hemoglobin undergoes a transition between the R and T states in both deoxy and liganded forms. The pH and IHP difference spectra for carp carboxyhemoglobin are shown in Figure 5 for the Soret region, again with a synthetic difference spectrum produced by differentiation.

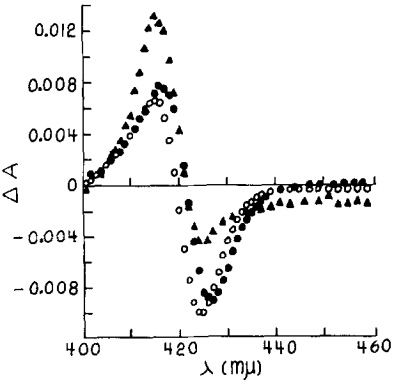

Figure 5. pH and IHP Difference Spectra for Carp Carboxyhemoglobin in the Soret Region. The pH difference spectrum of carp carboxyhemoglobin (○) 40 μM in heme at pH 4.5 (0.05 M citrate-phosphate) and pH 7.5 (0.050 M potassium phosphate) at 25° for a 1.0 mm light path. The difference spectrum of 40 μM carp carboxyhemoglobin ± 500 μM IHP (▲) at pH 6.0 (0.05 M citrate-phosphate) and 25° for a 1.0 mm light path. The derivative spectrum of carp carboxyhemoglobin (●).

The shape of the difference spectrum is different in the two experimental cases, apparently because IHP decreases the absorbance at the Soret maximum as well as moving the band towards longer wavelengths. Detailed comparisions of the pH and IHP difference spectra and of the effect of pH on the IHP difference spectrum suggest that there are at least two effects which produce similar but not identical spectra. The effect of pH is quantitatively greater and continues to modify the spectrum at least as far as pH 4.5 while the effect of IHP passes through a maximum and has declined to a small value by pH 5.5. The implication seems to be that the R-T transition is already complete at pH 5.5, which is in agreement with the functional experiments already referenced. Carp hemoglobin is known to bind anions strongly at least to pH 5.0 (6) so the alternative that IHP does not bind below pH 5.5 seems unlikely.

(vii). A strong Adams and Schuster spectrum is observed on changing the temperature of a solution of oxyhemoglobin. The effect is closely the same in tris, phosphate, and borate buffers and does not therefore depend upon a change in binding of phosphate to hemoglobin. Further, since tris buffers have a large temperature coefficient of pH and borate buffers a small one, it does not depend on pH either. The results are illustrated in Fig. 6. These static spectra may correspond to the very rapid unresolved absorbance change of Brunori and Schuster (10) which was seen in experiments by the temperature jump method. Work on the effect of temperature on the spectra of other derivatives and on other heme compounds is in progress.

(viii). The finding that an Adams and Schuster spectrum can be generated by differentiation means that the observed spectrum is produced by a small shift in the position of the bands. Correspondingly, it seems unlikely that such a shift will have a unique chemical cause, thus complicating its interpretation. An example of such complexity is offered by the spectra of mixtures of oxy- and deoxyhemoglobins in the presence of IHP. The basic findings are that IHP does not alter significantly the absorption spectrum of deoxyhemoglobin, but does, of course, produce the Adams and Schuster spectrum upon

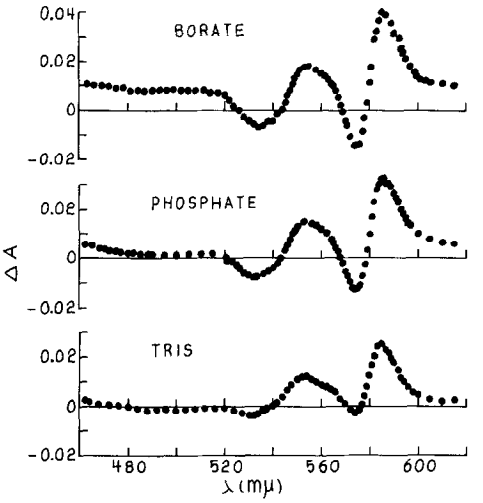

Figure 6. Temperature Induced Difference Spectra for Oxyhemoglobin in the Visible Region. Borate difference spectrum: 0.050 M sodium borate, pH 9.1, 322 μM heme, 30.6° minus 0.0°. Phosphate difference spectrum: 0.100 M potassium phosphate, pH 7.0, 273 μM heme, 30.0° minus 0.0°. Tris difference spectrum: 0.050 M tris, pH 7.0, at 20°, 170 μM heme, 32.7° minus 0.0°.

addition to oxyhemoglobin, though under our different experimental conditions (0.050 M tris, 0.100 M sodium chloride, pH 7.0, 20°) of smaller amplitude than they reported. On attempting to fit the spectra of partly saturated hemoglobin-oxyhemoglobin mixtures with the spectra of oxy and deoxyhemoglobins, significant residuals were observed whose non-random distribution showed that the oxyhemoglobin component of the partly saturated mixtures differed from the 100% oxyhemoglobin-IHP standard in having a much stronger Adams and Schuster spectrum which increased systematically on a per heme basis as the percent saturation was reduced. In this instance the spectral shift is probably not due to an R-T transition as the amplitude continues to increase even below 20% saturation where liganded hemoglobin should be almost entirely in the T-state.

Quite extensive quatitative studies would be needed to settle the matter, and in view of the several origins of the Adams and Schuster spectrum a definite answer may not be obtainable by optical absorption spectroscopy

alone. There seems every reason to expect analogous difficulties to recur frequently in accurate quantitative work with hemoglobin.

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