Haem-globin equilibrium studies by fluorimetry

The fluorescence of 1-dimethylaminonaphthalene-5-sulphonamide groups attached to the amino groups on the surface of myoglobin or haemoglobin molecules is effectively quenched by resonance-energy transfer[1] to the neighbouring haem group. The transfer is a sensitive function of the intermolecular separation and ceases completely when the haem group dissociates from the globin molecule.

Thus photometry of labelled haem-protein solutions provides a method for investigating haem-globin equilibria depending only on molecular proximity, and capable of employing protein concentrations as low as 10^{-9} M. The fluorescence intensity ratio between the dissociated and intact systems F_0/F and the pK's are included in Table I.

TABLE I

Substance	F_0/F	*Acid pK*	*Alkaline pK*
Myoglobin Fe^{++}	32	4.2	11.6
Myoglobin Fe^{+++}	15	4.8	12.0
Haemoglobin Fe^{++}	60	4.15	11.4
Haemoglobin Fe^{+++}	40	4.8	12.1

The quenching efficiency changes with the iron valency because of the altered overlap integral of the fluorescence and haem absorption spectra. The haem-globin equilibrium can be easily demonstrated near either of the pK values by the addition to the buffered system of an excess of unlabelled native globin prepared by the acid-ketone method[2]. The equilibrium exchange:

$$G + HG^* \longrightarrow HG + G^*$$

rapidly liberates most of the fluorescent globin. The rate of this globin turnover can be used to measure the activation energy of the haem-globin dissociation. With metmyoglobin at the acid pK value, E was measured at 3.1 kcal/mole, and 8.5 kcal/mole for the alkaline dissociation, over the temperature range 5°–50°. Similar values were obtained for methaemoglobin. While the alkaline pK value showed a marked concentration dependence, the value for the acid pK was constant over the concentration range 10^{-6}–10^{-9} M. This suggest that aggregation of both haem and globin occur after dissociation according to the scheme

$$2\,HG \rightleftharpoons H^2 + G^2$$

where H^2 and G^2 represent haem and globin dimers respectively. Direct evidence for myoglobin globin polymerisation was obtained by measurements of fluorescence polarization, while haem dimers have been demonstrated[3] in acid solution. In contrast, it appears that at pH 12 both globin and haem remain monomeric. Some properties of the haem-binding site were investigated by observing the effect of specific reagents for protein groups on the recombination velocity measured at the pK values. Some results are listed in Table II.

Substances capable of complexing with haem, such as pyridine, caffeine, histidine, and bovine serum albumin were ineffective. In general, only those reagents combining with the amino groups of the globin or altering the surface charge distribution altered the haem-binding efficiency.

Abbreviations: H, haem; G, globin; G*, globin labelled with a fluorescent residue.

TABLE II

Reagent	*Group involved*	*Result*
Acetic anhydride	$-NH_2$	No recombination
Diazoacetamide	-COOH	No recombination
Detergents	Charged groups	No recombination
6 *M* Urea	Protein structure	No recombination
Mercurials	-SH	No effect
Diazonium compounds	Histidine and Tyrosine	No effect
Bromoacetic acid	Histidine and Tyrosine	No effect
Halogens	Histidine and Tyrosine	No effect
Carbon dioxide	$-NH_2$	No effect

The effect of modifications of the haematin side-chain and of iron removal on the recombination was investigated at neutral pH. The low haematin concentrations employed (10^{-8} *M*) enabled methylated-haem derivatives of low solubility to be examined. The relative combining efficiencies expressed as the equilibrium constants of the reaction are listed in Table III for several haem derivatives.

TABLE III

Compound	*Relative K*	*Groups modified*
Haematin	100.0	—
Protoporphyrin	33.0	Fe^{+++} absent
Mesohaematin	9.3	$C_2H_5^-$ for $C_2 = CH-$
Deuterohaematin	7.05	H– for $C_2 = CH-$
Methylhaematin	0.8	–COOMe for $-COO^-$
Methylmesohaematin	0.65	–COOMe for $-COO^-$
Methyldeuterohaematin	0.5	–COOMe for $-COO^-$
Methylprotoporphyrin	0.32	–COOMe for $-COO^-$

Table III brings out the importance of the charged carboxyl groups and to a lesser extent the vinyl groups, in recombination with globin. The surprisingly high efficiency of protoporphyrin uptake suggests that the iron atom plays a relatively minor role in the binding process. No uptake of protoporphyrin by native myoglobin or haemoglobin, or by globin titrated with haematin, could be demonstrated, indicating that protoporphyrin and haematin compete for the same binding site.

Protoporphyrin remained fluorescent after adsorption onto globin, and energy transfer from the naphthalene dye groups and native tryptophan chromophores of globin could be shown to take place.

From the criterion of energy transfer, it could also be shown that "mixed" haemoglobins containing both protoporphyrin and haematin can be formed.

The intrinsic values of F_0/F can be calculated for several symmetric dispositions of from 1 to 4 haem groups per haemoglobin molecule, so that the mechanism of recombination can be investigated by kinetic or equilibrium measurements. One interesting case is haemoglobin reconstitution from a dissociated system containing only one half of the normal haem complement. The several distributions possible depend on the site affinities and the globin dissociation. The F_0/F value was measured as 2, indicating that all the available haem combined with one half of the globin.

More uniform distributions give $F_0/F \ll 2$. This result suggests that recombination becomes progressively easier as the adjacent sites are filled, so that the full quartet is achieved when possible.

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The salt-sensitive step in immune hemolysis

The hemolysis of red blood cells (E) by antibody (A) and complement (C′) is believed to result from the following sequence of reactions[1]:

$$E + A \rightleftharpoons EA \quad (1)$$

$$EA + C'_{1,4} \xrightarrow{Ca^{++}} EAC'_{1,4} \quad (2)$$

$$EAC'_{1,4} + C'_2 \xrightarrow{Mg^{++}} EAC'_{1,4,2} \xrightarrow[37^\circ]{\text{rapid}} EAC_{1,4} \quad (3a)$$

$$EAC'_{1,4,2} + C'_3 \longrightarrow E^* \quad (4)$$

$$E^* \longrightarrow \text{ghost} + \text{hemoglobin} \quad (5)$$

where C'_1, C_2, C_3, C_4 refer to the components of complement and E^* refers to a damaged cell which spontaneously hemolyzes. The overall reaction of sensitized cell (EA) with C′ has been shown to be very sensitive to the concentration of NaCl in the reaction mixture[2]. In studying the precise steps at which various inhibitors of the immune hemolytic reaction operate it was observed that reaction step 4, $EAC'_{1,4,2} + C'_3 \longrightarrow E^*$, is extremely sensitive to the effect of NaCl concentration, and, in fact, the effect of NaCl concentration on the overall hemolytic reaction can be quantitatively accounted for by its effect on step 4.

Sheep erythrocytes, rabbit hemolysin and triethanolamine-buffered saline were prepared as described previously[3]. In determining the effect of salt, varying quantities of fresh guinea serum diluted 1:100, buffered saline and either distilled water or 0.3 *M* NaCl were added to give a volume of 1.2 ml. The amounts of distilled water or 0.3 *M* NaCl were chosen so as to give NaCl concentrations in the final reaction mixture varying from 0.130 *M* to 0.188 *M*. To the 1.2 ml were added $1.5 \cdot 10^8$ sensitized cells in a volume of 0.3 ml. Exactly the same procedure was used in studying the step, $EAC'_{1,4,2} + C'_3 \longrightarrow$ hemolysis, except $EAC'_{1,4,2}$ was used in place of EA and 0.005 *M* EDTA was also added to the reaction mixture. By binding Ca++ and Mg++ EDTA prevented reactions 2 and 3. In all instances the reaction mixtures were incubated for 30 min at 37°; at the end of this time, 0.5 ml buffered saline was added, the tubes were centrifuged and the amount of hemolysis determined by reading the supernatants in a Junior Coleman spectrophotometer at 550 mμ. The volume of complement required for 50% hemolysis of EA and $EAC'_{1,4,2}$ was determined in

Abbreviation: EDTA, ethylenediaminetetraacetic acid.