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Haptoglobin–Hemoglobin Interaction. Stoichiometry*

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ABSTRACT: The fully saturated and the partially saturated intermediate products of the reaction between haptoglobin type 1-1 and cyanmethemoglobin were identified by polyacrylamide gel electrophoresis. Quantitative estimates of the protein constituents of each product were made by scanning gels in the ultraviolet for total protein and in the Soret band for hemoglobin. These measurements indicated that the fully saturated complex consisted of one molecule each of haptoglobin and hemoglobin; the intermediate complex consisted of one molecule of haptoglobin and one-half molecule of hemoglobin. A scheme involving sequential addition of half-molecules of hemoglobin fits the observations.

The reaction of haptoglobin 1-1 (Hp 1-1)[1] with excess hemoglobin yields a stable complex of one molecule of haptoglobin with one molecule of hemoglobin. An additional intermediate complex appears when less than an equivalent amount of hemoglobin is added (Allison and Rees, 1957). The intermediate compound is probably a complex of one molecule of haptoglobin and one-half molecule of hemoglobin (Laurell, 1959; Shim *et al.*, 1965; Hamaguchi, 1967; Ogawa *et al.*, 1968). The reaction between haptoglobin and hemoglobin probably involves one-half molecules of hemoglobin, *i.e.*, hemoglobin dissociated into $\alpha\beta$ subunits of molecular weight 32,250 (Bunn, 1967, 1969; Chiancone *et al.*, 1968; Giblett, 1968; Nagel and Gibson, 1967). The existence of the intermediate compound is thus well documented, but there

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[1] Abbreviations used are: Hp, haptoglobin; CNMetHb, cyanmethemoglobin.

have been no reports on the relative amounts of intermediate and fully saturated compounds which are produced when hemoglobin is added to haptoglobin.

We have measured the quantitative relationships and compositions of the fully saturated complex (C_{II}), the intermediate complex (C_I), and the free haptoglobin (C_0) formed with varying ratios of haptoglobin to hemoglobin. These compounds are readily resolved into separate bands by polyacrylamide gel disc electrophoresis. We scanned the gels at 280 mμ to estimate the total protein (Hp and Hb) and then scanned again at 416 mμ (Soret band) to determine the hemoglobin content of each band. We determined the 280 mμ:416 mμ absorbance ratio for the hemoglobin and then, using the total protein absorbances at 280 mμ, calculated the amount of hemoglobin and of haptoglobin in each band. Thus the molecular composition of the complexes could be determined. We find that the compositions of C_{II} and C_I are constant and independent of the amount of hemoglobin added to the haptoglobin. From the hemoglobin:haptoglobin ratios, we conclude that the intermediate complex has only one-half as much hemoglobin as the fully saturated complex.

Methods and Materials

Preparation of Haptoglobin. Haptoglobin type 1-1 was prepared from single-donor specimens of plasma from the Clinical Center Blood Bank. It was freed of extraneous protein by chromatography on DEAE-cellulose followed by gel filtration on Sephadex G-200. The methods, which are modifications of techniques previously published (Connell and Shaw, 1961; Gordon and Bearn, 1966), were briefly as follows.

Plasma was adjusted to pH 4.7 and dialyzed against 100 volumes of 0.01 M sodium acetate buffer (pH 5). A precipitate was discarded and 100 ml of the supernatant solution placed on a column (2.5 × 30 cm) of DEAE (10 g) previously equilibrated with the same buffer used for dialysis. The column was developed with a linear gradient of sodium acetate 0.01–0.30 M. The fraction containing the haptoglobin was adjusted to pH 7, concentrated to 5 ml, placed on a Sephadex G-200 column (2.5 × 90 cm), and eluted with 0.1 M Tris-HCl buffer (pH 7.4). The fractions containing haptoglobin were individually analyzed by disc gel electrophoresis to identify those few fractions which were free of other proteins. The purification process did not afford large amounts of highly purified haptoglobin, but routinely provided one or two fractions containing sufficient Hp 1-1 for the performance of the experiments indicated below.

Preparation of Hemoglobin. Twice-recrystallized human hemoglobin, 0.3 g (Gallard-Schlesinger, Lot No. A4083), was dissolved in 6 ml of five-times-concentrated Drabkin's reagent[2] to yield CNMetHb; 0.5-ml portion of this CNMetHb solution was subjected to electrophoresis by the technique of Raymond (1962) using a 6-mm gel slab and a Tris-EDTA–borate buffer [10.8 g of Tris, 0.93 g of Na_2EDTA, 0.55 g of boric acid per l.], pH 9.2. After the main CNMetHb band had migrated approximately 10 cm, the gel was removed and the main CNMetHb band cut out. The gel was macerated in a Waring blendor with 25 ml of H_2O. The supernatant solution contained a dilute solution of purified CNMetHb. Three such batches were pooled, concentrated to 0.6 ml and electrophoresed a second time to provide a small amount of cyanmethemoglobin A, essentially free of detectable contaminants.

Methods of Electrophoresis. Disc electrophoresis, modified after Davis (1964), was performed using tubes 90-mm long with an inside diameter of 4.5 mm. Other conditions of operation were the same as we have described earlier (Pastewka *et al.*, 1966), using 7.0% running gel and 2.5% spacer gel. The sample was diluted in 40% sucrose and layered onto the spacer gel because it had been found that the photocatalyzed gelation of the sample gel caused electrophoretically detectable changes in the hemoglobin.

Method of Scanning. Scanning was performed in the Gilford scanning attachment to the Gilford spectrophotometer (Model 240) using a quartz cuvet. The gels were first scanned at 1-mμ intervals from 413 to 420 mμ to verify that the absorption peak of the hemoglobin occurred at 416 mμ. The gels were then scanned at about 1 cm/min at 416 mμ followed by a scan at the same rate at 280 mμ. The composition of each mixture was evaluated by measurement of the area under the curve. The area was calculated as the product of the peak height (in absorbance units) times the peak width (in centimeters) at the half-absorption point.

Estimation of the Hemoglobin-Binding Capacity of the Haptoglobin Preparation. Increasing amounts of CNMetHb were added to constant volumes of the haptoglobin solution and mixed thoroughly. The mixtures were allowed to stand in the dark at room temperature for 5 min, after which aliquots of the mixtures, containing 20–30 μg of CNMetHb, were subjected to electrophoresis. The resulting gels showing only C_{II} and free CNMetHb were scanned at 416 mμ. From the areas under the curves the percentage of the total CNMetHb present in C_{II} was determined and the microgram of CNMetHb per microliter of Hp 1-1 calculated. The Hp 1-1 preparation (1 μl) used in this study bound 2.0 μg of CNMetHb when fully saturated.

Results

Experimental calculations required knowledge of the 280:416-mμ absorbance ratio for CNMetHb in the gel system. The ratio was determined from scans of gel electrophoretic patterns of solutions containing (a) varying amounts of CNMetHb and (b) solutions of haptoglobin containing an excess of CNMetHb. The average of 14 determinations yielded an $A_{280}:A_{416}$ ratio of 0.25. Similar measurements made on CNMetHb solutions in the Cary spectrophotometer, in which the complicating factors of the gel were not present, gave a ratio of 0.28, in close agreement with the value observed in gels. It has been reported that the characteristic hemoglobin absorbance peaks (Waks and Alfsen, 1966; Clark, 1966) are depressed when the hemoglobin is bound to haptoglobin. We found a depression of less than 2% using CNMetHb, which we have disregarded. We conclude that the factor 0.25 applied to the 416-mμ absorbance will measure the contribution of CNMetHb to the 280-mμ absorbances of the complexes.

Description of Mobilities and Nomenclature. When hemo-

[2] This reagent contained 1.0 g of potassium ferricyanide, 0.25 g of potassium cyanide, and 5 g of sodium bicarbonate per liter of solution.

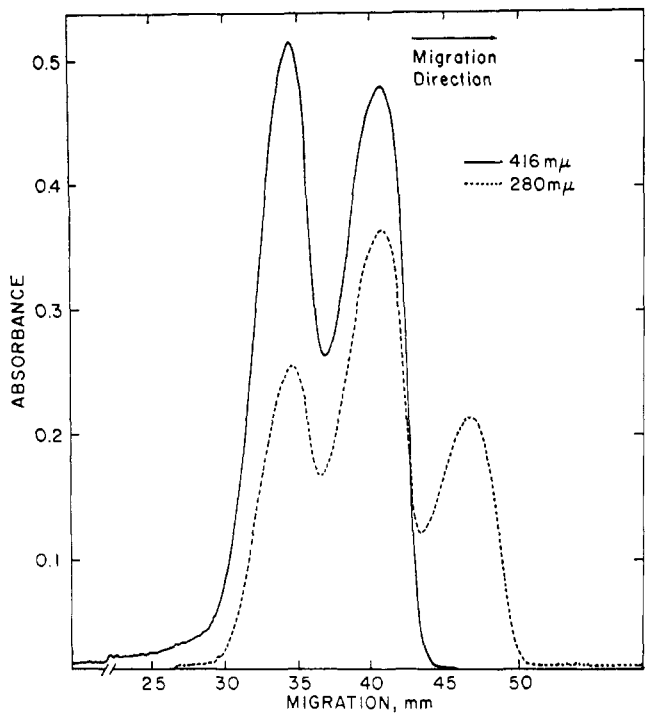


FIGURE 1: Densitometric tracings of unstained gel at 416 mμ and 280 mμ. In the figure the tracings are superimposed. The absorbance at 280 mμ is due both to haptoglobin and to hemoglobin, whereas the absorbance at 416 mμ is due only to hemoglobin. The most rapidly migrating protein zone at about 47 mm is C_0 (free haptoglobin); the hemoglobin-containing band migrating at approximately 40 mm is C_I (the intermediate complex); the slowest migrating band is C_{II} (the fully saturated haptoglobin–hemoglobin complex). The different proportions of haptoglobin and hemoglobin in C_I and C_{II} are apparent from the ratios of the absorbance at 416 mμ and 280 mμ for these two species.

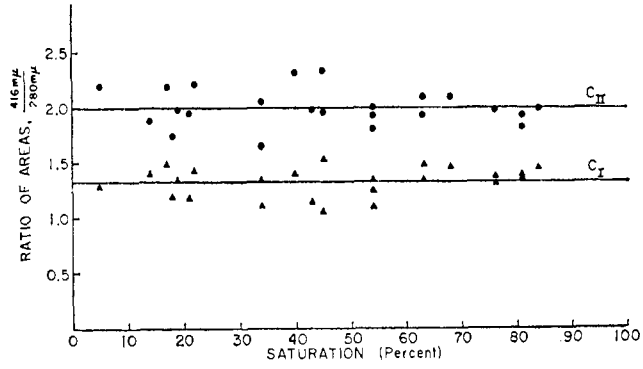


FIGURE 2: Characteristic values for the ratio of the areas 416 mμ/280 mμ for the complexes C_{II} and C_I. Densitometric data like that in Figure 1 was treated as described in the text to yield measures of the area arising from C_{II} and C_I. The figure indicates that these ratios are independent of the saturation percentage. The mean value for the ratio for C_{II} and for C_I agree closely with those expected for a 1:1 and a 1:2 molecular ratio of hemoglobin to haptoglobin for C_{II} and C_I, respectively.

globin is added to haptoglobin in less than saturating amounts, three ultraviolet-absorbing peaks containing protein can be separated by gel electrophoresis. The most rapidly migrating of these is free haptoglobin (C_0), the compound of intermediate mobility is the intermediate complex (C_I), and the slowest migrating species is the haptoglobin–hemoglobin complex (C_{II}). The curves of the scans of such a gel at 416 and 280 mμ are shown in Figure 1.

Ratio of CNMetHb to Total Protein in C_{II} and C_I. If C_{II} and C_I are of constant composition, the ratio of the hemoglobin to haptoglobin or the ratio of the hemoglobin to total protein (*i.e.*, 280-mμ absorbing substances) should be constant irrespective of the amount of hemoglobin added. Figure 2 is a plot of the ratios of the areas under the 416- and 280-mμ peaks and shows a reasonable constancy for each complex. The expected (see below) value of 2.0 for C_{II} and lower value of 1.3 for C_I are shown.

Relationship between the Amount of CNMetHb Added and the Distribution of Hp into C_0, C_I, and C_{II}. For each amount of CNMetHb added, the distribution of Hp into C_0, C_I, and C_{II} was calculated from the gel scans using the areas measured at 280 mμ. The contribution of CNMetHb to the 280-mμ area of C_I and C_{II} was determined by multiplying the 416-mμ areas by the factor 0.25. Each CNMetHb contribution was subtracted from its corresponding 280-mμ area to obtain the 280-mμ area representing the haptoglobin in C_I and C_{II}.

From these calculations we find (Figure 3) that the percentage of free haptoglobin decreases with increasing amounts of hemoglobin and falls to zero at the saturation point. At 1:1 molar ratio of CNMetHb to Hp, only C_{II} is present. At low levels of saturation the conversion of C_0 is predominantly into C_I, with very little conversion into C_{II}.

Distribution of CNMetHb. The ratio of CNMetHb in C_I to total CNMetHb added is shown in Figure 4. These data were determined from the areas observed at 416 mμ.

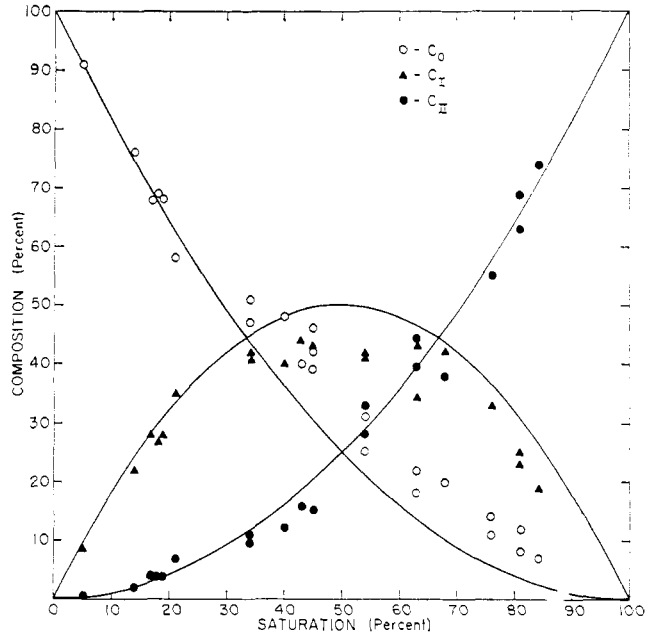


FIGURE 3: The distribution of haptoglobin into C_0, C_I, and C_{II} as a function of saturation with hemoglobin. The indicated points are experimental data; the lines drawn through the data points are calculated from the equation in the text. Replicate determinations were not averaged but are shown plotted on the figure.

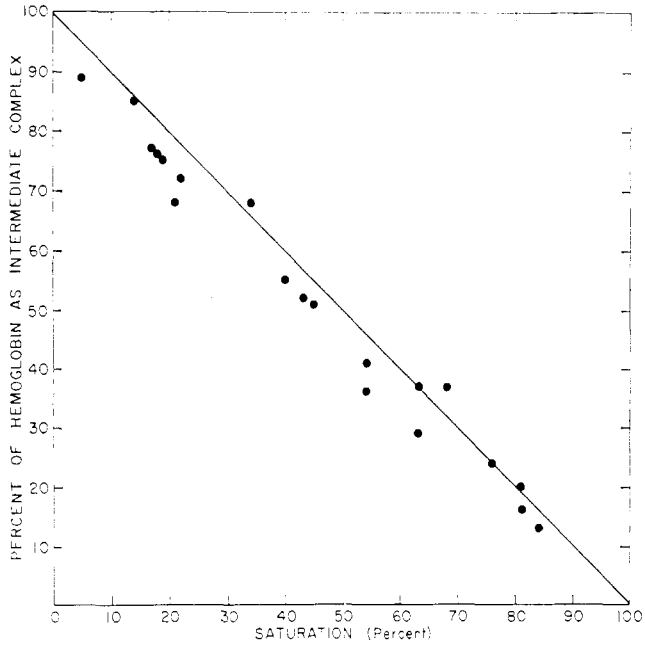


FIGURE 4: The distribution of hemoglobin between C_I and C_{II} as a function of saturation per cent. At low levels of saturation nearly all of the added hemoglobin is bound as (the intermediate complex) C_I but at higher levels an increasingly large percentage is bound as C_{II}. As in previous figures the line connecting the points is drawn from the theoretical equation.

At low levels of saturation, the added CNMetHb is present chiefly in the intermediate complex. As the amount of CNMetHb was increased, the percentage in C_I decreased.

Discussion

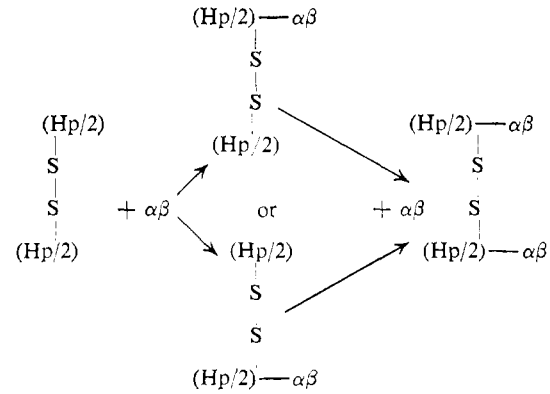
The addition of CNMetHb to a stoichiometric excess of haptoglobin produces two complexes, each of whose electrophoretic mobilities is less than that of free haptoglobin. Each complex has a definite, constant composition with respect to Hp and CNMetHb irrespective of the relative amounts of reactants.

Quantitation of these relationships by gel electrophoresis gave data of relatively high degree of accuracy, but as the scatter of points in the figures shows, the limit of accuracy is probably of the order of 10%. The use of ratios obtained from scans at two different wavelengths adds an element of precision. We earlier carried out a similar study in which the Soret band was measured using a blue filter and the protein distribution was estimated from Amido-Schwarz-stained gels, using a red filter. Those studies were substantially less satisfactory from the point of view of agreement between replicates. The use of CNMetHb in contrast to carbonmonoxyhemoglobin gave much more satisfactory results because of greater stability of the absorption in the Soret region. Lombart *et al.* (1965) measured absorbances at 280 and 540 mμ and concluded that one molecule of rabbit or rat haptoglobin combined with one molecule of horse CNMetHb.

The view that C_{II} consists of one molecule of haptoglobin and one molecule of hemoglobin, and that C_I consists of one molecule of haptoglobin and one-half molecule of hemoglobin requires that the percentage of CNMetHb in C_{II} be twice the percentage of CNMetHb in C_I. The mean and standard deviation of 24 estimates of this ratio using data taken from Figures 2, 3, and 4 was 2.07 ± 0.29. In addition, the molecular composition of each complex was verified by calculating the theoretical $A_{416}:A_{280}$ using experimentally determined extinction coefficients for CNMetHb ($\epsilon_{280}^{1\ mg/ml}$ 2.0 and $\epsilon_{416}^{1\ mg/ml}$ 8) and reported values for Hp ($\epsilon_{280}^{1\ mg/ml}$ 1.2 [Herman-Boussier *et al.*, 1960; Lombart *et al.*, 1965] and $\epsilon_{416}^{1\ mg/ml}$ essentially 0) and molecular weights of 64,500 (Braunitzer, 1964) for CNMetHb and 100,000 for Hp 1-1 (Herman-Boussier *et al.*, 1960; Cheftel and Moretti, 1966). The ratios of 2.1 for C_{II} and 1.4 for C_I agree with the experimentally found values of 2.0 and 1.3.

We postulate that the haptoglobin–hemoglobin interaction proceeds (very rapidly) according to Scheme I, where (Hp/2)

SCHEME I



stands for a one-half molecule subunit of haptoglobin, joined to its partner by a disulfide bond (model of Shim and Bearn, 1964), and $\alpha\beta$ is the dimer unit (mol wt 32,250) of CNMetHb. The association constants have been reported to be extremely high, perhaps as much as 10^9 (Clark, 1966) and certainly the dissociation of this complex has never been noted. If the reaction is considered to be reversible, standard methods for treating multiple equilibria (Tanford, 1961) may be used to obtain an estimate of the expected distribution of each compound. If, as seems more probable, the reaction is not reversible, the simplest alternate assumption is that each of the two sites on haptoglobin is equally available for binding hemoglobin. In the latter case, the relative amounts of C_0, C_I, and C_{II} are given by the terms p^2, $2pq$, and q^2, respectively, in the expansion of $(p + q)^2$, where q is the fractional saturation and $p = 1 - q$. Because of the very low level of free hemoglobin both of these treatments lead to the same distribution for C_0, C_I, and C_{II}. These calculated values have been used as the basis for the theoretical lines on the figures.

The participation of the $\alpha\beta$ subunit of hemoglobin is consistent with evidence of others for the existence of $\alpha\beta$ subunits in the concentrations, and ionic strength employed in the present study readily accounts for the apparent increase in size from C_0 to C_{II} observed by Shim *et al.* (1965) and is consistent with the diminished electrophoretic mobility of the complexes.

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