Sedimentation Analysis of the Interacting Mixture of Dextran Sulfate and Low Density Lipoprotein

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The reversible interaction between dextran sulfate (D) and the low density lipoprotein of human serum (P) was investigated by sedimentation velocity. Analysis of the velocity patterns of dextran sulfate—lipoprotein mixtures revealed that the maximum number of binding sites on dextran sulfate molecule is approximately 6. It was also shown that the species of the complex formed is affected by the mixing ratio of the two constituents: at the molar ratio (P/D) 0.69, the complex exists in average as $DP_{1.6}$ and at 0.98 as $DP_{2.2}$. The linear increment of sedimentation coefficient of the complex due to the binding of one lipoprotein molecule was 7.8S. Finally, the mechanism of precipitation of the complexes was discussed.

The authors^{1,2)} have shown that soluble and insoluble complexes were reversibly formed by the interaction of dextran sulfate, D, with the low density lipoprotein, P, of human serum in various media and that the size of the complexes was affected by the ratio of the two components in the mixture. At high P/D ratios insoluble complexes were produced, but with decrease of the ratio the insoluble complexes rapidly dissolved indicating the operation of rapidly established equilibria. It was also found that the complex formation was abolished by increasing salt concentration. This suggests that the intermolecular links between constituent units in the complexes are predominantly electrostatic in nature. Detailed studies on the nature of the interaction between the two components, D and P, have been recently reported by Nishida *et al.*^{3~5)} In the present communication, the effect of P/D ratio

on the size of the complexes is investigated by sedimentation velocity, and the mechanism of the complex formation is discussed.

THEORY AND METHODS

Several items of evidence suggest that the soluble complexes formed by the reversible interaction between P and D in the presence of excess D are of the type DP_i ($i=1, 2, 3, \dots, n$). First, the number of negative charge on D, a polyanion with 1.8 sulfate groups per glucose unit,^{1,2)} is obviously very much greater than the number of positive charge⁶⁾ on P. In addition, the hydrated density of the soluble complexes formed at a P/D ratio of 1 (molar ratio 0.172) was found from sedimentation in sucrose media of various density to be 1.10 g/ml which is in good agreement with that calculated for the complex $DP_{2.7}$. Finally, the molar ratio of P to D in the insoluble complexes is approximately 5.6:1. Thus, occurrence of complexes of the type D_jP ($j=1, 2, 3, \dots, m$) in the presence of excess D is very unlikely, though it is possible that the formation of huge network, D_jP_i ($j \gg 1, i \gg 1$), takes place at high P/D ratio where precipitation of the com-

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plexes occurs. Therefore, it appears that in the present work only equilibria leading to the formation of complexes, DP_i , need be considered.

According to Tiselius,⁸ it is possible to define the constituent sedimentation coefficient of all forms of P (bound and unbound) as

$$\bar{s}_P = \frac{1}{(P_0)} \left[(P)s_P + \sum_{i=1}^n i(DP_i)s_{DP_i} \right] \quad (1)$$

where $\bar{s}_P$ is the constituent sedimentation coefficient of P, (P) is molar concentration of unbound P, s_P is sedimentation coefficient of P, (DP_i) and s_{DP_i} are molar concentration and sedimentation coefficient of complex DP_i , respectively, and (P_0) is the total molar concentration of P. Following Smith and Briggs⁹ and Alberty and Marvin,¹⁰ Eq. (1) can be simplified as

$$\bar{s}_P = \frac{1}{(P_0)} \left[(P)s_P + ((P_0) - (P)) \left(\bar{s}_D + q \frac{n-r}{n} \right) \right] \quad (2)$$

where q is the increment of sedimentation coefficient of the complex due to binding of one P molecule, n is the maximum number of the binding sites on the D molecule, r is the number of moles of bound P per mole of D and $\bar{s}_D$ is the constituent sedimentation coefficient of D. $\bar{s}_D$ and r are respectively defined by

$$\bar{s}_D = s_D + qr \quad (3)$$

and

$$r = [(P_0) - (P)] / (D_0) \quad (4)$$

where s_D is sedimentation coefficient of D and (D_0) is total molar concentration of D. It should be noted that in the derivation of Eq. (2) Smith and Briggs¹¹ and Alberty and Marvin¹² assumed that the n binding sites are identical and independent and also the increment of sedimentation coefficient due to binding of P is given by

$$s_{DP_i} = s_D + qi \quad (5)$$

It should be noted that the linear increment (Eq. (5)) of sedimentation coefficient is still valid for a macromolecule—macromolecule interaction provided that i is not very large. Application of Eq. (2) for the sedimentation analysis of protein-small molecule interactions was discussed in detail by Schachman.¹¹

In this communication it is shown that Eq. (2) can be modified for the analysis of macromolecule—macromolecule interactions under the condition specified below. If $(D_0) \gg (P_0)$, at equilibrium practically all of P would be in bound form, i.e.,

$$(P) \rightarrow 0 \text{ as } (P_0)/(D_0) \rightarrow 0.$$

The range of $(P_0)/(D_0)$ where (P) becomes negligible

is determined by the magnitude of the association constant (see Appendix). Combination of this condition with Eq. (2), (3) and (4) gives an expression for $\bar{s}_P$ valid when the $(P_0)/(D_0)$ ratio is infinitesimally small.

$$\bar{s}_P = (s_D + q) + \left[q \left(1 - \frac{1}{n} \right) \right] (P_0)/(D_0) \quad (6)$$

From Eq. (6), it is evident that the parameters, q and n , can be obtained by extrapolation of the $\bar{s}_P$ vs $(P_0)/(D_0)$ plot. Thus, if $\bar{s}_P$ is measured at sufficiently low $(P_0)/(D_0)$ ratio, the plot should approach linearity. This was found to be the case in the present system.

Human serum low density lipoprotein was prepared by an ultracentrifugal method¹² using a Spinco model L ultracentrifuge and dextran sulfate by the method of Ricket *et al.*¹³ P and D were exhaustively dialyzed against phosphate buffer (pH 7.4, $I=0.1$) in which subsequent analyses were made. The physical properties of P and D were given elsewhere;^{1,2} the molecular weight of P and D are 2.3×10^6 and 4.0×10^5 , respectively and the sedimentation coefficients at zero concentration in phosphate buffer are 6.1S and 10.7S, respectively. The complex formation was effected by mixing the two components at various P/D weight ratios (0.1~10.0), the concentration of D being kept constant at 1.25 mg/ml in each mixture.

Velocity sedimentation was carried out in a Spinco model E ultracentrifuge at 52,640 rpm at 20°C in phosphate buffer. Schlieren patterns were measured using a Nikkon comparator and the square root of the second moment of observed boundaries were calculated from at least 18 points of equal interval.

RESULT AND DISCUSSION

Figure 1 shows the sedimentation velocity patterns obtained with mixtures with various $(P_0)/(D_0)$ ratio. Each mixture exhibits two boundaries representing free D, the slower boundary, and a faster moving reaction boundary which becomes progressively asymmetric on the leading side and broadens as the ratio increases. At the highest $(P_0)/(D_0)$ ratio (Fig. 1e), formation of insoluble aggregates occurred as indicated by the appearance of turbidity. The constituent sedimentation coefficient, $\bar{s}_P$, is given by the rate of movement of the median bisector of the gradient in the constituent

FIG. 1. Traces of Schlieren Patterns (plots of refractive index gradient *vs* distance) Obtained in the Sedimentation Velocity of Mixtures of Low Density Lipoprotein, P, and Dextran Sulfate, D (sedimentation from left to right).

The molar ratio of mixing, $(P_0)/(D_0)=R$, used in each experiment is indicated, the concentration of D being 1.25 mg/ml in each mixture. The values (in Svedberg unit) under the base line indicate sedimentation coefficients of the maximum ordinate position (s_H) and the apparent range of sedimentation coefficients of each reaction boundary. All patterns were recorded 14 min after reaching speed (52,640 rpm) at bar angle 55° .

TABLE I. $\bar{s}$ VALUES OF THE REACTION BOUNDARIES OF THE DEXTRAN SULFATE-LIPOPROTEIN MIXTURES

$(P_0)/(D_0)$	$\bar{s}(S)$	$(P_0)/(D_0)$	$\bar{s}(S)$
0.017	16.3 ₅	0.232	17.5 ₉
0.058	16.7 ₇	0.344	18.7 ₀
0.099	16.9 ₇	0.688	21.2 ₀
0.139	17.1 ₀	0.987	25.6 ₀
0.172	17.3 ₀	1.720	20.0 ₀

concentration of P, which must occur in the region of the faster moving boundary.¹⁴⁾ The observed faster moving boundary is the algebraic sum of the contributions of all components and thus it is not possible to define uniquely the required median bisector needed for the evaluation of $\bar{s}_P$. It is clear that the maximum ordinate position does not provide a suitable approximation since it fails to reflect the increasing asymmetry of the boundary as the $(P_0)/(D_0)$ ratio increases (Fig. 1): indeed sedimentation coefficients calculated using the maximum ordinate positions, s_H , were insensitive to changes in the $(P_0)/(D_0)$ ratio. Accord-

FIG. 2. The Plot of $\bar{s}$ *vs* $(P_0)/(D_0)$.

ingly, the rate of movement of the square root of the second moment of the faster moving boundary, $\bar{s}$, was taken as a measure of $\bar{s}_P$.

Figure 2 shows the plot of $\bar{s}$ *vs* $(P_0)/(D_0)$ which is linear up to $(P_0)/(D_0)=0.69$. When the ratio becomes 0.98, $\bar{s}$ deviates significantly from linearity indicating either that (P) is no longer negligible or that the formation of complexes of the type D_jP_i ($j>1, i>1$) commences. The

low value of $\bar{s}$ (Table I) observed at $(P_0)/(D_0) = 1.72$ is obviously due to partial precipitation of the complexes. Since insoluble complexes, consisting of a higher proportion of P, are removed from solution phase in the early stage of sedimentation as evidenced by the anomalously small area under the reaction boundary (Fig. 1e), the $(P_0)/(D_0)$ ratio of the remaining solution would be lowered and hence the use of the initial mixing ratio as a parameter of the mixture is no longer valid. The values of q and n calculated according to Eq. (6) are 7.8S and 5.5, respectively. As the molar ratio of P to D in the insoluble aggregates is approximately 6, precipitation of the soluble complex, DP_6 (or DP_5), involves no further binding of P. Thus, it appears that the driving force toward precipitation of DP_6 , which carries both positive and negative charges, is similar to that operating in the precipitation of proteins in isoelectric region.

In Table I were given $\bar{s}$ values of several mixtures for numerical comparison. It is of interest now to see the size change of the soluble complexes as a function of $(P_0)/(D_0)$. For comparison, writing $\bar{s}$ values of the soluble complexes formed at the $(P_0)/(D_0)$ ratios, 0.69 and 0.98 (Fig. 2), after Eq. (5); $21.2S = 8.5S + 7.8S \times 1.63$ and $25.6S = 8.5S + 7.8S \times 2.19$, it is clear that the complex, in average, exists as $DP_{1.6}$ at the ratio 0.69 and as $DP_{2.2}$ at 0.98.

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APPENDIX

For the interaction described by $D + iP_i \rightleftharpoons DP_i$ ($i=1, 2, 3, 4 \dots n$), the following relation¹⁵⁾ is valid provided that the n binding sites are identical and independent.

$$\frac{r}{n-r} = K(P) \quad (1')$$

where K is the intrinsic association constant. Combination of Eq (4) and (1') yields

$$(P)^2 + \left[n(D_0) - (P_0) + \frac{1}{K} \right] (P) - \frac{(D_0)}{K} \frac{(P_0)}{(D_0)} = 0$$

or

$$(P) = \frac{1}{2} \left[\left(\alpha^2 + \frac{4(D_0)}{K} R \right)^{1/2} - \alpha \right] \quad (2')$$

where $\alpha \equiv n(D_0) - (P_0) + 1/K$ and $R \equiv (P_0)/(D_0)$. It is obvious from Eq. (2') that

$$(P) \rightarrow 0 \text{ as } R \rightarrow 0.$$

Indeed, if K is sufficiently large (noting that in the present experiment (D_0) is kept constant) (P) becomes insignificant at finite value of R as found in the present system.