The Structure of Bovine Serum Albumin at Low pH*

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ABSTRACT: Low-angle X-ray scattering, diffusion coefficients, and intrinsic viscosities have been calculated for several models of bovine serum albumin at low pH. These models are composed of spherical subunits, and include trimers, tetramers, and monomer–dimer mixtures in which the monomer is trimeric.

Good agreement with experiment at pH 3.6 is obtained if a linear trimer model is used, in which the radius of the central sphere is 26.6 A, that of the two flanking spheres is 19.0 A, and adjacent subunits are touching. These results suggest that no substantial change in over-all dimensions of the protein has occurred by pH 3.6. At lower pH, expansion is considerable.

At pH values below about 4, bovine serum albumin (BSA)[1] undergoes a striking, reversible structural transition. Since the discovery of this phenomenon in 1952, an enormous number of studies has been undertaken to elucidate the nature and details of the transition. (See Foster (1960) for a comprehensive review with references.) Many aspects of the process are well understood in broad outline, but the question of the exact structure of the protein at acid pH, and how it differs from that before the transition, has not yet been satisfactorily answered in quantitative terms. The experimental evidence from various laboratories which should help to answer this question has thus far seemed to give rise to incompatible results. It is the purpose of this work to compute the low-angle X-ray scattering curves, the intrinsic viscosity, and the diffusion coefficients of the various plausible models for BSA at low pH, to examine the agreement of these computations with experiment, and to show that the data are all consistent with a particular model for the protein.

Models

The models that have been suggested for BSA at pH 3.6 are either ellipsoids of revolution of nonconstant density or subunit models. Luzzati *et al.* (1961) have thoroughly investigated the low-angle X-ray scattering from BSA solutions at pH 5.3, where the protein has its native configuration, and at pH 3.6. On the basis of this study they propose an ellipsoid of revolution model consisting of a compact core which comprises about 65% of the protein and an extended envelope which contains the remaining 35%. This model is some 300 A long and is represented by model A in Figure 1.

Models B and C represent two conceivable spherical subunit models. The subunit structures were suggested by early work of Weber (1952) and of Harrington *et al.* (1956) on fluorescence depolarization, and more recently by the studies of Foster (1960) on detergent binding and Weber and Young (1964a,b) on the pepsin-catalyzed hydrolysis of BSA in acid. Weber and Young found that pepsin digestion at pH 3.0 results in subunits of mol wt 30,000 and 11,000–12,000. They proposed that intact BSA consists of one heavy fragment and two or three identical light fragments linked together by peptide chains. Consequently both trimer and tetramer models have been considered (Figure 1, B and C, respectively).

In the subunit models all the segments are assumed to be of equal density and impenetrable to solvent (for hydrodynamic purposes) and of constant and equal electron density (for X-ray scattering purposes). The segments are taken to be spherical with radii R_I for the small spheres and R_{II} for the large sphere. The distance between the centers of two adjacent subunits is $2L$, while the distance of closest approach of adjacent spheres is indicated by δ. It is assumed that the links connecting the segments do not contribute to the hydrodynamic or scattering properties. $2L$ will doubtless be a function of such solution variables as pH and ionic strength, but will be treated here simply as a parameter whose value is to be determined by the best fit to the data.

Models B and C are both represented as linear models because calculations, not presented here, of the properties of various bent models indicate that linear configurations give low-angle X-ray scattering in slightly better accord with experiment. Further, it has been found that calculated properties are not very sensitive to where the large subunit is located in the chain. Model D in Figure 1 is a plausible structure of a dimer of BSA if the monomer is composed of three subunits.

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[1] Abbreviation used in this work: BSA, bovine serum albumin.

Theory

Scattering Properties. Luzzati and co-workers (1961) have shown that the observed X-ray scattering is consistent with that which would be observed from two concentric ellipsoids. To test subunit models we need to calculate the low-angle X-ray scattering from a rigid assembly of spheres. In such a calculation we must take into account the scattering from each sphere individually, and the interference of scattering from each pair of spheres. It is well known (see, *e.g.*, James, 1962) that the intensity of scattering of a point beam of X-rays of wavelength λ through an angle 2θ by a single sphere of radius R is

$$I(h) = CR^6\Phi^2(hR) \tag{1}$$

where C is a constant, $\Phi(x)$ is the scattering function for spheres

$$\Phi(x) = 3(\sin x - x\cos x)/x^3 \tag{2}$$

and

$$h = 4\pi \sin\theta/\lambda \tag{3}$$

Similarly, the contribution to scattered intensity by interference between two spheres of radii R_i and R_j whose centers are separated by a distance L_{ij} is (James, 1962)

$$I(h) = CR_i^3R_j^3\Phi(hR_i)\Phi(hR_j)(\sin hL_{ij})/hL_{ij} \tag{4}$$

By combining (1) and (4), we find that the trimer, model B, exhibits a scattering curve, normalized to unit intensity at zero scattering angle, expressed by

$$\begin{aligned} i_3(h) = (2 + r^3)^{-2}\{&2\Phi^2(hR_I) \times \\ &[1 + (\sin 4Lh)/4Lh] + r^6\Phi(hR_{II})^2 + \\ &4r^3\Phi(hR_I)\Phi(hR_{II})(\sin 2Lh)/2Lh\} \end{aligned} \tag{5}$$

where $r = R_{II}/R_I$. The tetramer, model C, has a normalized scattering curve

$$\begin{aligned} i_4(h) = (3 + r^3)^{-2}\{&\Phi(hR_I)^2[3 + (4\sin 2Lh)/2Lh + \\ &(2\sin 4Lh)/4Lh] + r^6\Phi(hR_{II})^2 + \\ &2r^3\Phi(hR_I)\Phi(hR_{II})[(\sin 2Lh)/2Lh + \\ &(\sin 4Lh)/4Lh + (\sin 6Lh)/6Lh]\} \end{aligned} \tag{6}$$

The six-subunit dimer, model D, with adjacent spheres touching, gives the scattering curve

$$\begin{aligned} i_6(h) = (4 + 2r^3)^{-2}\{&2\Phi(hR_I)^2 \times \\ &[2 + (2\sin hL_{13})/hL_{13} + (2\sin hL_{14})/hL_{14} + \\ &(\sin hL_{16})/hL_{16} + (\sin hL_{34})/hL_{34}] + \\ &2r^6\Phi(hR_{II})^2[1 + (\sin hL_{25})/hL_{25}] + \\ &4r^3\Phi(hR_I)\Phi(hR_{II})[(2\sin hL_{12})/hL_{12} + \\ &(\sin hL_{15})/hL_{15} + (\sin hL_{24})/hL_{24}]\} \end{aligned} \tag{7}$$

where

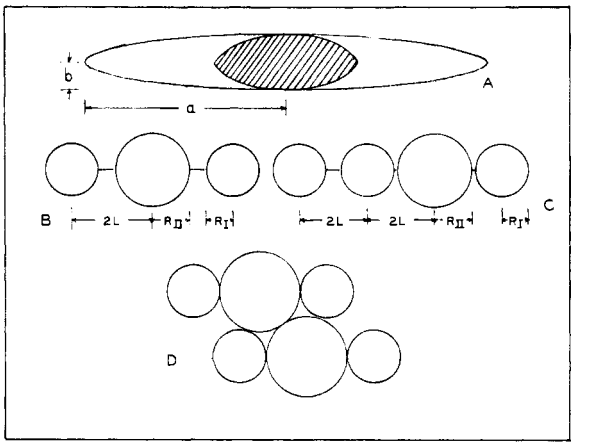

FIGURE 1: Models for BSA at pH 3.6.

$$\begin{aligned} L_{12} = R_I(1 + r) = L_{24},\ L_{13} = 2R_I(1 + r) \\ L_{14} = 2rR_I = L_{25},\ L_{15} = (1 + 2r + 9r^2)^{1/2}R_I \\ L_{16} = 2(1 + 2r + 4r^2)^{1/2}R_I \\ L_{34} = 2(1 + 2r)^{1/2}R_I \end{aligned} \tag{8}$$

The scattering i_{md} observed from a monomer–dimer mixture in which the weight fraction of dimer is f is

$$i_{md}(h) = (1 - f)i_m(h) + fi_d(h) \tag{9}$$

where i_m and i_d are the normalized scattering intensities for monomer and dimer, respectively.

The above equations have been derived for an incident beam of point cross section. Experimentally it is usually necessary to use a different geometry, in which the beam may be idealized as infinitely narrow and infinitely high. In this case the observed intensity, $j(h)$, will be (Guinier and Fournet, 1955)

$$j(h) = 2\int_0^\infty i(\sqrt{h^2 + y^2})\mathrm{d}y \tag{10}$$

Methods for extracting molecular parameters from data obtained with infinite slit geometry have been developed by Guinier and Fournet (1947) and by Luzzati (1960).

From (2) it may be seen that, as x gets very large compared to unity, $\Phi(x)$ will vary asymptotically as $3x^{-2}$, with superimposed oscillations of pseudoperiod $2\pi/x$. On squaring and averaging, the $\cos^2 x$ term gives a factor of 0.5. If (5), (6), and (7) are expanded in powers of h^{-1}, terms that die off more rapidly with h than h^{-4} are neglected, and the results substituted in (10), we obtain for the trimer, tetramer, and six-unit dimer, respectively

$$i_3(h) \approx 9\pi(2 + r^2)[2R_I^2(2 + r^3)]^{-2}h^{-3} \tag{11}$$

$$i_4(h) \approx 9\pi(3 + r^2)[2R_I^2(3 + r^3)]^{-2}h^{-3} \tag{12}$$

$$i_6(h) \approx 9\pi(4 + 2r^2)[2R_I^2(4 + 2r^3)]^{-2}h^{-3} \tag{13}$$

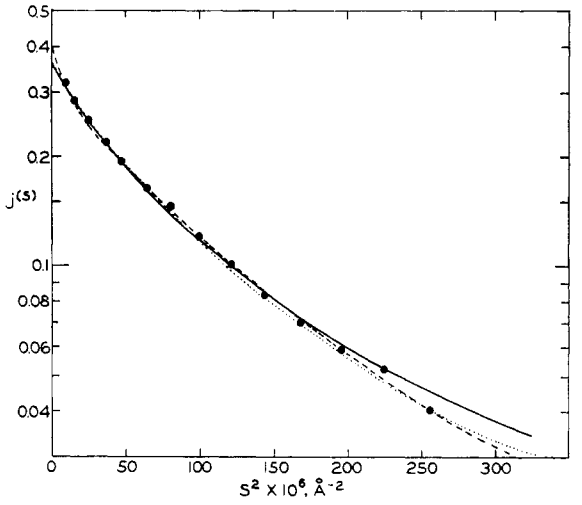

FIGURE 2: Guinier plot of low-angle X-ray scattering from BSA at pH 3.6. Experimental points (●) are those of Luzzati *et al.* (1961). Curves for model B (———), model C (— —), and an 80–20% mixture of models B and D (----) are calculated with the dimensions shown in Table I.

According to these equations, $h^3j(h)$ should be a constant for large h (Porod, 1951a,b), depending only on the radii of the segments and not on the distances between them. An ellipsoid of revolution model can also be shown (Beeman *et al.*, 1957) to give a horizontal plot of $h^3j(h)$ *vs.* h^3. Following Luzzati *et al.* (1961), we shall use in the following calculations not h but the related variable s, where $s = h/2\pi$.

Hydrodynamic Properties. Methods of calculating intrinsic viscosity and diffusion coefficients for ellipsoids are well known. The hydrodynamic properties of subunit models may also be easily calculated. The diffusion coefficient for an assembly of rigid spheres may be calculated by a generalization of a result due to Kirkwood (1949, 1954). He has demonstrated that for a chain of n identical elements of frictional coefficient ζ, the translational diffusion coefficient is

$$D = kT\left[1/n\zeta + (1/6\pi\eta n^2)\sum_{l=1}^{n}\sum_{\substack{s=1\\ s\neq l}}^{n}\langle L_{ls}^{-1}\rangle_{av}\right] \quad (14)$$

where η is the viscosity of the solvent, and the average is taken over all intramolecular configurations. It can be shown (Bloomfield, V., 1965, unpublished) that the appropriate generalization of (14) for a chain of nonidentical segments is

$$D = kT\left\{\left(\sum_{l=1}^{n}\zeta_l\right)^{-1} + \left[6\pi\eta\left(\sum_{l=1}^{n}\zeta_l\right)^2\right]^{-1}\sum_{l=1}^{n}\sum_{\substack{s=1\\ s\neq l}}^{n}\zeta_l\zeta_s\langle L_{ls}^{-1}\rangle_{av}\right\} \quad (15)$$

where ζ_l is the frictional coefficient of segment l.

For the rigid models, composed of spherical subunits, under examination, (15) becomes

$$D = kT/6\pi\eta\left[\left(\sum_{l=1}^{n}R_l\right)^{-1} + \left(\sum_{l=1}^{n}R_l\right)^{-2}\sum_{l=1}^{n}\sum_{\substack{s=1\\ s\neq l}}^{n}R_lR_sL_{ls}^{-1}\right] \quad (16)$$

where the Stokes law expression, $\zeta_l = 6\pi\eta R_l$, has been used.

It can be shown that D calculated in this way is equal to that of an ellipsoid of revolution with the same axial ratio but with dimensions decreased by 7%. The intrinsic viscosity for the subunit models is also assumed to be that for the same equivalent ellipsoid of revolution. Thus

$$[\eta] = (4\pi/3)ab^2N_a\nu(a/b)/(1.07)^3M \quad (17)$$

where N_a is Avogadro's number, M the molecular weight, $\nu(a/b)$ is the shape factor for viscosity calculated by Simha (1940), $b = R_{II}$, and $2a$ is the total length of the molecule.

This treatment of $[\eta]$ assumes in effect that the Scheraga–Mandelkern (1953) β-function has the value 2.12×10^6 appropriate to ellipsoids of low axial ratio. It is possible that a more exact calculation of $[\eta]$ would lead to a β closer to the experimental value (see below) of 2.07×10^6. Computed in this manner $[\eta]$ would then be 7% lower than that calculated from (17). Compared to the differences found in $[\eta]$ among the various models, this difference is slight.

Results

Scattering. Figure 2 is a Guinier (1939) plot of observed and calculated scattered intensity against s^2 from BSA at pH 3.6 and a protein concentration of 1.59%. The large dots represent the experimental points obtained by Luzzati *et al.* (1961). The curve for the trimer is calculated, using (10), from (5); that for the tetramer from (6); and that for the monomer–dimer mixture from (5) and (7). This last was calculated assuming 20% by weight of dimer, a value corresponding to the molecular weight of 81,000 found by Luzzati and co-workers for this preparation. The molecular dimensions used in these calculations are shown in the second column of Table I. Numerical computations were performed on an IBM 7094 digital computer. The calculated curves have been adjusted to coincide with the experimental point for the smallest value of s.

Agreement between the shapes of the experimental curve and of those calculated for the subunit models is at least as good as, if not better than, that of the ellipsoid model. The calculated curve is rather sensitive to the dimensions assumed; calculations with several other sets of dimensions differing by only 5–10% from those listed in Table I gave markedly poorer fit.

TABLE I: Dimensions and Hydrodynamic Properties.

Model	Dimensions (A)	$D \times 10^7$ (cm²/sec)	$[\eta]$ (cc/g)
A	$a = 150$, $b = 19.8$	3.92	17.7
B	$R_I = 20.0$, $R_{II} = 27.2$, $2L = 50.0$	5.78	5.44
C	$R_I = 21.0$, $R_{II} = 29.6$, $2L = 50.0$	4.61	11.3
B	$R_I = 19.0$, $R_{II} = 26.6$, $2L = 45.6$[a]	6.11	4.62
Expt		5.93[b]	4.5[c]
		5.42[d]	5.94[d]

[a] These dimensions of B were determined by optimizing fit of calculated to experimental X-ray scattering curves from an 80% B, 20% D mixture. [b] Harrington *et al.* (1956). [c] Tanford *et al.* (1955). [d] Champagne (1957).

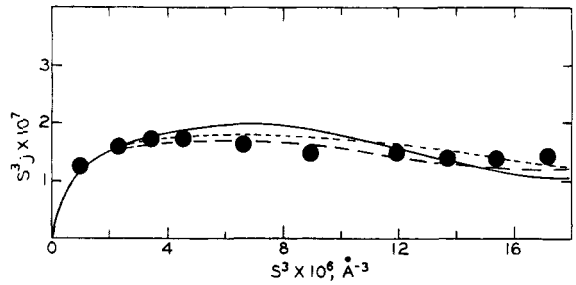

FIGURE 3: Plot of $s^3j(s)$ *vs.* s^3 for BSA at pH 3.6, showing experimental points (●) of Luzzati *et al.* (1961), and curves for model B (———), model C (— —), and an 80–20% mixture of models B and D (---) calculated with the dimensions shown in Table I.

Figure 3 is a plot of s^3j *vs.* s^3, suggested by Porod (1951a,b), which emphasizes larger angle scattering. The tetramer seems to give the best fit. The trimer, by itself, does not agree too well with the experimental points, but a 3-subunit monomer–dimer mixture is well within experimental error. Williams and Foster (1960) have demonstrated that BSA has a very strong tendency to dimerize around pH 3.5, so that the assumption of appreciable dimer in the X-ray scattering experiments is reasonable, and, indeed, was suggested by Luzzati and co-workers (1961) themselves. As mentioned above, the ellipsoidal model would also be expected to give an essentially horizontal scattering curve at large s on this plot. Evidently, these calculations alone are insufficient to distinguish definitely among the various models shown in Figure 1, although the poor agreement of the scattering due to trimer alone with experiment in the s^3j *vs.* s plot weighs against this species being the only source of scattering.

Hydrodynamic Properties. In the third and fourth columns of Table I are presented the diffusion coefficients and intrinsic viscosities of the various models, calculated according to (16) and (17) with the dimensions used to compute the scattering curves. D and $[\eta]$ for the ellipsoidal model were calculated by Luzzati *et al.* (1961). At the bottom of Table I are experimental values of D and $[\eta]$ at pH 3.6. The only direct detailed study of D in the acid pH range is that of Champagne (1957). From the study of Harrington *et al.* (1956) of the dependence of sedimentation coefficient on pH in acid solution, and use of the equation

$$S = DM(1 - \bar{v}_2\rho)/RT \qquad (18)$$

with $M = 66{,}000$ and $\bar{v}_2 = 0.734$ for BSA, the other value of D in Table I has been calculated. The intrinsic viscosity as a function of pH in the acid range has been studied by Champagne (1957) and by Tanford *et al.* (1955). Because of the close attention paid by the latter group to a time-dependent aggregation phenomenon, the value of 4.5 cc/g at pH 3.6 may be more truly representative of the intrinsic viscosity of monomeric BSA.

On comparing the calculated and experimental results it is immediately evident that both the ellipsoidal model A, as pointed out by Luzzati *et al.* (1961), and the tetramer model C are too large hydrodynamically. Similar calculations, not shown here, have demonstrated that it also is impossible to obtain simultaneous satisfactory agreement of scattering and hydrodynamic behavior for a two subunit, dumbbell type model. This structure was suggested by Harrington *et al.* (1956) and recently has received support from the enzymatic degradation studies, performed at basic pH, of Adkins and Foster (1965). The presence of 20% dimeric material was assumed in the scattering calculation, while the intrinsic viscosity for a dumbbell was calculated from the results of Riseman and Ullman (1951).

The larger trimer model B, in the second line of Table I, has a diffusion coefficient in good agreement with experiment, though the intrinsic viscosity is considerably larger than that found by Tanford and coworkers (1955). If we consider a trimer of slightly smaller dimensions (fourth line of Table I), we get an $[\eta]$ in much better agreement with that of Tanford *et al.* (1955), and a D only slightly greater than that calculated from the sedimentation coefficient of Harrington *et al.* (1956). The dimensional differences between this trimer and the larger one are slight, about an angstrom difference in segment size and the spheres touching rather than 2.8 A apart, but the resulting differences in hydrodynamic and scattering properties are considerable.

A Scheraga–Mandelkern (1953) β can be calculated from $[\eta]$ and D. Champagne's values give $\beta = 2.08 \times 10^6$, while combination of the results of Tanford *et al.* (1955), and Harrington *et al.* (1956) gives $\beta = 2.07 \times 10^6$. As mentioned above, use of the latter value instead

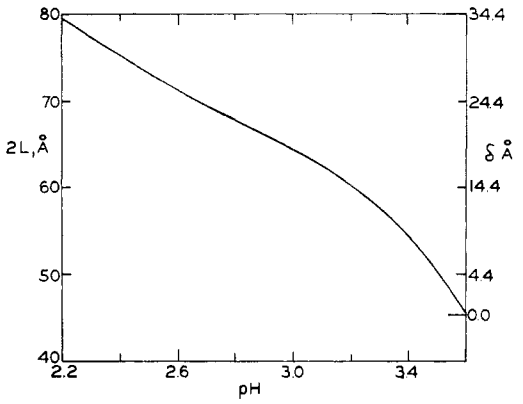

FIGURE 4: Center-to-center distance, $2L$, and distance of closest approach, δ, of adjacent spheres of model B as a function of pH, calculated from the data of Harrington *et al.* (1956) and eq 16 and 18.

of 2.12×10^6 appropriate to ellipsoids would predict $[\eta]$ of 4.31 cc/g for the smaller trimer and 5.07 cc/g for the larger trimer. The smaller model would still give considerably better agreement with experiment.

Discussion

It thus appears, on the basis of these studies, that a trimer consisting of two spherical subunits of radius 19.0 A and one of 26.6 A, with adjacent spheres touching, gives the best agreement for monomeric BSA at pH 3.6. The volume of this trimer is 136,000 A^3, and its surface to volume ratio is 0.132 A^{-1}. These values do not agree well with those obtained experimentally by Luzzati *et al.* (1961): 245,000 A^3 and 0.17 A^{-1}, respectively. However, the experimental values undoubtedly reflect the presence of significant amounts of dimer and are highly dependent on the higher angle scattering which is subject to considerable experimental uncertainty because of its low intensity at the low concentration employed. Moreover, the maximum volume that a particle with the molecular weight and intrinsic viscosity of BSA at pH 3.6 could have is 197,000 A^3, according to the Einstein theory for the viscosity of suspensions of spheres. A nonspherical particle would have even a smaller volume.

There are two additional pieces of experimental work which may be cited in support of this model. First, support for the values of R_I and R_{II} obtained above comes from diffusion and fluorescence depolarization measurements of Weber and Young (1964a) on the separated subunits. The rotational relaxation times ρ and Kirchoff's expression $\rho = 4\pi\eta R^3/kT$ give $R_I = 18.8$ A and $R_{II} = 26.6$ A. The diffusion coefficients and the Stokes–Einstein relation $D = kT/6\pi\eta R$ give $R_I = 19.8$ A and $R_{II} = 26.9$ A. This agreement between the model and the experimental results is remarkable.

Second, support for the close approach of adjacent subunits is obtained from the solvent perturbation studies of Herskovits and Laskowski (1960, 1962). These workers found that difference spectral changes of BSA in high ionic strength occurred with sucrose from pH 4.2 to 3.8; *i.e.*, this is the region of change of accessibility of tyrosyl chromophores. Thus, by pH 3.6 the molecule should have expanded only very slightly.

Decreasing the pH further, however, results in substantial separation of the subunits, as shown in Figure 4. If we assume that the considerable increase in frictional resistance of BSA below pH 4 is due solely to increase in the intersegment distance $2L$ without change in R_I or R_{II}, it is possible to calculate $2L$ as a function of pH from the sedimentation data of Harrington *et al.* (1956) and (16) and (18). Plots of $2L$ and δ *vs.* pH are shown in Figure 4. That the subunits themselves do not change size with pH is suggested by the fact that the rotational relaxation times of the subunits are essentially constant between pH 2 and 7 (Weber and Young, 1964a).

Summary

The major results of this study are the following: (1) a model for BSA at pH 3.6 which agrees well with low-angle X-ray scattering and hydrodynamic measurements is a covalently bonded trimer, with two spheres of radius 19.0 A separated by one of radius 26.6 A. About 20% of dimeric BSA was apparently present in the scattering experiments. (2) Adjacent subunits are just touching at pH 3.6, but are separated by about 34 A at pH 2.2. (3) A tetramer model with three small subunits and one large one is too large hydrodynamically. Since only about 54,000 of the 66,000 molecular weight is accounted for by the three subunits of a trimer, the remainder may reside in polypeptide chains connecting the subunits.

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The Isolation and Characterization of 2-Hydroxyphenazine from *Pseudomonas aureofaciens**

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ABSTRACT: 2-Hydroxyphenazine was isolated from culture supernatant liquid of *Pseudomonas aureofaciens* by solvent extraction, ion-exchange chromatography, and crystallization.

The compound was identified by comparison with chemically synthesized 2-hydroxyphenazine by paper chromatography and electrophoresis, infrared and ultraviolet spectrophotometry, and X-ray diffraction. Some preliminary studies on the biosynthesis of the compound are presented.

In the course of studies on the biosynthesis of phenazine-1-carboxylic acid (Levitch and Stadtman, 1964), a contaminant was observed during the early stages of isolation of the acid. Ultraviolet spectra of alkaline solutions showed maxima at 365 and 252 mμ, characteristic of the 1-carboxylic acid derivative, and an additional peak at 275 mμ. Partition of the mixture between chloroform and 1% aqueous sodium bicarbonate indicated that the contaminating compound was somewhat less acidic than phenazine-1-carboxylic acid, although a complete separation could not be effected by this procedure.

Haynes and his co-workers (1956) had described a phenolic fraction from *P. aureofaciens* which was insoluble in sodium bicarbonate solution, but gave no other chemical data. Kluyver (1956) described a red pigment which was isolated from chloroform extracts of *P. aureofaciens* cultures by column chromatography on alumina. Elemental analysis of this compound suggested to Kluyver an empirical formula of $C_{13}H_8N_2O_3$, for which he postulated a structure of (1) a hydroxyphenazine-1-carboxylic acid or (2) a phenazinecarboxylic acid *N*-oxide. Since the compound which exhibited an absorption maximum at 275 mμ in our alkaline extracts was also red and had weakly acidic properties, the possibility existed that this compound, the phenolic fraction of Haynes *et al.*, and the red pigment of Kluyver were the same compound. In this paper we report the isolation and characterization of this compound and some preliminary studies on its biosynthesis.

Experimental Procedure

Growth of the organism and cultural conditions for large-scale production (about 20 l. of medium) of phenazine compounds by *P. aureofaciens* are as described by Haynes *et al.* (1956). The chloroform extraction procedure was described in a previous publication (Levitch and Stadtman, 1964). The chloroform solution containing the phenazine compounds was extracted with a minimal amount of 0.1 N sodium hydroxide solution, which was then adjusted to pH 7.0 with 1 N HCl plus enough 1 M sodium phosphate to bring the phosphate concentration to 0.01 M. The substances in the

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