For very small values of (OAA) [high -log (OAA)], the term

$$\phi_{12}/\phi_1 + \frac{\phi_{12}}{K'_I}$$

will dominate the denominator, and for sufficiently small (OAA) values 1/slope will again approach zero. There must, therefore, be a maximum value of 1/slope in the range of (OAA) somewhere. Since (OAA) is confined to the denominator, when the derivative of the denominator with respect to (OAA) becomes zero, *i.e.*, when

$$\phi_1/(\phi_1 K'_I + \phi_{12}) - \phi_{12}/\left(\phi_1 + \frac{\phi_{12}}{K'_I}\right)\frac{1}{(\text{OAA})^2} = 0$$

1/slope has the maximum value.

Thus, the plot of 1/slope versus −log (OAA) will be a bell-shaped curve with a *maximum* when

$$(\text{OAA})^2_{max} = \phi_{12}K'_I/\phi_1 \qquad (3)$$

Substituting the above equation in equation (1), the maximum value of 1/slope is

$$1/\text{slope}_{max} = \left(\sqrt{\phi_1} + \sqrt{\phi_{12}/K'_I}\right)^{-2} \qquad (4)$$

If $(\text{OAA})_1$ is the oxalacetate concentration at which

$$(1/\text{slope})/(1/\text{slope}_{max}) = 1/2$$

on the left hand side of the bell-shaped curve and $(\text{OAA})_2$ is the concentration of oxalacetate at the corresponding point on the right hand side of the curve then

$$(\text{OAA})_1 = \frac{\phi_1 K'_I + \phi_{12}}{\phi_1} \qquad (5)$$

and

$$(\text{OAA})_2 = \frac{\phi_{12}}{\phi_1 + \frac{\phi_{12}}{K'_I}} \qquad (6)$$

Since the values of $(\text{OAA})_{max}$, $1/\text{slope}_{max}$, $(\text{OAA})_1$, and $(\text{OAA})_2$ can be read directly from the curve of 1/slope versus −log (OAA) (Fig. 3), ϕ_1, ϕ_{12}, and K'_I can be calculated by using equations (3), (4), (5), and (6). A correction in the values of $(\text{OAA})_1$ and $(\text{OAA})_2$ obtained from the bell-shaped curve is necessary if the negative logs of their values differ by less than three units. Correct values given in the Table were obtained by computer calculation.

A similar treatment for the intercept can be derived readily.

ACKNOWLEDGMENT

The authors are grateful to Dr. W. W. Cleland for valuable advice and for making available computer programs facilitating statistical analysis of the data.

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The Use of Deuterated DPNH Mixtures as an Aid in Establishing Dehydrogenase Mechanism*

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Received August 10, 1962

The use of a mixture of the stereoisomers of DPND as a coenzyme in dehydrogenase reactions may provide information permitting a distinction between reaction mechanisms. In the case of an equilibrium mechanism, there is a linear relationship between the reciprocal of the initial velocity (v) and the reciprocal of either the substrate (S) or coenzyme (C) concentration. For steady-state mechanisms, however, $1/v$ varies linearly with $1/C$, but not with $1/S$. Data are presented for two enzymes, yeast alcohol dehydrogenase and rabbit muscle lactate dehydrogenase, which obey different mechanisms.

The kinetic equations for an enzyme acting on a mixture of two substrates have been derived for unimolecular reactions. Whether a steady-state (Reiner, 1959) or a rapid-equilibrium (Thorn, 1949) mechanism is postulated, the following expression holds:

$$v = \frac{V_1 S_1}{S_1 + K_1(1 + S_2/K_2)} + \frac{V_2 S_2}{S_2 + K_2(1 + S_1/K_1)} \qquad (1)$$

where S_1 and S_2 are the concentrations of the two substrates, K_1 and K_2 are the Michaelis constants, and V_1 and V_2 are the maximum velocities obtainable with each substrate individually.

When the concentration of S_1 is any fraction a of

* This work was performed under the auspices of the U. S. Atomic Energy Commission.

S_t, the total concentration, and that of S_2 is $(1 - a)\,S_t$, the following expression is obtained:

$$\frac{1}{v} = \frac{aK_2 + (1 - a)K_1}{aV_1K_2 + (1 - a)V_2K_1} + \frac{K_1K_2}{aV_1K_2 + (1 - a)V_2K_1} \cdot \frac{1}{S_t} \quad (2)$$

so that from a Lineweaver-Burk plot the apparent V_{max} and K_m can be established.
Thus

$$V_{max} = \frac{aV_1K_2 + (1 - a)V_2K_1}{aK_2 + (1 - a)K_1}$$

and

$$K_m = \frac{K_1K_2}{aK_2 + (1 - a)K_1}$$

If the individual values of K_m and V_{max} for each substrate are known, together with the mole fraction of each component, the values for the apparent V_{max} and K_m can be predicted.

The problem of a bimolecular reaction involving enzyme, substrate, and a mixture of coenzymes apparently has not been considered. In the course of studies in this laboratory on the effect of deuteration of DPNH on various dehydrogenases, some observations on the behavior of DPND prepared by chemical reduction of DPN in D_2O suggested that a consideration of various mechanisms of bimolecular reactions with a coenzyme mixture would be profitable. It will be shown that the use of a coenzyme mixture may in some cases provide a simple means for establishing enzyme mechanisms of dehydrogenases and may be a useful adjunct to the procedures recently reviewed by Baker and Mahler (1962).

Methods

Coenzymes.—DPND was prepared by reduction of DPN by hydrosulfite in D_2O (Lehninger, 1957). This method of preparation yields a product containing a mixture of the α- and β-DPND stereoisomers. The α-form predominates, making up 56% of the total, according to Fisher *et al.* (1953), or 72%, according to Pullman *et al.* (1954). Both enzymes employed are α-stereospecific (Vennesland, 1956), so that with some of the coenzyme molecules there would be a primary isotope effect, and with the remainder a secondary effect.

Enzymes.—Yeast alcohol dehydrogenase and rabbit muscle lactate dehydrogenase were commercial preparations (Sigma), which were used without further purification.

Assay.—The assay was based on the rate of reduction of DPNH, measured at 340 mμ at 25° in a Beckman spectrophotometer equipped for automatic recording. For both enzymes, 0.03 M potassium phosphate buffer, *p*H 7.4, was used, with concentrations of DPNH or DPND from 2×10^{-5} to 2×10^{-4} M. Concentrations of acetaldehyde ranged from 1.48×10^{-4} to 8.88×10^{-4} M, and those of pyruvate from 1.07×10^{-4} to 5.33×10^{-4} M. Initial rates were obtained by extrapolation from the chart readings.

Analysis.—The data were treated by the method of Dalziel (1957). For each concentration of substrate employed, the reciprocal of the velocity was plotted against the reciprocal of the coenzyme concentration. Both the slopes and intercepts of the five or six lines thus obtained were then plotted against the reciprocal of the substrate concentration, and from the slopes and intercepts of the secondary plots the characteristic reaction constants could be determined. Curves were fitted by the method of least squares, except for those shown in Figure 4.

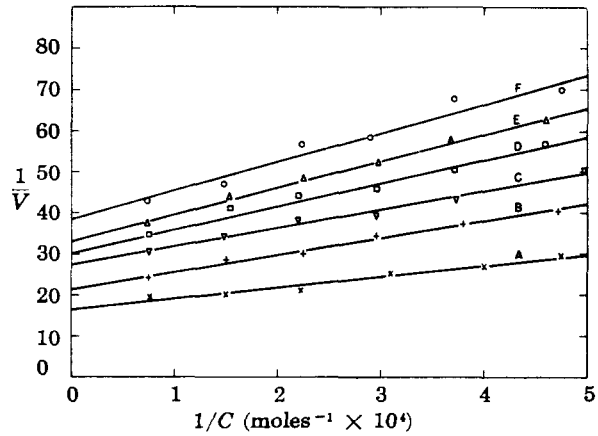

Fig. 1.—Kinetic data for yeast alcohol dehydrogenase. The ordinate is the reciprocal of the optical density change per unit time. Letters on the lines represent acetaldehyde concentrations used (see Fig. 3). Standard errors are shown in Figure 3.

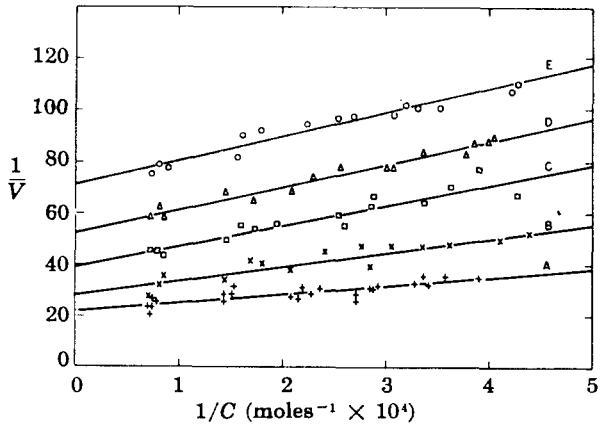

Fig. 2.—Kinetic data for rabbit muscle lactic dehydrogenase. Composite data from three experiments. (See also Fig. 1.)

Results

The plots of $1/v$ *vs.* 1/DPND for alcohol dehydrogenase and lactate dehydrogenase are shown in Figures 1 and 2. The corresponding secondary plots are shown in Figures 3 and 4. It is clear that straight lines can be readily fitted to the sets of points on Figure 3, but not to those on Figure 4. A secondary plot for lactate dehydrogenase with DPNH is shown in Figure 5, in order to show that with a single coenzyme linear relationships are obtained between the reciprocal of the pyruvate concentration and the slopes and intercepts.

Discussion

A number of mechanisms for bimolecular reactions have been considered by various authors (Segal *et al.*, 1952; Alberty, 1953, 1958; Dalziel, 1957; Reiner, 1959; Baker and Mahler, 1962). The general form (Dalziel, 1957) of the rate equation is:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{C} + \frac{\phi_2}{S} + \frac{\phi_{12}}{SC} \quad (3)$$

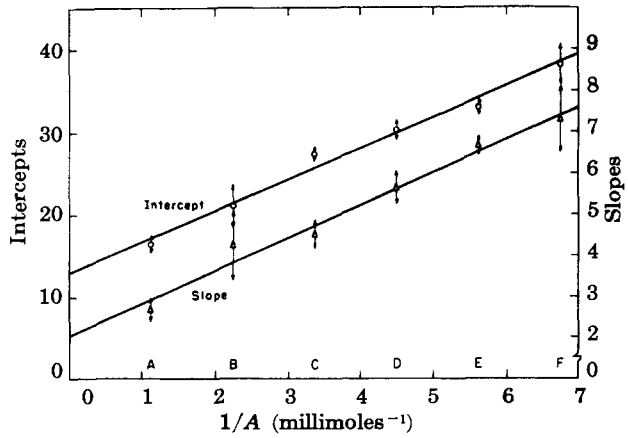

FIG. 3.—Slope and intercept plots (from Fig. 1) for alcohol dehydrogenase. Vertical arrows represent standard errors. Letters on the abscissa designate the concentration of acetaldehyde employed in Figure 1.

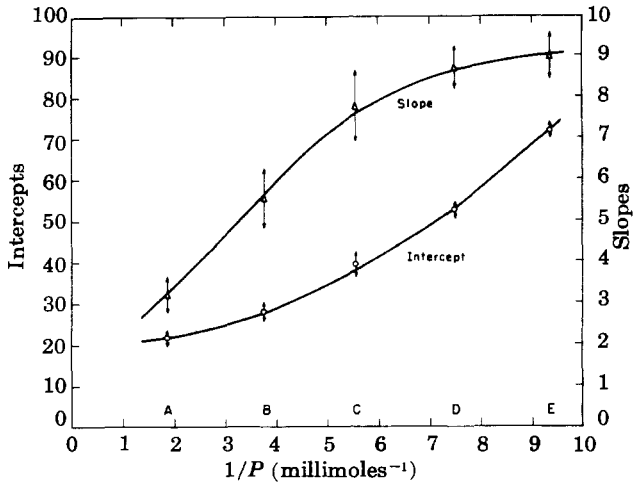

FIG. 4.—Slope and intercept plots for lactic dehydrogenase. (See also Fig. 3.)

where the ϕ's are constants, the significance of which depends on the mechanism involved. When two molecular species of coenzyme, in this case α-DPND and β-DPND, are employed, the equation must be modified.

1. Rapid Equilibrium.—In this mechanism, it is assumed that equilibrium is rapidly established among the enzyme (E), substrate (S), and coenzymes (C_α and C_β), so that a number of equilibria exist:

$$K_{1\alpha} = \frac{(E)(C_\alpha)}{(EC_\alpha)} \quad K_2 = \frac{(E)(S)}{(ES)} \quad K_{3\alpha} = \frac{(EC_\alpha)(S)}{(ESC_\alpha)} \quad K_{4\alpha} = \frac{(ES)(C_\alpha)}{(ESC_\alpha)}$$

$$K_{1\beta} = \frac{(E)(C_\beta)}{(EC_\beta)} \qquad K_{3\beta} = \frac{(EC_\beta)(S)}{(ESC_\beta)} \qquad K_{4\beta} = \frac{(ES)(C_\beta)}{(ESC_\beta)}$$

The over-all reaction velocity of the mixture when $C_\alpha = aC_t$ and $C_\beta = (1 - a)C_t$ is:

$$\frac{1}{v} = \frac{(1-a)K_{1\alpha} + aK_{1\beta}}{aV_\alpha K_{4\beta} + (1-a)V_\beta K_{4\alpha}} \cdot \left[1 + \frac{K_{4\alpha}K_{4\beta}}{(1-a)K_{4\alpha} + aK_{4\beta}} \cdot \frac{1}{C_t} + \frac{aK_{3\alpha}K_{4\beta} + (1-a)K_{3\beta}K_{4\alpha}}{(1-a)K_{4\alpha} + aK_{4\beta}} \cdot \frac{1}{S} + \frac{K_2K_{4\alpha}K_{4\beta}}{(1-a)K_{4\alpha} + aK_{4\beta}} \cdot \frac{1}{C_tS}\right] \quad (4)$$

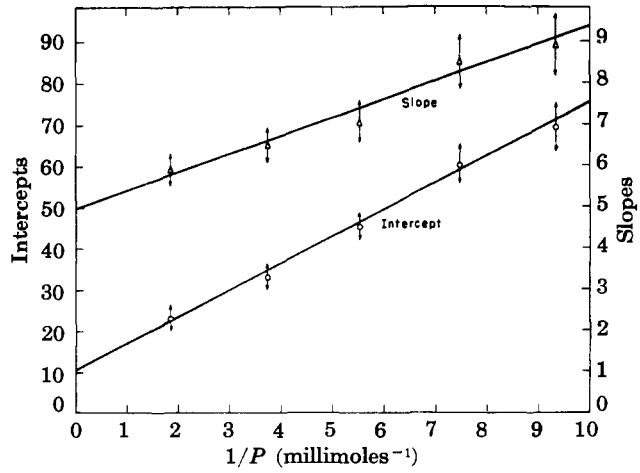

FIG. 5.—Slope and intercept plots for lactic dehydrogenase with DPNH rather than α,β-DPND as coenzyme. Based on composite data from seven experiments. The enzyme concentration was 0.6 that used in Figures 1 and 3.

By comparison with the expression for one coenzyme (*e.g.*, Alberty, 1953):

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1 + \frac{K_4}{C} + \frac{K_3}{S} + \frac{K_1K_3}{CS}\right] \quad (5)$$

it can be shown that the apparent values of the constants are as follows:

$$V_{max(app)} = \frac{aV_\alpha K_{4\beta} + (1-a)V_\beta K_{4\alpha}}{(1-a)K_{4\alpha} + aK_{4\beta}}$$

$$K_{1(app)} = \frac{K_{1\alpha}K_{1\beta}}{(1-a)K_{1\alpha} + aK_{1\beta}}$$

$$K_{2(app)} = K_2$$

$$K_{3(app)} = \frac{aK_{3\alpha}K_{4\beta} + (1-a)K_{3\beta}K_{4\alpha}}{(1-a)K_{4\alpha} + aK_{4\beta}}$$

$$K_{4(app)} = \frac{K_{4\alpha}K_{4\beta}}{(1-a)K_{4\alpha} + aK_{4\beta}}$$

This mechanism has been postulated for yeast alcohol dehydrogenase (Dalziel, 1957; Shiner *et al.*, 1960), and the fact that linear secondary plots were obtained supports this hypothesis.[1]

2. Consecutive Bimolecular Reactions.—In the case of the random bimolecular reaction, the kinetic equation for a single coenzyme is nonlinear with respect to either $1/C$ or $1/S$, so that the use of the coenzyme mixture is not necessary to differentiate this mechanism from any of the others.

However, for the ordered bimolecular reactions, kinetic equations of the form shown in equation (3) result from a variety of mechanisms. Alberty (1953) and Dalziel (1957) have shown that a study of the interrelationships among the ϕ constants and the equilibrium constants may provide some differentiation among mechanisms, but these relationships do not differentiate between mechanism I and the ordered bimolecular reaction with two ternary complexes:

$$E + C \underset{k_2}{\overset{k_1}{\rightleftarrows}} EC$$

[1] Mahler and Douglas (1957), in their study of yeast alcohol dehydrogenase, assumed that $K_2 = K_3$ and $K_1 = K_4$, an assumption not supported by subsequent observations from the same laboratory (Shiner *et al.*, 1960).

$$EC + S \underset{k_4}{\overset{k_3}{\rightleftarrows}} ESC \underset{k_6}{\overset{k_5}{\rightleftarrows}} EC_0S_R \underset{k_8}{\overset{k_7}{\rightleftarrows}} EC_0 + S_R$$

$$EC_0 \underset{k_{10}}{\overset{k_9}{\rightleftarrows}} E + C_0$$

where C_0 is the oxidized coenzyme and S_R is the reduced substrate. Mahler and co-workers (Shiner *et al.*, 1960; Baker and Mahler, 1962) have pointed out that for reactions obeying the equilibrium mechanism I, the replacement of DPND by α-DPNH should give another set of ϕ constants with the following relationships:

$$\frac{\phi_{1H}}{\phi_{1D}} = \frac{\phi_{12H}}{\phi_{12D}} \text{ and } \frac{\phi_{2H}}{\phi_{2D}} = \frac{\phi_{0H}}{\phi_{0D}}$$

which would not hold for the steady-state mechanism described above. However, the double - reciprocal method gives rather large errors, and apparent inequalities may be *statistically* equal.

When the α,β-DPND mixture is employed, the following expression is obtained:

$$\frac{E_0}{v} = \frac{\left(\frac{\phi_{12}}{S} + \phi_1\right)\left(\frac{\psi_{12}}{S} + \psi_1\right)}{(1-a)\left(\frac{\phi_{12}}{S} + \phi_1\right) + a\left(\frac{\psi_{12}}{S} + \psi_1\right)} \cdot \left[\frac{1}{C_t} + \frac{a\left(\frac{\phi_2}{S} + \phi_0\right)}{\left(\frac{\phi_{12}}{S} + \phi_1\right)} + \frac{(1-a)\left(\frac{\psi_2}{S} + \psi_0\right)}{\left(\frac{\psi_{12}}{S} + \psi_1\right)}\right] \quad (6)$$

The ϕ constants represent various combinations of kinetic constants for one species of coenzyme:

$$\phi_0 = \frac{k_5k_7 + k_5k_9 + k_6k_9 + k_7k_9}{k_5k_7k_9}$$

$$\phi_1 = \frac{1}{k_1}$$

$$\phi_2 = \frac{k_4k_6 + k_4k_7 + k_5k_7}{k_3k_5k_7}$$

$$\phi_{12} = \frac{k_2}{k_1} \cdot \phi_2$$

The ψ constants are the corresponding combinations of the kinetic constants for the other form of the coenzyme.

The same relationship is obtained for mechanisms postulating none, one, or three ternary complexes, although the significances of the ϕ and ψ constants are of course different.

The equation is linear with respect to $1/C$, but not to $1/S$. Hence the data presented in Figures 2 and 4 suggest that a mechanism of this sort is valid for rabbit muscle lactate dehydrogenase. Zewe and Fromm (1962), by the use of kinetic studies with product inhibition, have concluded that the mechanism of the reaction involves an ordered binding with no ternary complexes. However, data from this laboratory (Thomson and Darling, 1962) indicate that their mechanism may not be entirely correct and that the mechanism postulated for beef heart lactate dehydrogenase by Schwert and his co-workers (Takenaka and Schwert, 1956; Novoa *et al.*, 1959; Novoa and Schwert, 1961) may also apply to the rabbit muscle enzyme.

It should be pointed out that isotopic substitution in the substrate, rather than the coenzyme, leads to an expression[2] that is linear with respect to both $1/S$ and $1/C$:

$$\frac{E_0}{v} = \frac{\phi_0\psi_2 + \psi_0\phi_2}{\phi_2 + \psi_2} + \frac{\phi_1}{C} + \frac{2\phi_2\psi_2}{\phi_2 + \psi_2} \cdot \frac{1}{S_t} + \frac{2\phi_{12}\psi_{12}}{\phi_{12} + \psi_{12}} \cdot \frac{1}{S_tC} \quad (7)$$

Thus a study of the reverse reactions, with a mixture of proto- and deuterio-ethanol or lactate, would not reveal differences in mechanism.

3. Termolecular Reaction.—For the sequence:

$$E + S + C \rightleftarrows ESC \rightleftarrows ES_RC_0 \rightleftarrows E + S_R + C$$

the kinetic equation is of the form:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_{12}}{SC} \quad (8$$

For the coenzyme mixture, the expression becomes:

$$\frac{E_0}{v} = \frac{a\phi_0\psi_{12} + (1-a)\psi_0\phi_{12}}{a\psi_{12} + (1-a)\phi_{12}} + \frac{\phi_{12}\psi_{12}}{a\psi_{12} + (1-a)\phi_{12}} \cdot \frac{1}{SC_t} \quad (9)$$

This mechanism could be readily detected without using the coenzyme mixture.

4. Reactions Involving Enzyme Alteration.—A scheme involving a change in the enzyme has been postulated (Alberty, 1953; Dalziel, 1957):

$$E + C \rightleftarrows EC \rightleftarrows E' + C_0$$

$$E' + S \rightleftarrows E'S \rightleftarrows E + S_R$$

Since there is no $1/CS$ term:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{C} + \frac{\phi_2}{S} \quad (10)$$

the use of the coenzyme mixture again is unnecessary. A linear relationship between $1/v$ and both $1/C$ and $1/S$ is obtained with the mixture:

$$\frac{E_0}{v} = \frac{\phi_1\psi_1}{a\psi_1 + (1-a)\phi^1}\left[\frac{1}{C} + \frac{a\phi_2\psi_1 + (1-a)\phi_1\psi_2}{\phi_1\psi_1} \cdot \frac{1}{S} + \frac{\alpha\phi_0\psi_1 + (1-a)\psi_0\phi_1}{\phi_1\psi_1}\right] \quad (11)$$

For the variation of this mechanism in which the binary complexes have no kinetic significance, the equation is the same as (11) except that there is no constant term, since $\phi_0 = \psi_0 = 0$.

Thus the use of the α,β-DPND mixture is useful for differentiating between mechanisms involving a rapid equilibrium among enzyme, substrate, and coenzyme (4) and those in which ordered consecutive bimolecular reactions are postulated (6). In the former case plots of $1/v$ against both $1/C$ and $1/S$ will be linear, whereas in the latter, a plot of $1/v$ against $1/S$ will not be linear. The cases in which the mixture is most useful for discrimination are generally those for which the tests devised by Alberty, Dalziel, and Baker and Mahler are of least value or most difficult to apply.

It should be pointed out that the DPND mixture need not consist of equimolar quantities of the two stereoisomers. Measurements have been made in this laboratory of the kinetic constants for the individual stereoisomers, and although the data for β-DPND are less satisfactory than one would like because of large standard errors, the values can be used

[2] In this equation, the two forms of the substrate are considered to be present in equimolar quantities, *i.e.*, $a = 1 - a$.

to predict the curves shown in Figure 4 with fair accuracy. The departure from linearity is obvious for ratios of α-DPND:β-DPND from 80:20 to 20:80. These data will be presented in detail elsewhere.

ACKNOWLEDGMENT

I wish to thank Mrs. Jacqueline Darling for technical and computational assistance.

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Synthesis, Characterization, and Racemization of Poly-L-serine*

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Received August 29, 1962

Poly-*O*-benzyl-L-serine (III) with an average molecular weight of 52,000 was obtained by the polymerization of *O*-benzyl-*N*-carboxy-L-serine anhydride (II) in bromobenzene with sodium methoxide used as initiator. Debenzylation of III with anhydrous hydrogen bromide in dioxane yielded optically pure poly-L-serine (IV). Poly-L-serine is insoluble in water and the common organic solvents; it dissolves, however, in 7.0 to 8.5 M aqueous lithium bromide. Racemization of poly-L-serine was found to occur in alkaline media. The kinetics of racemization in concentrated aqueous lithium bromide solutions was investigated. The data obtained suggest that the racemization is base-catalyzed and that each of the seryl residues of the polymer undergoes optical inversion independently by a similar first-order reaction. The x-ray and infrared data indicate that the optically pure poly-L-serine (IV) has a β-conformation in the solid state. Optical rotatory dispersion data suggest that IV does not attain an α-helix conformation in aqueous lithium bromide or in aqueous lithium bromide–2-chloroethanol mixtures.

Poly-L-serine may serve as a suitable high-molecular-weight model compound in the elucidation of the physicochemical and biological properties of proteins with a high serine content, such as silk fibroin (Lucas *et al.*, 1958) and phosvitin (Mecham and Olcott, 1949; Williams and Sanger, 1959). The synthesis of a low-molecular-weight, optically inactive, water-soluble poly-DL-serine was described by Frankel *et al.* (1953). *O*-Acetyl-*N*-carboxy-DL-serine anhydride (Bamford *et al.*, 1956; Katchalski and Sela, 1958) was used as the starting monomer, and the resulting poly-*O*-acetyl-DL-serine was deacetylated with aqueous ammonia. A different procedure for the preparation of poly-DL-serine was described by Okawa and Tani (1954). *O*-Benzyl-*N*-carboxy-DL-serine anhydride was the monomer used, and the poly-*O*-benzyl-DL-serine obtained was debenzylated in dioxane with anhydrous hydrogen bromide. The synthesis of a water-soluble optically active polypeptide of serine reported to be poly-L-serine was recently described by Fasman and Blout (1960). *O*-Acetyl-*N*-carboxy-L-serine anhydride was polymerized in dioxane and the resulting poly-*O*-acetyl-L-serine was deacetylated with sodium methoxide in nitrobenzene. The marked solubility in water of the polypeptide obtained by Fasman and Blout seemed somewhat surprising in view of the insolubility in water of silk fibroin and of the fiber produced by *Chrysopa flava*, which is known to contain an exceptionally high percentage (~ 40%) of L-serine (Parker and Rudall, 1957; Lucas *et al.*, 1957). Furthermore, phosvitin, in which approximately half of the amino acid residues are L-serine, yields a water-insoluble product after exhaustive enzymatic dephosphorylation (Rabinowitz and Lipman, 1960). Since the deacetylation of poly-*O*-acetyl-L-serine was carried out by Fasman and Blout (1960) in an alkaline medium, the partial racemization of seryl residues had to be considered. Such a racemization has in fact been found to occur when proteins are treated with strong base (Daft and Coghill, 1931; Groh and Nyilasi, 1952; Nyilasi and Kovats, 1952; Nyilasi, 1956–57; Neuberger, 1948). Preliminary results, obtained in this laboratory, have shown that D-serine can be detected in acid hydrolysates of "poly-L-serine" samples obtained according to Fasman and Blout (1960). The synthesis of optically pure poly-L-serine was therefore undertaken.

In the following section we describe the synthesis of

* This investigation was supported by Grant A-3083 of the National Institutes of Health, United States Public Health Service.

† Taken in part from Ph.D. thesis submitted by Z. Bohak to the Hebrew University, Jerusalem, 1961.