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High Concentration Active Enzyme Centrifugation: Analysis of Active Polymeric Forms at up to 10 000-Fold Higher Concentrations than with Conventional Methods[†]

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ABSTRACT: This paper describes the theoretical basis, experimental technique, and experimental evaluation of a new method of analysis called "high concentration active enzyme centrifugation". It extends by up to four orders of magnitude the upper concentration limits at which the technique of "active enzyme centrifugation" can be used for analysis of enzyme structure. This new theory is largely based on certain properties of Gaussian curves which we have described in previous publications [Wei, G. J., & Deal, W. C., Jr. (1976) *Anal. Biochem. 75*, 113–121; *Anal. Biochem.* (1978) *87*, 433–446]. One of the most important aspects of this development is that it extends the concentration range upward so that experiments can be performed on enzymes in the active polymeric forms corresponding to their in vivo states. Furthermore, this expansion includes the range in which most enzymes go through all their association–dissociation transitions from one polymeric form to another. Hence, the method can be used to define the various concentration-dependent transitions and also to ascertain which of the various polymeric forms of an enzyme are active, under various conditions. This method also retains the many favorable characteristics inherent in the active enzyme centrifugation technique. In studies with lactate dehydrogenase, the results from this method of *band* sedimentation were identical within experimental error (about 1.5%) with results from conventional *boundary* sedimentation velocity studies.

The method of "active enzyme centrifugation" (reacting enzyme centrifugation), a band sedimentation technique introduced by Cohen and co-workers (Cohen, 1963; Cohen & Mire 1971a,b), provides the unique capability of analyzing both enzyme structure and activity at the same time. This avoids hazardous extrapolations and correlations between activity properties measured with low enzyme concentrations and structural properties measured with high enzyme concentrations. This very powerful technique has a number of unique advantages over other conventional types of physical analysis of macromolecular structure. It has extreme sensitivity (requires only minute quantities (nanograms) of enzyme), analyzes the active form of the enzyme, and does not require purified enzyme. However, the extreme sensitivity of the technique provides not only one of its greatest and most unique advantages but also its greatest limitation; measurements above approximately 0.1 μg/mL are excluded for some enzymes with present methods of analysis (Cohen & Mire, 1971a).

Obviously, it would be of great interest to analyze the concentration region from 0.1 μg/mL up to 1000 μg/mL and higher, since this is the concentration region in which most enzymes exist in vivo and also the range in which most enzymes undergo shifts in their association–dissociation equilibria.

We have developed a theory for such analysis based largely on the theoretical properties of Gaussian curves described in previous publications (Wei & Deal, 1976, 1978). We have tested the theory with experiments using our computer-controlled ultracentrifuge scanner system (Wei & Deal, 1977; Wei, G. J., Deal, W. C., Jr., Gasper, E., & Johnson, C. S., unpublished results). This development removes any theoretical upper limits on the enzyme concentration which can

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[†] From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received October 10, 1978; revised manuscript received December 20, 1978.* This is paper 5 in a series entitled "Physical Methods for Analysis of Macromolecules". Papers 1–4 are respectively Behnke et al. (1975), Wei & Deal (1976–1978). This research was supported in part by the National Science Foundation (PCM 76-24595) and the Michigan State University Agricultural Experiment Station (Hatch 1273, Publication No. 8751).

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0006-2960/79/0418-1129$01.00/0

be used in active enzyme centrifugation experiments. However, some practical experimental upper limits remain. Special experimental techniques, developed to overcome severe convection problems, now allow experiments at concentrations as high as 1200 μg/mL, which is about 10 000 times the amount previously allowable with some enzymes, such as the lactic dehydrogenase used in this study. This paper describes the theory and experimental procedures for this technique, which we call *high concentration active enzyme centrifugation*. In addition the paper presents an experimental evaluation of this new band sedimentation theory, by means of a study of lactic dehydrogenase by both band and conventional boundary sedimentation velocity techniques.

Methods

Ultracentrifugal Technique for High Concentration AEC. The experiments were done in a Model E analytical ultracentrifuge equipped with a new absorption optical system (Wei & Deal, 1977), a computer-controlled scanner system, and an automated data acquisition and analysis system (Wei, G. J., Deal, W. C., Jr., Gasper, E., & Johnson, C. S., in preparation).

For all experiments we used cells with a type I double-sector band-forming centerpiece and sapphire windows. First, 15 μL of water and enzyme sample, respectively, were loaded into the two circular chambers. After the cell was assembled, the first main sector was filled with 0.35 mL of 0.1 mM NADH to provide a solvent base line. This solution was used instead of water to minimize the difference between sample and solvent and thereby allow greater sensitivity electronically. The second sector was filled with assay mix of the same volume. Appropriate dilutions of a stock of enzyme were made with buffer to the desired concentration 30 min or less before an experiment. The legend for Figure 2 describes the experimental conditions, as well as the complete assay mix.

A slit setting of 200 μm was used for both the photomultiplier slit and monochrometer exit slit. The photomultiplier power supply voltage (approximately 320 V) was set to give a detector signal voltage of 6 V in a region of the cell where the enzyme had already passed. The light source was a 200-W Hanovia 901-B1 Hg–xenon lamp powered by a Schoeffel Instruments Corp. LPS 255-SSP starter and a Hewlett Packard 6269B DC power supply set at 26 V and 8.5 A. The wavelength was set at 366 nm. The resulting magnification factor was 2.052. The scan range across the cell was from 6.2 to 7.1 cm from the center of rotation, corresponding, respectively, to 6000 and to 12 000 stepping motor steps (one step equals 1.547 μm). An increment of 30 steps (95.25 μm) was used between positions at which data were collected. Three samples and a reference counterbalance were run simultaneously in a four-cell titanium AN-F rotor. The inner and outer reference edges of 5.7 and 7.3 cm corresponded to approximately 2800 and 13 100 steps, respectively.

The major problem in working with bands of enzyme at high concentration is sinking; the enzyme band reaches a region of negative density gradient and then sinks until it reaches a region of sufficiently positive density gradient. Extreme sinking can be detected empirically by increasing sedimentation rate during an experiment; moderate sinking may be revealed only by curvature in log R vs. t plots.

Two features have been incorporated into our experimental protocol to minimize this problem. The first is a 5–20 min "holding" period at about 5000 rpm, to allow establishment of a concentration gradient in the solvent. The speed selected is a compromise between the lowest speed possible, to minimize sedimentation, and a speed high enough to ensure complete layering of the enzyme solution. The time at the "holding" speed is kept as short as possible, but long enough to allow sufficient gradient to be established to yield stable sedimentation, as judged empirically.

The second feature is that the rotor speed selected for the experiment is the slowest value compatible with a good, measurable movement of the enzyme band between scans. Both of these steps serve the same purpose—to allow the solvent to create a density gradient as far down the cell as possible before the enzyme band moves appreciably. The concentration gradients of the sucrose solvent initially form at the meniscus and bottom of the cell and move toward the center of the cell with time. Hence, if any part of the solution has an unsatisfactory density gradient, it is most likely to be the region in the center of the cell. Other aspects of the experimental procedures are similar to previously published procedures (Cohen & Mire, 1971a; Kemper & Everse, 1973).

Data were analyzed either locally in our laboratory computer with simple methods or at the campus computer center with more extensive methods. The data and results were handled only by computer-controlled hardware or computer software programs, beginning with data collection through to the finished graphs. Scans were made at 4–6-min intervals. In each scan, 50 data values were collected at each r point and the average of the 50 values was stored in central memory. Between scans the data were dumped onto a disk file. Then after the experiment, the disk file was processed into a form compatible with the Michigan State University CDC 6500 central computer and transferred via telephone lines to that computer for final data analysis. First, the data were smoothed by a computer-programmed digital filtering process; the main effect of this program is to smooth out the base lines. Then the apparent radial position of the band at each scan time was determined by six different methods: inflection point, half-height, and four methods based on difference curves (see below). Then a statistical program for curve fitting was carried out. The inflection point method and the maximum method from the simple difference curves generally gave the best precision.

The inflection point was determined by taking the derivative of the concentration curve and searching for the maximum on the derivative curve with a simple seven-point average technique. The average value at a point is equal to the sum of the seven points at, and symmetrically about, the point of interest. This is done to reduce the effect of noise spikes on the chosen inflection point.

To find the midpoint by the half-height method, it was necessary to get the two plateau values on each side of the boundary. This was accomplished by first determining the level-off points (one on each side of the boundary) and then calculating the average plateau values using the middle-half portion of the plateau regions (those between the level-off points and the extreme limits of the curve).

Cohen and co-workers (Cohen et al., 1967) have described in detail the four methods based on differences between successive scans: (1) moment method with simple difference curve; (2) moment method with rigorous (diffusion-corrected) difference curve; (3) maximum method with simple curve; (4) maximum method with rigorous curve. For the difference methods in our programs, we used eq 43 through 51 of that paper (Cohen et al., 1967). In order to avoid the "edge effect" introduced by using the "infinite boundary" approximation, every scan curve is checked to make sure a plateau region exists on each side of the boundary.

Before a linear least-squares fitting was done to obtain the best average s and/or D values, each set of log R vs. t data

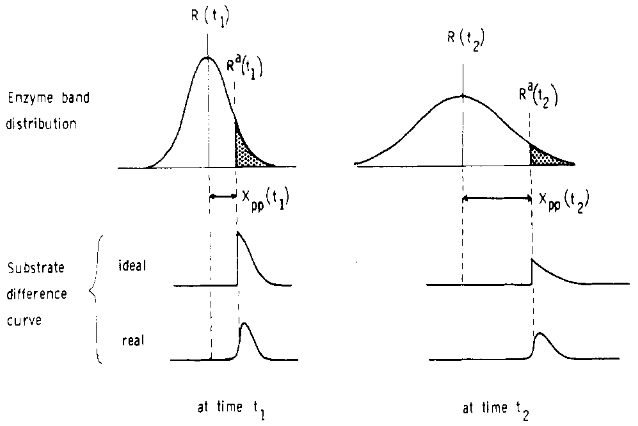

FIGURE 1: A schematic description of the partition point, $R^a(t)$, in the actual enzyme band (upper pattern) and its relation to the ideal and the real peaks of the substrate difference curve. $R^a(t)$ and $R(t)$ represent the distance from the center of rotation to the partition point position and to the true center of the enzyme band, respectively. The theoretical values are calculated assuming an ideal curve (center patterns). The actual points measured are the inflection points on the trailing (left) side of the real curves (bottom patterns). The value, x_{pp}, is the value which must be subtracted from the apparent measured point (R^a) to yield the true center, $R(t)$, of the enzyme band.

was first screened by an iterative process to exclude data with abnormally large deviations. In this screening process, the origin was set at the first data point, and slopes and the average deviations in slope of all of the rest of the points were calculated. Then those points with a deviation above a preset first-exclusion level (highest level) were removed, and the process was successively repeated at lower exclusion levels until all remaining points were "good" (i.e., within 2% of the average). The origin was also checked by comparing the calculated intercept with the experimental point. If the test was unsatisfactory, the calculated intercept was used as a new origin and the whole process was repeated.

Results

Nature of the Error in Conventional AEC[1] Analysis with Excess Enzyme. The present upper limit on enzyme concentration is related to the fact that the enzyme is measured indirectly; in the experiment, a band of enzyme is sedimented through an assay solution and the rate of movement of the enzyme is followed by means of absorption measurements, at various times, of the substrate (or product) concentration as a function of radial distance in the cell (Figure 1). Theoretically, the velocity of the *center of the enzyme band*, R, must be measured in order to deduce the velocity of individual macromolecules; however, the experimentally measured quantity (R^a), in the product (or reactant) distribution, will reflect the movement of the center of the enzyme band only if no more than a few percent of the substrate is converted to product (Cohen & Mire, 1971a). Furthermore, in a system with a considerable excess of enzyme over that required to convert all substrate to product, the experimentally measured quantity will reflect the velocity of some (changing) point near the leading edge of the band (Figure 1). Diffusion-driven broadening of the band will therefore result in erroneously high values[2] of the sedimentation coefficient (Cohen & Mire, 1971a).

A theory to quantitatively account for the spreading of the leading edge with time would allow calculation of the *band center* from the experimentally measured *point in the leading edge* of the enzyme band, and accurate sedimentation coefficients could be calculated from such experiments with "excess enzyme".

For this paper, the concentration range where the enzyme is in substantial excess covers most of the region of interest to us. Therefore, we will use a model system with a several-fold excess of enzyme for the theoretical development.

Definition of the Partition Point Parameters, R^a and x_{pp}, for the r and the x Coordinate Systems, Respectively. In such a system with excess enzyme, the enzyme band can be divided into two fractions, namely, the *leading fraction*, which reacts with substrate (Figure 1, upper left, shaded), and the *trailing fraction* (Figure 1, upper left, unshaded), which does not react with substrate (because all substrate has been converted to product before that fraction of enzyme gets to any given point). We define the abscissa value at the vertical line dividing these two fractions as the partition point and give it the symbols $R^a(t)$ and x_{pp} in the radial and in the x coordinate systems,[3] respectively. It is very significant because (1) it corresponds very closely to the actual experimentally measured point in such experiments and (2) it is a theoretical mathematical parameter for the calculation of the true center, $R(t)$, of the enzyme band from eq 1 where R and x are in real units and

$$R^a(t) - R(t) = x_{pp} = x'_{pp}\sigma \tag{1}$$

units and x' is in normalized units, i.e., real units divided by σ, the standard deviation.

General Theory of Band Sedimentation. A "conventional" theory of band sedimentation velocity and diffusion has been developed by Vinograd and co-workers (Vinograd et al., 1963; Vinograd & Bruner, 1966a,b). Like many other theoretical developments in sedimentation analysis (Fujita, 1962), the theory has its origin in analyses (Carlslaw & Jaeger, 1959) of the differential equations describing heat flow, in this case of the spreading of heat from an infinitely thin disk source down a cylindrical conductor. Vinograd and co-workers showed (Vinograd et al., 1963) that sedimentation coefficients calculated by their theory of band sedimentation were identical, within experimental error, with values calculated by the well-established boundary sedimentation velocity technique.

Assumption 1. As previous workers (Vinograd et al., 1963) have done, we assume that, within a short period after the layered enzyme band sediments away from the meniscus, it has, and remains in, a Gaussian form (Wei & Deal, 1976, 1978) such that, at any time t, the normalized concentration distribution is described by eq 2, where σ is the standard

$$C(r) = \frac{C(R)}{\sigma(2\pi)^{1/2}}e^{-x^2/2\sigma^2} \tag{2}$$

deviation of the curve, $x = (r - R)$ is the real (i.e., not normalized) abscissa coordinate, with *origin at the center of the enzyme band*, r is the radial abscissa coordinate, with origin at the center of rotation, R is the radial distance of the center

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[1] Abbreviation used: AEC, active enzyme centrifugation.

[2] Comparison in Figure 1 of the two upper diagrams, at two different sedimentation times, t_1 and t_2, shows that the partition point, $R^a(t)$, moves faster than the center of the enzyme band, $R(t)$, because the distance, x_{pp}, between the two points is greater at t_2 than at t_1. Hence estimates of velocity based on the raw conventional data will be too large.

[3] In this analysis we deal with two sets of time variables: (1) a *diffusion* time, based on a time of zero when layering begins, and (2) a *sedimentation* time, based on a time of zero at the first absorption scan measurement; for difference curve sedimentation analysis, there is yet a third time, t_θ, which is the average time between two scans at times t_i and t_{i-1}. There are also two coordinate systems: (1) a *moving* diffusion coordinate system, with x as abscissa variable and origin at the *center of the enzyme band*, and (2) the usual *fixed* sedimentation coordinate system, with r as abscissa variable and origin at the *center of rotation* of the rotor.

of the enzyme band from the center of rotation, and $C(r)$ and $C(R)$ are respectively the concentration of enzyme at any point r and the concentration at R, the center of the enzyme band, at the given time t. The spreading of the sedimenting band with time in a sector-shaped cell obeys (Vinograd et al., 1963) eq 3, where σ_t and σ_{t_0} are the standard deviations of the curves

$$\sigma_t^2 = \frac{R_t}{R_{t_0}}\left[\left(\frac{R_t}{R_{t_0}}\right)\sigma_{t_0}^2 + 2D(t - t_0)\right] \tag{3}$$

at times t and t_0, respectively; R_t and R_{t_0} are the radial distances from the center of rotation to the center of the enzyme band at times t and t_0, respectively; t is the time and t_0 is the zero time with respect to diffusion.[3] For brevity and clarity, we omit the letter t in the time subscripts for σ and R values in some equations.

Overall Calculation Sequence. In order to calculate valid sedimentation coefficients for the excess enzyme system, we need to calculate true band center values, $R(t)$, from calculated values of $x_{\text{pp}}(t)$ and experimentally determined values of apparent band center positions, $R^{\text{a}}(t)$, as shown in eq 1. The desired values of $x_{\text{pp}}(t)$ are obtained (eq 1) from calculated values of $x'_{\text{pp}}(t)$ and $\sigma(t)$ (eq 3).[4]

Calculation of $x'_{\text{pp}}(t)$ from Appropriate Relative Area (t) under Normalized Gaussian Curve (t). For a given scan time t, the theoretical calculation of the partition point, $x_{\text{pp}}(t)$, depends on the indirect calculation, from experimental data, of the fractional area (defined below) to the right of the partition point at time t. In order to make this calculation, we utilize the fact that Gaussian functions, such as that for our enzyme band (eq 2), have well-defined, fixed relationships between the value of a given normalized x' coordinate and the relative area fractions under the curve on either side of the coordinate. For the directly related function, the normal curve of error, tables of values of area, Ar, as a function of x'_i values are readily available or can be calculated by computer;[5] note that the area of the normal curve of error pertains only to some fraction of the right one-half of the curve.

We assume that the enzyme concentration distribution (eq 2) is identical with its mass distribution if both distributions utilize normalized abscissa coordinates.[6] Then, the relative area under the curve to the right of the partition point, x'_{pp}, at a given time is simply the ratio of the "partition-point mass" (E_{pp}) of enzyme to the total mass (E_{T}) of enzyme. The partition-point mass is defined as that mass of enzyme needed to convert all the substrate to product. This ratio is therefore the fraction, f_{E}, of the total enzyme needed to convert all substrate in that particular volume element. Consequently, the fraction, f_{E}, is the area, Ar, from $x' = x'_{\text{pp}}$ to $x' = +\infty$, so that

$$f_{\text{E}} = E_{\text{pp}}/E_{\text{T}} = \text{Ar}(x'_{\text{pp}} \rightarrow \infty) \tag{4}$$

However, we need the complement, f_{E}^{c}, of the relative area, f_{E}. The complement area is the relative area under the curve from $x' = 0$ to $x' = x'_{\text{pp}}$. Since the Gaussian normal curve of error is symmetrical, the relative area in the total right half of the curve ($x' = 0$ to $x' = +\infty$) is 0.5. Therefore

$$f_{\text{E}}^{\text{c}} = 0.5 - f_{\text{E}} = 0.5 - E_{\text{pp}}/E_{\text{T}} = 0.5 - \text{Ar}(x'_{\text{pp}} \rightarrow \infty) = \text{Ar}(0 \rightarrow x'_{\text{pp}}) \tag{5}$$

Once $\text{Ar}(0 \rightarrow x'_{\text{pp}})$ is known, the corresponding value of x'_{pp} is found in standard tables or calculated by computer.[5]

Calculation of E_{pp}. From the previous definition, it is obvious that E_{pp} is a function of the substrate concentration, centrifuge cell dimensions, enzyme sedimentation coefficient, rotor speed, and rotor temperature.

For the calculation of E_{pp}, it is convenient to define the substrate concentration in terms of a total possible absorbance change, ΔA, and then define E_{pp} by an equation involving various parameters and that absorbance change. This total possible absorbance change, ΔA, is determined empirically, by means of a direct end-point spectrophotometric assay carried out in the presence of excess enzyme.

Now, the required change in absorbance (ΔA) caused by a band of sedimenting enzyme molecules is equal to the product of the time (Δt) needed for the band to pass through a given point, the amount of substrate (U) turned over per unit time per unit volume, the extinction coefficient (ϵ_{s}), and the optical path length (h) of the cell, so that

$$\Delta A = \Delta t U \epsilon_{\text{s}} h \tag{6}$$

Furthermore, since U = (specific activity) × (enzyme concentration) = $a[\text{E}]$ and Δt = (thickness of the band)/(sedimentation velocity)

$$\Delta t = (V_{\text{e}}/\text{cross-sectional area})/(s\omega^2 r)$$

$$\Delta t = V_{\text{e}}/(hr\theta s\omega^2 r)$$

where

V_{e} = volume of the enzyme sample

θ = sector angle of the cell

then

$$\Delta A = \left(\frac{V_{\text{e}}}{(hr\theta s\omega^2 r)}\right) a[\text{E}]\epsilon_{\text{s}} h$$

or

$$\Delta A = aE_{\text{pp}}\epsilon_{\text{s}}/s\omega^2 r^2\theta \tag{7}$$

A more rigorous proof is given in the Appendix.

From eq 7

$$E_{\text{pp}} = s\omega^2 r^2\theta(\Delta A)/a\epsilon_{\text{s}}) \tag{8}$$

All parameters on the right side of the equation are known quantities except r (for which an approximate average value, r = 6.7 cm, may be used) and s (for which the uncorrected s value, from the first pass of the program, may be used).[4]

Calculation of σ_0. We use the approximation, which is quite good, that σ_0 is equal to one-half of the initial band width. The calculation uses eq 9a which is derived from simple radius–volume relationships in a sector-shaped cell.

$$\sigma_0 = \text{half-thickness of initial band} = 0.5(r_{\text{s}} - r_{\text{e}}) \tag{9a}$$

where

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[4] For the most accurate work, an iterative approach should be used for calculating input values of s for the E_{pp} calculation and for input values of $R(t)$ for the $\sigma(t)$ calculation according to eq 3.

[5] Abscissa values are given only to one or two decimal places in the readily available tables of area of normal curves of error (Beyer, 1968, 1973; Superintendent, 1941; Weast & Selby, 1964, 1967). We have calculated by numerical integration the area quantity (see below) for the normalized abscissa coordinate x', in increments of 0.001 for values of x' up to 2.70 (area = 0.4965), and in increments of 0.0002 for values of x' greater than 2.70. (Note that $x' = x_{\text{real}}/\sigma$.)

$$\text{Ar}(0 \rightarrow x'_i) = \frac{1}{(2\pi)^{1/2}}\int_0^{x'_i} e^{-x'^2/2}\,\mathrm{d}x$$

[6] The two normalized distributions, c vs. x' and mass vs. x', are identical for rectangular sedimentation cells since the cross-sectional area of such cells is constant at all r values. The approximation of our sector-shaped cell by a rectangular shaped cell will not introduce an appreciable error.

$$r_s = \left(r_b^{\,2} - \frac{2V_s}{\theta h} \right)^{1/2} \qquad \text{(9b)}$$

$$r_e = \left[r_b^{\,2} - \frac{2(V_s + V_e)}{\theta h} \right]^{1/2} \qquad \text{(9c)}$$

and where the terms are defined as follows: r_b, r_s, and r_e, radial distances of cell bottom, substrate–enzyme interface, and enzyme–air interface, respectively (r_b = 7.2 cm for standard cell); V_s and V_e, volumes of substrate and enzyme, respectively, in centrifuge cell; θ, sector angle (radians) of cell compartment; and h, cell height (1.2 cm for standard cell).

Computer Calculation Procedure for High Concentration AEC. Our high concentration active enzyme centrifugation method of analysis is essentially a two-pass process.[4] The first pass of the data is through our modified conventional active enzyme centrifugation analysis program, to provide a detailed calculation of various experimental parameters which are either needed for the second pass program or which are not influenced by the second pass program. The main data output from the first pass program is sets of values of "apparent" (R^a) band positions and their associated scan times, t_θ; in addition, this first pass also provides needed values of various parameters including initial band thickness, initial band position (R_0), and estimates of s and D.

The main purpose of the second pass program is to calculate, for each time t, the value of x_{pp} and then calculate, for that t value, the correct band-center position, R, from the value of R^a and x_{pp}. For convenience, the program then calculates both a new "corrected" value for s (= $s(R)$) and an uncorrected value for s (=$s(R^a)$), respectively, from graphs of log R vs. t and log R^a vs. t, respectively. In addition, in order to provide, for information only, an idea of the relative magnitude of the change introduced by using the high concentration AEC theory, the program calculates the ratio of the slopes of the two graphs mentioned above; the resulting value is then the ratio of $s(R_{\mathrm{uncorr}})$ to $s(R_{\mathrm{corr}})$ and is designated the "effect of correction". It should be emphasized that this "effect of correction" value is informational and not operational.

Second Pass: High Concentration AEC Input Parameters, Source, and Definitions. The calculation procedure requires input of the following values: ΔA, change in absorbance/unit volume in end-point assay with excess enzyme; $a\epsilon_s$, enzyme specific activity (absorbance change $\mathrm{s}^{-1}\,\mu\mathrm{g}^{-1}$) (assay just before experiment); E, enzyme initial concentration, as applied in cell sector (μg/mL); V_e, enzyme volume introduced into cell; s_{app}, estimate of sedimentation coefficient (from first pass); D_{app}, estimate of diffusion coefficient (estimated from M through s estimated, using empirical curves of s vs. M and D vs. M for enzymes of known values); t_0, time of layering of the enzyme (start of centrifugation) with respect to first scan used (t_0 is negative); rpm, rotor speed; θ, sector angle-degrees (converted to radians by program); R_{av}, average R of scan, distance from center of rotation, cm (from first pass); R_0, initial center of band, distance from center of rotation, cm (from first pass); Thic, thickness of enzyme band initially, before diffusion (calculated by computer from input data in first pass); N, number of sets of $R^a(t)$, t_θ values; t_θ, times of scan (difference curve time) in minutes (converted to seconds by program); $R^a(t)$, partition point values, measured from center of rotation (initial[4] measured "apparent" center of the band, from which the true positions ($R(t)$) of center of enzyme band are to be calculated using eq 1).

Second Pass: High Concentration AEC Output Parameters. The second pass output parameters, which have all been defined in the text, are E_{app}, E_T, f_E, and $f_E{}^c$, and sets of values, at each time $t_\theta(i)$, are $R^a(t)$, $R(t)$, ln $R^a(t)$, ln $R(t)$, $s(R^a)$, and $s(R)$. The program does a least-squares data analysis, excludes bad data, and calculates a final best $s(R^a)$, $s(R)$, and the ratio $s(R)/s(R^a)$.

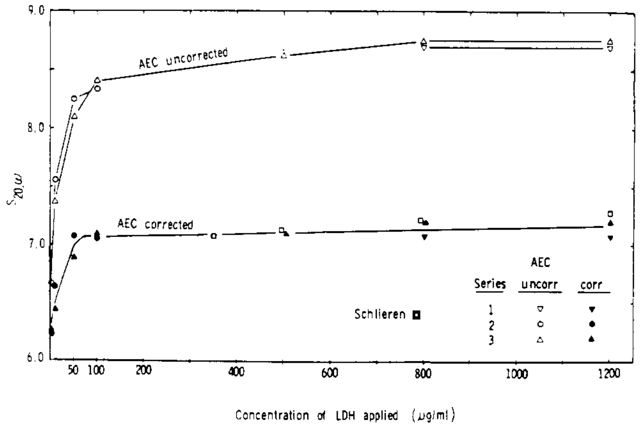

FIGURE 2: Experimental test of the high concentration AEC theory. Sedimentation coefficient values obtained for rabbit muscle lactic dehydrogenase by three different methods are compared. The upper curve represents results (open symbols) using the conventional AEC method, which yields $s(R^a)$ values, referred to as AEC(uncorrected) values. The lower curve represents results (closed symbols) using the high concentration AEC method described in this paper; this analysis yields $s(R)$ values, referred to as AEC(corrected) values. These theoretically calculated values are to be compared with the experimental results obtained with the well-established method of conventional moving boundary sedimentation velocity, using the schlieren optical system. In all the AEC experiments, the assay solution contained 0.1 M phosphate buffer, pH 7.4, 0.69 mM NaCl, 0.4 mM NADH, 2 mM sodium pyruvate, and 4% sucrose. All experiments were done at room temperature (21–24 °C). A four-cell titanium rotor was used throughout at a speed of 50 740 rpm. The corrected data were obtained as described in the text by applying a correction to each apparent peak position and fitting the equation ln $R = s\omega^2 t$, to get the slope. For the conventional boundary sedimentation, cells with 12-mm centerpieces were used for the samples at 1.2 and 0.8 mg/mL. Cells with 30-mm centerpieces were used for the two lower concentrations, 0.5 and 0.35 mg/mL.

Application of High Concentration AEC to Rabbit Muscle Lactate Dehydrogenase. Rabbit muscle lactate dehydrogenase was chosen for study because it has a sedimentation coefficient of about 7 S, which is typical of a great number of enzymes. Under the particular set of conditions used in the experiments to be described, the partition-point mass of enzyme, E_{pp}, is estimated to be about 3 ng.

Figure 2 shows the sedimentation coefficient values calculated from three separate series of band sedimentation velocity experiments using the high concentration active enzyme centrifugation technique, covering the concentration range from 0.5 μg/mL (7.5 ng applied) up to 1200 μg/mL (18 μg applied); the highest concentration is 6000-fold above the partition-point concentration. Series 3 is the most reliable because it was performed after the other two series and incorporated the improvements gained from experience with the first two series. The data designated "AEC uncorrected" are the results obtained with conventional AEC methods, which yield R^a values. The data designated "AEC corrected" are the results obtained with the high concentration AEC method, which yields R values. In addition, Figure 2 shows the results from conventional boundary sedimentation velocity experiments carried out over 75% of the same concentration range using the schlieren optical system.

In the range above 100 μg/mL, sedimentation coefficients from active enzyme centrifugation data calculated using uncorrected radial distance values (i.e., R^a values) are approximately 15–20% (1.4 S units) higher than the sedimentation coefficient values using the corrected radial distance values (i.e., R values).

The sedimentation coefficient values using the corrected radial distances are quite close to the values from the conventional moving-boundary experiments using the schlieren optical system (maximum difference = 0.15 S). The most reliable data, that from series 3, is virtually coincident with the schlieren experimental data. In addition, it can be seen from the two high concentration AEC experiments carried out at 800 μg/mL (or 1200 μg/mL) that the experimental variation in results within high concentration AEC experiments (series 1 and 3) is greater than the difference between the results (series 3) with the new technique and those with conventional boundary analysis. Hence, results from this new method of analysis of *band* sedimentation agree with the results from *conventional* boundary sedimentation, within the experimental error. This is excellent agreement, especially in view of the fact that the conventional boundary experiments had to be carried out without one of the components (4% sucrose) required for the band sedimentation.

A value of 7.0 ± 0.4 S has been reported (Cohen & Mire, 1971b) for this enzyme in the sample range of 20–50 ng in a 0.1 M glycine buffer at pH 10.0. We measured a value of $s^0_{20,w}$ = 7.2 S using the schlieren optical system. Our high concentration AEC values for the enzyme in 0.1 M phosphate buffer at pH 7.4, with 4% sucrose, agree well with that value.

The sedimentation coefficient values from both corrected and uncorrected data clearly show (Figure 2) that LDH goes through a dissociation transition in the concentration region below about 60 μg/mL. It should be noted that the observation of a sedimentation coefficient value markedly below that of the corresponding native enzyme is not conclusive evidence for active subpolymers; in a rapidly equilibrating system, active native polymers would be present throughout the band. The data are not sufficient to allow definitive conclusions regarding whether the dissociation products are active or not and whether dimers or monomers, or both, are present. It is outside the scope of this presentation to pursue these questions in detail. Detailed analyses of this sort have been carried out on several enzymes (Wei, G. J., & Deal, W. C., Jr., in preparation; Johnson, C. S., & Deal, W. C., Jr., in preparation).

The Effect of the High Concentration AEC Theory at Different Enzyme Concentrations. For rabbit muscle LDH under the conditions used in these studies, the "effect of the correction" is virtually constant in the higher concentration region, from 100 to 1200 μg/mL. This is qualitatively evident from Figure 2; the differences are shown quantitatively in Table I. In series 3, for example, the effect of correction varies only about 2% in going from 1200 μg/mL (EC = 0.826) to 100 μg/mL (EC = 0.847).

However, it should be noted that the effect of correction for a given concentration varies from experiment to experiment; e.g, the ratio for 1200 μg/mL varies from 0.803 in series 1 to 0.826 in series 3. Therefore, it is not feasible to calculate a single "effect of correction" for a series of experiments, even if most of the experimental procedures will be identical for the entire series of experiments. Certain key variables are not easy to fix as constants for an entire series of experiments; these include the specific activity of the sample and the time of diffusion at low speeds before data collection begins. Furthermore, the correction depends on the actual radial distance of the peak at the time of the particular scan; it would be very difficult to ensure that this would be the same for corresponding scans in two separate experiments.

Table I: Enzyme Concentration Dependence of the "Effect of Correction" in High Concentration AEC Experiments[a]

series	appl concn (μg/mL)	$10^3 f_E$[b]	$S_{20,w}$ (uncorr r)	$S_{20,w}$ (corr r)	EC (effect of corr)
1	1200	0.132	8.73	7.00	0.803
	800	0.198	8.72	7.07	0.792
2	100	1.759	8.35	7.07	0.848
	50	3.521	8.26	7.09	0.858
	10	17.610	7.58	6.65	0.879
	1	176.100	6.65	6.24	0.939
	0.5	352.000	5.96	5.80	0.972
3	1200	0.151	8.74	7.23	0.826
	800	0.227	8.75	7.20	0.822
	500	0.331	8.62	7.10	0.823
	100	1.653	8.41	7.11	0.847
	50	3.342	8.09	6.90	0.852
	10	16.710	7.38	6.48	0.876
	1	167.000	6.66	6.27	0.939

[a] The effect of correction value, EC, was calculated using the theory and methods described in the text. See the legend of Figure 3 for experimental conditions. [b] The quantity f_E is defined as $f_E = E_{pp}/E_T$, where E_{pp} is the amount of enzyme required to use up all the substrate in a unit cross-sectional volume in the centrifuge cell under the given conditions of experiment and E_T is the total enzyme actually present in the given experiment. Note that f_E is the reciprocal of the enzyme capacity ratio, E_T/E_{pp}, which is an approximate measure of the fold-excess enzyme capacity, with respect to that required to use up all the substrate. In some cases, the value of f_E is somewhat different for the same enzyme concentration in different experiments; this is a result of slight variations in the empirically determined specific activities of the various enzyme samples, which were prepared at different times, from different lots of stock enzyme.

As shown in Figure 2 and Table I, the "effect of correction" varies markedly with enzyme concentration in the range below about 100 μg/mL. In series 2, there is a 12% change in going from 100 to 0.5 μg/mL. In series 3, there is an 11% change in going from 100 to 1 μg/mL. Note that, at 0.5 μg/mL, the effect of correction is only slightly greater than 2%, so the effect of correction is virtually negligible.

Effects of Errors in Experimental Input Parameters on the Resulting Values of $s(R)$. Since this theory is based on calculation of enzyme *band-center* positions, by correcting mainly for fold-excess enzyme and for diffusion-controlled broadening of the curve, it is obvious that the key parameters which will influence the calculation are the values of f_E, D, and t, time of diffusion. Therefore, it is of interest to know what effect errors in these quantities will have on the resulting sedimentation coefficient values, $s(R)$. As shown in Figure 3, the error in the overall results (ordinate) is not very sensitive at all to sizable errors in f_E or D. We might expect a typical worst error in f_E to be of the order of 30% and a typical worst error or uncertainty in D to be of the order of 5–10%. The graphs in Figure 3 show that, in each case, such errors in the input values would introduce less than 1% error in the overall results. Similar calculations reveal that the effect of a typical worst error (10%) in t, the diffusion time, leads to an error of less than 1% in the overall result (not shown). So errors in these values probably contribute far less than 1.5–2% error in the overall results.

Use of Other Methods to Determine Apparent Band Positions (R^a). All the results described in this paper thus far have been based on the use of the simple difference curve method to provide the initial-approximation values for apparent enzyme position (R^a) from the experimental scan data. The apparent boundary positions are presumed to correspond to

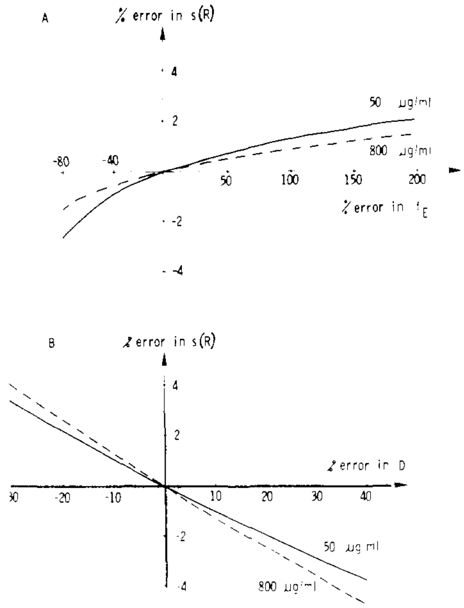

FIGURE 3: The % error introduced in high concentration AEC values of $s(R)$ by given errors in f_E (A) or D (B). The % error in $s(R)$ is calculated from the change in the value of EC. See text and Table I.

the peak of the difference curve. It is of interest to use the same experimental scan data and to see the effect of application of other methods to determine apparent boundary positions.

These other methods include the rigorous difference curve method, the inflection-point method, and the half-height method.

Table II shows the "apparent" sedimentation coefficient results, $s(R^a)$, obtained from the apparent boundary position values obtained by various methods. In virtually all the data, the agreement between results from the different methods is better than 1%, and, in some cases, it is much better than that. Furthermore, the precision of all the methods is excellent, with typical standard deviations of less than 1%. Therefore, at least in this system, the other methods give results close to those using the most reliable method, the difference curve method.

Discussion

New Concentration Range Possible with High Concentration AEC. With the high concentration AEC method, it is now possible to make valid determinations of sedimentation coefficients of active polymeric forms at enzyme concentrations far above those which will utilize all the substrate in the cell. The useful range of enzyme concentration has been extended by four orders of magnitude for enzymes such as LDH.

Assumptions. Assumption 1 is reasonably correct for a homogeneous component with constant s and D, ignoring the secondary effects arising from the interaction between the constituents of the solvent. The distortion of the Gaussian distribution caused by the cylindrical geometry is expected to be small and is not taken into account (Vinograd et al., 1963).

Implicit in our treatment is a second assumption, namely, that the rate of substrate turnover is linearly proportional to enzyme concentration (i.e., the specific activity of the enzyme does not change with enzyme concentration and the reaction is zero order with respect to substrate concentration) and, therefore, the peak of the difference curve is a good approximation for the position of the partition point. We have also neglected the reverse enzyme reaction for the purpose of simplicity. Both of these assumptions are minor and have neglible effects, except in an extremely narrow concentration region (where f_E is near 0.5). The direct result is an underestimation of the partition point; the measured partition point has a smaller value than it would have had if all the enzyme in the leading edge of the band had been maximally active (fully saturated). However, previous analysis has shown that the results are not very sensitive to errors in f_E, so this will be a minor effect throughout most of the concentration curve. Essentially the same error occurs at all measured band positions, so that the error affects mostly the intercept of the ln R vs. t plot, but not the slope, from which s is obtained.

Table II: Comparison of Different Methods of Determining Apparent Boundary Positions, $R^a(t)$, as Reflected in s Values Calculated[a]

concn of appl enzyme (µg/mL)	sedimentation coeff values ($s_{t,b}$) calcd by the method of			
	max of simple[b] difference curve	max of rigorous[b] difference curve	half-height	inflection point
1200	8.33 ± 0.07	8.38 ± 0.06	8.47 ± 0.05	8.48 ± 0.04
800	8.33 ± 0.11	8.39 ± 0.17	8.02 ± 0.13	8.38 ± 0.16
500	7.70 ± 0.02	7.73 ± 0.04	7.82 ± 0.03	7.84 ± 0.04
100	7.50 ± 0.06	7.51 ± 0.06	7.56 ± 0.04	7.54 ± 0.05
50	7.31 ± 0.04	7.22 ± 0.06	7.27 ± 0.06	7.29 ± 0.07
10	6.67 ± 0.04	6.60 ± 0.06	6.72 ± 0.07	6.67 ± 0.04
1	6.02 ± 0.04	5.93 ± 0.04	5.93 ± 0.06	5.97 ± 0.06

[a] Part of the series 3 data for rabbit muscle LDH. See Figure 2 for other experimental details. The rotor temperatures were 21.9, 21.4, and 24.0 °C, respectively, for the 1–50, 100–500, and 800–1200 µg/mL samples, respectively. The uncertainties in the values are standard deviations calculated from least-squares fits of the various sets of data. The results at a given concentration are comparable since they are derived from the same c vs. r scan data. Note that these results are "apparent" values from AEC experiments at high concentrations; they were calculated using uncorrected (R^a) values. [b] A "rigorous" difference curve is obtained from each set of two successive curves by correcting the first (in time) of the two curves for diffusion of the NADH substrate molecules before calculation of the difference curve. A "simple" difference curve is obtained without such correction. See Cohen & Mire (1971a,b) for details.

It should also be noted that the relative error introduced in the results by the above assumptions is greatest when the partition point lies near the center of the band. However, under such circumstances, the absolute value of the change introduced by the high concentration AEC theory is very small, so any error is essentially negible.

Limitations. This method is very valuable because it extends the valid enzyme concentration range of AEC by several thousand fold.[7] With the high concentration AEC theory, the theoretical upper limits on AEC are totally removed. But there remain practical experimental limits on concentration, which have already been discussed.

Also, the method has one inherent limitation. Since only a small fraction of the enzyme band near the leading edge is functional, the substrate boundary does not contain information on the distribution of the whole enzyme band. Hence, if the enzyme sample contains more than one active species and they resolve into multiple bands during the sedimentation process, this method will detect the fastest moving species only.

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[7] As mentioned in the Results section, this theory is not valid for that very small concentration range where $f_E \geq 0.5$. In this region the measured R^a value is not a good approximation to the theoretical R^a value defined by the f_E value and the corresponding partition point. Experimentally, this corresponds to an extremely narrow concentration range on the very low concentration end of the scale, the region where more than one-half of the total enzyme is required to use up all the substrate.

In general this method is useful[7] when the sample is homogeneous and has a simple Gaussian distribution. The presence of rapidly equilibrating species (Gilbert, 1955, 1959) would complicate analysis. Nearly Gaussian curves might be produced for a monomer-dimer equilibrium mixture, and hence a fair approximation to a weight-average sedimentation coefficient might be obtained. For more complex systems, the calculated results would only be approximations subject to considerable uncertainty.

It should be noted that assumption 1 implies a constant s value, at a given concentration (i.e., within the effective concentration range in a given experiment). This does not exclude the possibility of different s values at substantially different initial concentrations. Use of an iterative approach[4] allows us to adapt our calculations to a system which has different s values at substantially different measured concentrations. In most of the experiments carried out thus far, the sedimentation coefficient has not changed with time, in a given experiment.

Pronounced concentration dependence might also produce asymmetry in concentration distribution; this would affect the results, because the theory assumes a Gaussian distribution. Convection at the leading edge of the enzyme band could also produce an asymmetric concentration distribution. Such effects would introduce uncertainty into the results and therefore should be kept in mind when interpreting results.

With multisubunit enzymes, there are two major causes of concentration dependence in sedimentation velocity experiments, namely, nonideality effects and shifts in association equilibria. In general, it is expected that nonideality effects will be negligible in high concentration AEC experiments since the working range of concentration is only 1.2 mg/mL. The rare exceptions will involve particles with extreme asymmetry.

Shifts in association equilibria are often quite pronounced over a narrow concentration range. Since results from high concentration AEC experiments in dissociation transition regions will be subject to somewhat greater uncertainty than results in nontransition regions, there will be a corresponding uncertainty in equilibrium constant values and transition points determined from such data. However, the s values for the associated and dissociated forms should be reliable, because they are obtained from plateau regions of the s vs. c curves, where there is virtually no concentration dependence. Furthermore, for most enzymes, this method is probably the best (and possibly the only) way to get estimates of equilibrium constant values and transition midpoints. To be able to get even this information is a major advance, made possible by this new technique.

The Band Theory Used to Calculate σ in the High Concentration AEC Theory. The required value of σ is obtained using eq 3, which is an equation derived for band sedimentation velocity experiments; it is based on an approximation (Vinograd et al., 1963) for a sector-shaped cell. The approximation appears to be quite good; it has given results within experimental error in numerous band sedimentation velocity experiments, both in the earlier work introducing the theory (Vinograd et al., 1963) and in the work presented here. More rigorous (Gehatia & Katchalski, 1959; Shumaker & Rosenbloom, 1965; Rubin & Kalchalsky, 1966) and more extended detailed treatments (Vinograd & Bruner, 1966a,b) yield equations which are correspondingly more complex (for review, see Fujita, 1975). The simpler theory seems quite adequate for our present purposes, since it gives results within experimental error. In fact, it has been shown (Shumaker & Rosenbloom, 1965) theoretically that, for the limiting case of very thin bands, the treatment (Vinograd et al., 1963) utilizing an approximation to the sector cell yields equations which are identical with the corresponding equations derived (Shumaker & Rosenbloom, 1965) by an exact treatment.

Conclusions

The agreement between the sedimentation coefficient values calculated using the high concentration AEC theory and the values calculated from conventional boundary sedimentation is very satisfactory. This provides evidence for the validity of the new theory at high enzyme concentrations. The absolute accuracy of the correction at lower concentrations is expected to be somewhat less than at high concentrations, but, even there, the corrected sedimentation coefficients should always be a better approximation to the true values than the uncorrected values.

A major value of this theory is that it makes up the concentration gap between the conventional centrifugation method (0.3 mg/mL and up) and the conventional AEC method, which is limited to low enzyme concentrations (below approximately 1 µg/mL for an enzyme with a specific activity of 60). With the high concentration AEC method, it is possible to study the active polymeric forms of enzymes in rapid equilibrium over the important concentration range in which most enzymes exist in vivo. Also, it is now possible, by use of this method, together with conventional sedimentation methods at higher protein concentrations, to obtain a fairly complete model description of the order and size of polymeric forms produced sequentially, as the newly synthesized polypeptide chains of a given enzyme undergo successive associations to yield the native enzyme, which may contain as many as 150 subunits in some cases.

Appendix: Derivation of Eq 7

The symbols used are defined as follows: $C_E(r,t)$, enzyme distribution at time t; $u(r,t)$, enzyme flux at position r and time t; X, cross-section area of cell = $r\theta h$; t_1,t_2, defines the time bracket in which the entire enzyme band passes through a unit cross-sectional volume; s, sedimentation coefficient of enzyme; ω, rotor speed; θ, sector angle of cell; r, radial distance coordinate; t, time; A, absorbance; C_s, substrate concentration; h, cell path length; ϵ_s, extinction coefficient of substrate; a_E, specific activity of enzyme; E_e, effective mass of enzyme.

We start by considering the flux of the enzyme molecules at any fixed position r^a as a function of time due to sedimentation (diffusion is ignored for simplicity):

$$u(r^a,t) = C_E(r^a,t)s\omega^2r^a$$

Then the total amount of effective enzyme is

$$E_e = \int_{t_1}^{t_2} u(r^a,t)X\,dt = \int_{t_1}^{t_2} C_E(r^a,t)s\omega^2r^ar^a\theta h\,dt$$

Therefore

$$E_e = s\omega^2(r^a)^2\theta h\int_{t_1}^{t_2} C_E(r^a,t)\,dt$$

or

$$\int_{t_1}^{t_2} C_E(r^a,t)\,dt = E_e/s\omega^2(r^a)^2\theta h$$

Assuming all enzyme molecules in the band have the same rate of substrate turnover and ignoring the sedimentation and the diffusion of the substrate:

$$\frac{dC_s}{dt} = a_EC_E$$

From Beer's law, $A = \epsilon_s h C_s$, so the rate of change of absorbance at position r^a is

$$\frac{dA}{dt_{r^a}} = \epsilon_s h \frac{dC_s}{dt} = \epsilon_s h a_E C_E$$

Then the total absorbance change due to the entire effective enzyme band is

$$\Delta A = \int_{t_1}^{t_2} \left(\frac{dA}{dt_{r^a}} \right) dt = \epsilon_s h a_E \int_{t_1}^{t_2} C_E \, dt = \epsilon_s h a_E E_e / (s\omega^2 (r^a)^2 \theta h) = \epsilon_s a_E E_e / (s\omega^2 (r^a)^2 \theta)$$

So eq 7 is obtained without assuming any specific distribution form for the enzyme band.

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