F. ACTIVE ENZYME CENTRIFUGATION

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Sedimentation coefficients (s values; units, seconds or 'Svedbergs' = 10^{-13} sec) can yield important information concerning mass and shape properties of both native and engineered biopolymers. In most circumstances their estimation calls for the isolation of the substance under investigation in either purified or, at least, paucidisperse form. For enzymes an elegant alternative possibility exists: their velocity of migration in an applied field can be monitored by means of spectral change linked to their distinctive catalytic property. In essence, their movement leaves a spectral 'footprint', and the migration of this 'footprint' can be followed. First devised by Cohen (238) and analysed in detail by Cohen and Mire (239), the method has since that time seen a modest degree of application. With the advent of the new generation of analytical ultracentrifuges (AUCs) with full on-line computer-based data analysis, this method, which is uniquely valuable in enabling quality physical information to be obtained about systems which have been perhaps only partially purified, and which can target the *active* component of a polydisperse system, can expect to find extended use.

34. BASIC PRINCIPLE OF ACTIVE ENZYME CENTRIFUGATION

The method is quite simple to apply, but a limited number of criteria must be satisfied if valid results are to be obtained. A solution column is set up in one sector of an AUC double-sector cell which contains all the substrates and cofactors necessary for spectral change to result from the introduction of the enzyme under study. For example, if the enzyme-catalysed reaction results in the uptake of protons, then a pH indicator such as phenol red will be present, and the solution will be (weakly) buffered to a pH (around 6.5) below the pK of the phenol red (7.3). The layering of a very small volume of enzyme on to the top of this solution column while the cell is being accelerated to speed causes a very thin zone of the enzyme to be formed, which at a sufficiently high centrifuge speed will sediment progressively down the solution column. As it does, the spectral color of the solution will be changed by the interaction of the reaction product with the indicator – in the example given above, the region above the migrating zone will become red, and the migration of the interface between the red upper region of the cell and the paler, more yellow, lower region can be followed and analysed to give a sedimentation rate (s value). In simple theory, this s value can be regarded as the sedimentation rate of the (fastest) active species of the enzyme. It is not necessary for the enzyme-catalysed reaction to yield a measurable spectral change directly: biochemical ingenuity can be used to couple almost any reaction to a spectrally significant change (e.g. the NAD^+/NADH couple; see Chapter 3).

Figure 6 shows the relationship between the change in extinction and the location of the zone of enzyme for a simple case. The change in the extinction caused by the enzyme can, of course, be either positive or negative.

35. PRACTICAL USE OF AN AUC TO GIVE ACTIVE ENZYME CENTRIFUGATION

It is assumed that the reader has the use of an AUC equipped with scanning absorption optics (e.g. a Beckman XL-A) and is competent in its basic use. The type of cell often called a 'Vinograd' cell (240) must be employed – or rather a centerpiece of this type. In this double-sector centerpiece are located one or more small drilled-out holes (*Figure 7*). The two sectors contain the full assay solution in one case, and an appropriate reference solution in the other. Both solutions will normally contain enough additional salt (e.g. 50–100 mM excess) or glycerol to ensure freedom from density inversion when the enzyme solution is overlayered. It is generally convenient for automated analysis to ensure that the change in extinction caused by the enzyme is *positive* (i.e. not as shown in *Figure 6*). This can be arranged by appropriate choice of reference solution or choice of channel.

Figure 6. Diagram showing the association between a migrating zone of enzyme and the presence of a boundary in the radial scan of the AUC cell at an appropriate wavelength.

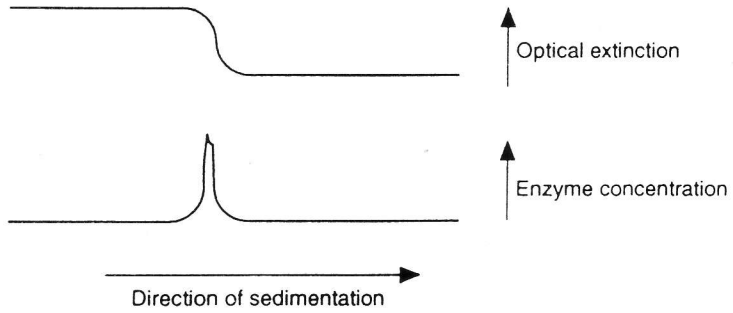
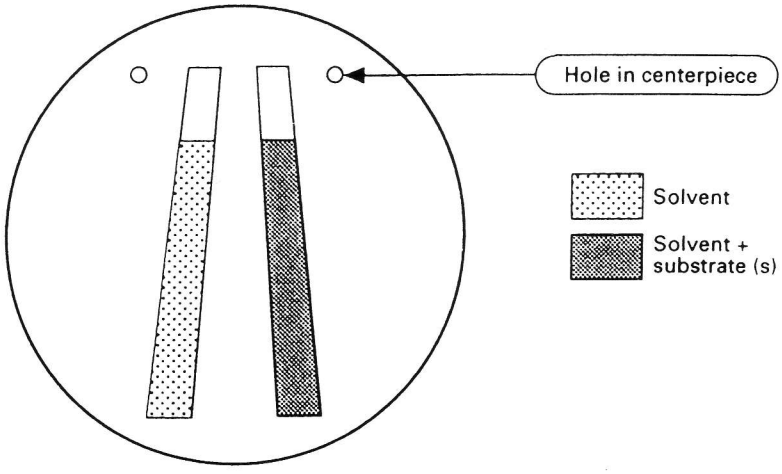


Figure 7. Diagram of a 'Vinograd' type AUC cell, with two drilled holes adjacent to the sectors.



The hole immediately adjacent to the sector containing the assay solution is filled *prior to assembly of the cell* with a small quantity of enzyme solution (usually 10 μl), using an accurate ('Hamilton') syringe to place the enzyme solution at the bottom of the hole. This is essential to avoid a trapped air bubble which would probably lead to loss of this solution during cell assembly and tightening. After the cell has been carefully assembled and torqued up, the assay and reference solutions are loaded, the rotor loaded and accelerated to a speed (usually 10 000 r.p.m. (8000 *g*) +) at which transfer of enzyme solution from the hole to the top of the column occurs. A quick radial scan is then performed at the selected wavelength to check for the presence of a level plateau region, indicating no leakage or convection. The rotor is then accelerated to operational speed, and automatic scans taken at intervals defined by the presumed range of *s* value under investigation.

After completion of the experiment, the *s* value associated with the migration of the active species can then be estimated by the normal methods – either by following the second moment of the migrating boundary, or (more simply) its first moment (point of inflection). For most cases the latter suffices.

36. CONDITIONS THAT MUST BE SATISFIED

The reader is referred to full treatments given in refs 239 and 241. The most important conditions to be met are as follows:

(i) all components of the assay system, including the agent of the spectral change and any coupling enzymes, must sediment much more slowly than the enzyme under study. Normally only coupling enzymes must be considered seriously here.

(ii) The enzyme must be loaded at a concentration such that a substantial *but not total, and generally not more than 50% maximal* spectral change occurs during passage of the zone. Although ultimately this is a matter of trial and error, a good start will be made if in dummy experiments in a spectrophotometer the enzyme in the cuvette *at its initial loading concentration* causes a spectral change of this magnitude within 5–10 min.

(iii) If the enzyme solution contains any substances (e.g. reaction products) which might themselves induce spectral change, then it is vital that the velocity of movement of the boundary exceeds by a reasonable margin the diffusion rate of such substances.

(iv) Care must be taken to ensure that the conditions employed do not inhibit any coupling enzymes used; for example, one could not use EDTA in the PK/LDH system described below, since Mg^{2+} is a vital cofactor.

37. EXAMPLES OF THE USE OF ACTIVE ENZYME CENTRIFUGATION

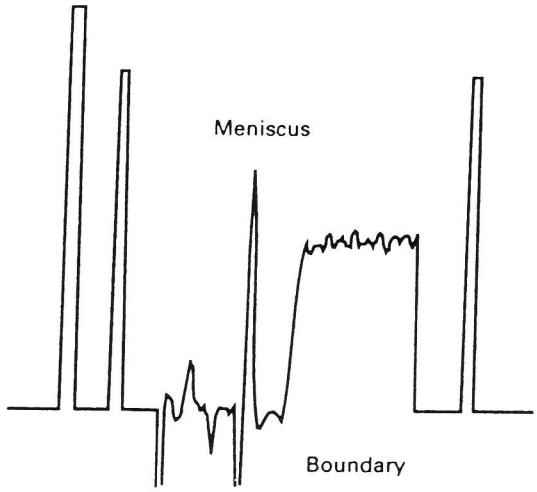
37.1. Native myosin filaments

These have been followed by their ATPase activity being linked (via pyruvate kinase and lactic dehydrogenase) to the NAD/NADH couple, at 340 nm (242; *Figure 8*). This was an early use of a multienzyme coupling system. The *s* value of these filaments (132S) had to be measured at above 12 000 r.p.m. (11 000 *g*) to avoid problems from diffusion of the ADP present in the preparation.

37.2. Solubilized (α+β) chains of Na^+/K^+ ATPase

Here active enzyme centrifugation has been employed to show that the monomeric species was active, no dimer needed to be formed (244). The activity was coupled to phenol red in this case, and scanned at 550 nm. The *s* value found (6.5±0.2S) was confirmed as being identical to that measured using the purified enzyme in conventional velocity analysis. A similar study has been performed on the monomer of the sarcoplasmic reticulum (SR) calcium pump in $C_{12}E_8$ solution (245).

Figure 8. Active enzyme sedimentation diagram of native thick filaments from vertebrate skeletal muscle (243).



37.3. Glutamate dehydrogenase

The activity of the wild-type enzyme was compared with that of two point mutants (R61E and F187D) to show that only the hexameric form of the enzyme is active (246). The activity was coupled to NADH *production* (i.e. the opposite to that used above (242)) and scanned at 340 nm.

Figure 9. Active enzyme sedimentation diagram of glutamate dehydrogenase in the presence of glutamate and NAD^+. Rotor speed, 25 000 r.p.m. (50 000 *g*); scan interval, 5 min. The line fitted follows the point of inflection of the sedimenting boundary in the same way as a conventional sedimenting boundary would be followed.

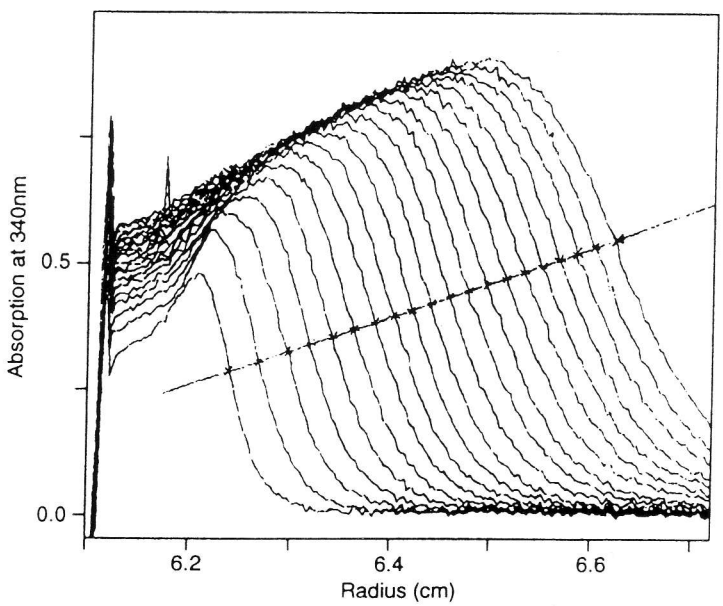


Figure 9 shows the active enzyme velocity profile for the R61E mutant in phosphate–chloride buffer (pH 7.0), yielding an $s_{20,w} = 14.3 \pm 0.2$S. In a buffer of higher pH (8.8) where the enzyme is entirely in its trimeric form, no active enzyme sedimenting boundary was observed.

38. REFERENCES

238. Cohen, R. (1963) *C. R. Acad. Sci.*, **256**, 3513.

239. Cohen, R. and Mire, M. (1971) *Eur. J. Biochem.*, **23**, 276.

240. Vinograd, J., Bruner, R., Kent, R., and Weigle, J. (1963) *Proc. Natl Acad. Sci. USA*, **49**, 902.

241. Kemper, D.I. and Everse, J. (1973) *Methods Enzymol.*, **XVII**, 67.

242. Emes, C.H. and Rowe, A.J. (1978) *Biochim. Biophys. Acta*, **537**, 125.

243. Emes, C.H. (1977) Ph.D. Thesis, University of Leicester.

244. Madden, C.S., Ward, D.G., Walton, T.J.H., Washbrook, R.F., Rowe, A.J. and Cavieres, J.D. (1994) in *The Sodium Pump: Structure, Mechanism, Hormonal Control and its Role in Disease* (E. Bamberg and W. Schoner, eds). Springer, Darmstadt, p. 445.

245. Martin, D.W. (1983) *Biochemistry*, **22**, 2276.

246. Pasquo, A. Britton, K.L., Stillman, T.J., Rice, D.W., Cölfen, H., Harding, S.E., Scandurra, R. and Engel, P.C. (1995) *Biochim. Biophys. Acta*, in press.