5

Methods for Off-line Analysis of Sedimentation Velocity and Sedimentation Equilibrium Patterns

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1. INTRODUCTION

The equilibrium or transient sedimentation velocity distribution of particles dispersed in an aqueous solute situated in a centrifugal accelerative field is routinely studied by means of either an optical trace recorded photographically or by chart recorder traces. Rayleigh interferometric fringe patterns have been widely used to record *equilibrium* distributions, in which the displacement of the parallel fringes is directly related to particle concentration differences. In this Chapter we will outline how we have developed a simple but highly efficient frameshift algorithm for automatic interpretation of these patterns[1]: results obtained from extensive use and further definition of this algorithm confirm its validity and utility.

We will also describe the use of algorithms we have developed for the interpretation of Fresnel fringe patterns yielded by an alternative optical system: Schlieren optics: these more complex patterns involving non parallel fringes can be analysed successfully, subject to certain conditions, with a precision similar to that obtained using Rayleigh interference optics. For the analysis of sedimentation equilibrium *or* sedimentation velocity records from chart paper rather than photographic output (as applies for example, to the use of the scanning absorption optics system on the Beckman (Palo Alto, U.S.A.) Model E or MSE (Crawley, U.K.) Mk II analytical ultracentrifuges or the scanning absorption and knife edge Schlieren optics on the MSE Centriscan ultracentrifuge), the use of a simple digitising table is sufficient to give acceptable precision combined with convenience of use. Some reference to these features will also be made in this Chapter.

Although the theory of sedimentation transport and equilibrium has been dealt with elsewhere in this volume, it is still instructive at this stage to consider some basic

formulae which indicate whether it is concentration or concentration gradient distribution a particular data capture regime is designed to record.

The basic equation governing the distribution of particles at *sedimentation equilibrium* in a centrifugal field is given at infinite dilution by[3]

$$\frac{dc}{dr} = \sigma c r \tag{1}$$

where c is the concentration of particles at radial position r, and σ is a "reduced" molecular weight (*cf* Chapter 7), given by

$$\sigma = \frac{M_r (1-\bar{v}\rho)\omega^2}{RT} \tag{2}$$

where M_r is the molecular weight, or, as biochemists like to use, the "relative molecular mass", $\bar{v}$ the partial specific volume ($\approx$ reciprocal density) of the particles, ρ the density of the fluid, R the gas constant, ω the angular velocity and T the temperature (in degrees Kelvin).

Eq. (1) is frequently used in its "integrated" form:

$$\frac{\Delta \log c}{\Delta(r^2)} = \frac{\sigma}{2} \tag{3}$$

Normally the parameter M_r will be the object of study. It may itself be a function of r, either directly as a result of polydispersity or indirectly as a result of depending upon c (which itself will vary with r), through non-ideality or associative phenomena.

The basic purpose of any analysing optical system is thus to record a pattern capable of being interpreted to yield *either* c *or* dc/dr as a function or r, at the stable, equilibrium distribution.

By contrast, *sedimentation velocity* is a transport method, and a time (t)-dependent series of distributions must be logged in this case. If the interface which is being monitored between solution and solvent is at a radial position r_b, then the sedimentation coefficient s (velocity per unit field) is given by

$$s = \frac{1}{\omega^2 r_b}\left(\frac{dr_b}{dt}\right) \tag{4}$$

Again it is often more convenient to use the integrated form:

$$s = \frac{1}{\omega^2}\left(\frac{\Delta \log_e(r_b)}{\Delta t}\right) \tag{5}$$

from which s is readily determined from the regression of $\log_e(r_b)$ on t.

2. DATA CAPTURE IN OFF-LINE MODE

Historically only a small minority of workers have used wholly on-line systems for data capture in the analytical ultracentrifuge. This has been related to the cost and complexity of the systems required. It may now be taken for granted, however, that on-line capture will be normal for scanning absorbance work, particularly with the advent of the new Beckman Optima XLA Analytical Ultracentrifuge, which incorporates as standard the logging of data to disk (formatted as ASCII files). Almost certainly the same will apply to Rayleigh Interferometric and to Schlieren patterns, as and when the modern instrument incorporates the appropriate optical systems. However, in the meantime - which is likely to extend for some years to come - it will be necessary (other than in laboratories possessing specialised devices) to evaluate distributions recorded using these optics in off-line mode. We consider now optimal procedures for doing this, and note that the interpretative algorithms involved may well be equally useful for analysis of on-line data.

Rayleigh Interferometric and Schlieren patterns are invariably recorded on photographic film. Modern film backings give adequate dimensional stability, although care must be exercised to avoid serious temperature changes during processing. 'Fine grain' emulsions offer sufficient (line) resolution in almost every case, normally around 100 cycles per mm. Absorption and refractometric scanning patterns are recorded onto chart paper. Because of the large spatial magnification used, resolution is not a problem in this case, although for several reasons ('shot noise' from sampling, window absorption and scattering, electronic noise) the attainable precision is normally lower than with the two other optical systems.

Analysis to Yield c *vs.* r Data for Equilibrium Distributions

The classical approach has been to use Rayleigh interference optics to give a pattern in which the displacements of the fringes in a direction (z) normal to radial is a linear function of the concentration increment at the radial position in question. The fringes are of course equi-spaced and parallel, and hence a scan across them in the z direction yields a sinusoidal intensity function whose phase is a measure of (the non integer part of) the fringe shift.

We have developed a simple but fast and stable algorithm for deriving the phase shift from the intensity function[1]. The latter is logged from the photographic record of the fringe pattern, using a commercial scanning densitometer, the LKB (Bromma, Sweden) 2202 laser densitometer. Then if Q fringes are contained within the window analysed, an iterative frameshift is performed within the data set, to maximise the Fourier coefficient of order Q. The method is thus a null method, which searches for the frameshift which will set the phase term to zero[1]. This algorithm, unlike earlier approaches in this area, yields estimates for the fringe increment whose precision is not a function of the latter parameter itself. The precision of the recorded

fringes may be gauged subjectively from Fig. 1 and using this system a precision of f/500 (f is a single fringe increment) is in our experience attainable[1].

Analysis to Yield dc/dr *vs.* r Data

The earliest optical method used to analyse distributions within the ultracentrifuge cell was the 'Schlieren' optical system, in which an analysing diaphragm is inserted into the back focal plane of the camera lens employed to image the cell. Shadows or other traces are produced whose displacement, again in the z direction, is proportional to the first derivative of solute concentration with respect to radial distance. Other than in the earliest work, a 180° phase plate has been used as the analysing diaphragm. The resulting single trace is rather broad as compared to an interference fringe (Fig. 2).

It has been universally considered that the precision with which this trace can be interpreted falls well short of what can be achieved using Rayleigh interference optics. Subjectively this is understandable. The Schlieren trace appears relatively broad, and only a single trace is yielded, thus making unavailable the reduction in noise/signal normally achieved from multiple records. Yet the principal optical components of the two optical systems are identical, and are used at the same working aperture. Insofar as distinctive components are introduced in either method, there is no reason to suppose that these limit the information transfer function, which one would expect to be very similar in both cases, given adequate interpretative algorithms.

We have therefore researched the possibility of developing the interpretation of Schlieren records to a much higher level than heretofore. There are a number of practical reasons for doing this. As detailed below, we find that with suitable developments of the methodology, and subject to certain relatively minor reservations, results from the Schlieren optical method can indeed be interpreted with a precision approaching those obtained by the Rayleigh interference method. The basis of this is the recording and interpretation of the more complex *Fresnel* fringe patterns generated by the Schlieren optical diaphragms. Several approaches to the interpretation of Fresnel fringe patterns can be defined. It seems likely that an optimal approach has yet to be delineated, but results to date are more than adequate to demonstrate the potential of work in this area.

3. FRINGE SHIFTS IN RAYLEIGH INTERFEROMETRIC PATTERNS

Our off-line data capture algorithm (ANALYSER written in Turbo Pascal) from c *vs.* r records has already been described in some detail[1,2]. ANALYSER[1], which is based on a frameshift Fourier algorithm, has now been upgraded to version ANALYSE2 which permits the analysis of data from a two-dimensional data acquisition system as opposed to a series of individual one-dimensional scans. Sophisticated search procedures have been incorporated to ensure that the system reproducibly and stably

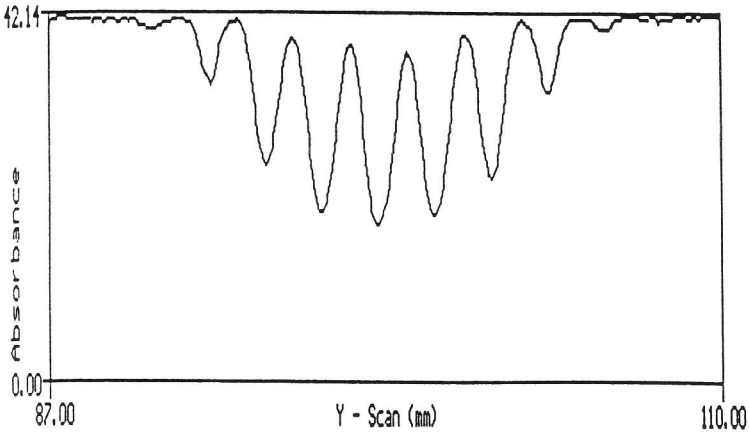


Figure 1. Digitised optical density values output from an LKB Ultroscan XL 2222 (two-dimensional scanner) scanning at a single radial position. 575 values were logged in this case at each of 175 radial positions in the cell, and these form the data set for subsequent analysis. Data for sedimentation equilibrium experiment on lipase, using Rayleigh interference optics.

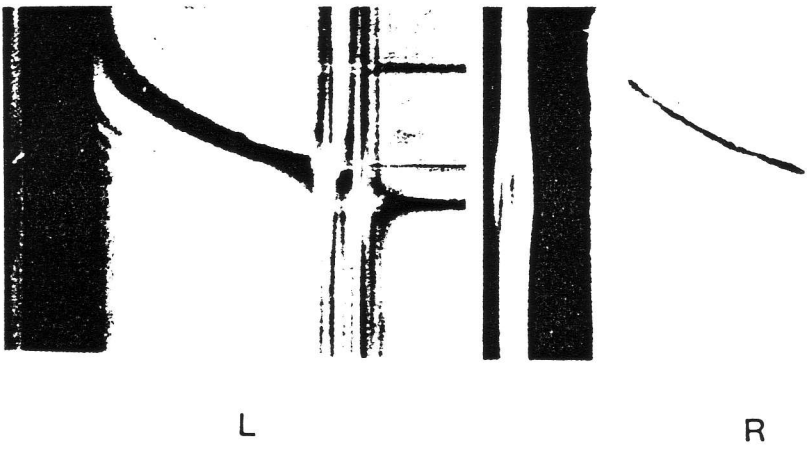


Figure 2. Phase plate Schlieren records of a solution at sedimentation equilibrium in an ultracentrifuge cell (MSE Mk II Analytical Ultracentrifuge). The solution column is 2 mm long in real space. From original negatives conventionally (L) and correctly (R) exposed.

finds the correct fringe intensity maximum in what is now a full two-dimensional record. As it is now possible to analyse data at up to 200 radial positions from a single experiment, rigorous tests can be performed to assess such factors as sample homogeneity and interactions (Fig. 3). The completed system is now in intensive use, and results on many systems have fully justified our initial estimates[1] of the precision attainable. What we would now like to focus on for the purpose of this Chapter is a new off-line data capture system for optical *Schlieren* records of *dc/dr vs.* r.

4. REFRACTOMETRIC OPTICS AND FRESNEL FRINGE PATTERNS

Refractometric Optics

The presence of an analysing diaphragm in the back focal plane of the camera lens imaging a cell, in this case located in an ultracentrifuge rotor, leads to the formation of a 'Schlieren' pattern in the image plane. The presence of a cylindrical lens results in a pattern in the image plane in which the z deflection of the trace at any radial position is linearly related to the refractive index change (dc/dr) in the conjugate locus in the cell plane (Lloyd[3]). Although any physical form of diaphragm can in principle be employed, it has long been customary to use a 180° phase plate (Wolter[4]), which causes no loss of transmitted light and has been considered on general principles (rigorous analysis appears not to have been performed) to maximise the information transfer function[3]. As a pure phase plate records no signal as $dc/dr > 0$, a thin line of metal evaporated onto the half wave step is normally added[3]. The latter *only* produces a trace for zero or very low dc/dr values. The optics of the transition region of dc/dr have yet to be been defined.

The resulting trace shows a well defined but rather broad line (Fig. 1). This represent the zero'th order fringe of an often poorly resolved Fresnel pattern. We have addressed ourselves to the definition at high resolution of the co-ordinates of this pattern, both by location of the zero'th order fringe, and by an alternative approach in which we derive and apply a relationship between Fresnel fringe *spacing* at defined r and the corresponding dc/dr and Δc values.

Definition of the Zero'th Order Fringe in a Fresnel Pattern

As noted above, the conventional approach here has been to record the Schlieren pattern using a modified phase plate diaphragm. The pattern is in principle symmetric with respect to the zero'th order fringe, which is located in the centre of the line trace. However, as was noted by Rowe and Khan[5], a simple knife edge diaphragm has a better established optical theory, and can yield results of a precision equal to that given by a phase plate. We have therefore explored the use of a simple knife edge diaphragm to generate the Fresnel pattern.

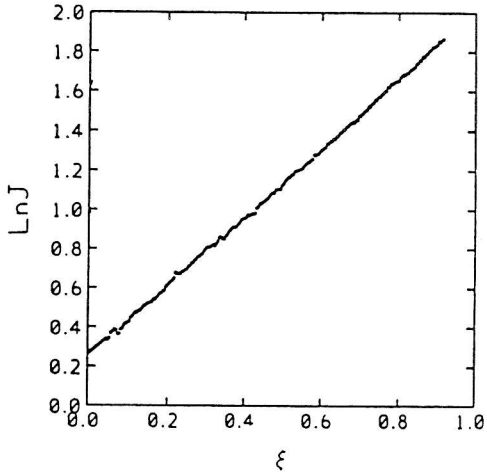


Figure 3. Plot of the logarithm of the solute concentration (expressed in absolute fringe numbers, J) *versus* the radial displacement (squared) parameter ξ. Data from a low speed sedimentation equilibrium experiment on recombinant hirudin, loading concentration 0.8 mg/ml. From the slope a weight averaged molecular weight of (7080±100) is computed (from sequence = 6964).

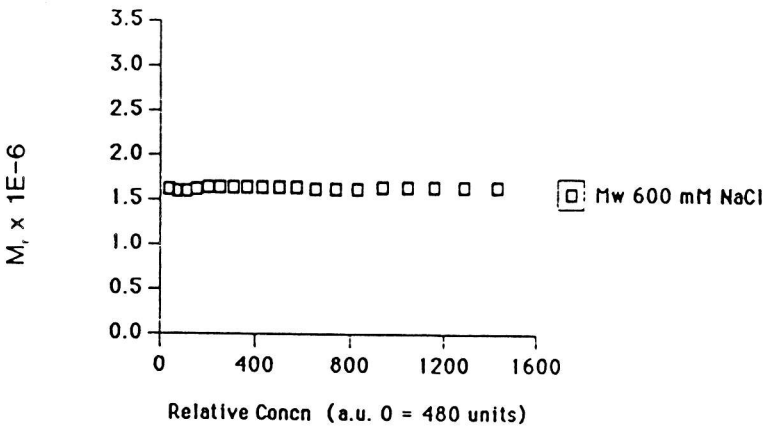


Figure 4. $M_{r,w}$ values computed (from an equilibrium solute distribution recorded using Schlieren optics) for the protein dynein at a cell loading concentration of 0.5 mg/ml. These point weight average values are computed by numerical integration of the dc/dr values to give c values, the constant of integration being found by a numerical manipulation based upon the equivalence of harmonically related averages.

We have in all cases adopted certain modified procedures for setting up of the ultracentrifuge cells and optics designed to maximise the information transfer function. These will be described elsewhere. They are not relevant to the interpretative algorithms described below, but are indispensable if results of the precision described are to be obtained in practice.

The Zero'th Order in a Phase Plate Generated Fresnel Pattern

The zero'th order in this case is simply the maximum density (minimal intensity) in the z scan across the line pattern (Trautman and Burns[6]). Unfortunately as normally recorded the line is rather broad and the precision in the estimation of the position of the maximum not high. This is the conventional reason behind the general opinion that Schlieren optics are inherently of low precision.

Several experimental procedures can be adopted which largely circumvent the problem of the width and lack of definition of the optical trace. The experimental procedure most relevant to the present discussion is the use of longer photographic exposures to bring the optical record *near to the centre of the line trace* into the linear part of the gamma curve of the recording material. It seems not to have been generally appreciated (though Lloyd[3] commented briefly on the matter) that giving a 'normal' exposure as gauged for the whole photograph seriously degrades the transfer function in the critical spatial region.

Locating the zero'th order in a suitably exposed photographic image (*i.e.* highly *over* exposed with respect to 'non-information') by analysis of successive radial z scans results in a very smooth data set. Fig. 4 below illustrates typical final results computed from data measured in this way.

These results are comparable to those which would be obtained using Rayleigh interferometric optics together with the Fourier algorithms described above, and would correspond to a precision of at least f/300 in the latter terms. There are however certain qualifications which must be made concerning the absolute *accuracy* as distinct from the *precision* of the results. As noted above, for low values of dc/dr, the optical behaviour of a compound phase plate is far from well defined. This can be circumvented by avoidance of such conditions. More seriously, it seems not to have been appreciated that the physical properties of the phase plate and in particular its phase angle are critical.

We have computed intensity distributions in the image plane for a phase plate of various angles, using the Cornu Spiral construction[6]. The treatment given by Trautman and Burns[6] assumes a phase angle of 180°. We have extended this to the more general case, and as the tabulated values for the Cornu Spiral are in some cases of insufficient resolution we have computed the co-ordinates from the relation[7]:

$$F(x) = \left(\frac{2}{\pi}\right)^{0.5} \left\{ x + \frac{jx^3}{3} + \left(\frac{j^2}{2!}\right)\left(\frac{x^5}{5}\right) + \ldots + \left(\frac{j^n}{n!}\right)\left(\frac{x^{2n+1}}{2n+1}\right) \right\}^{0.5} \quad (6)$$

for n = 0, 1, 2, ... and the spiral is plotted in the complex plane ($j=\sqrt{-1}$).

The results are shown in Fig. 5 below for phase angles of 180°, 150° and 120°.

It is clear that the location of the true geometrical edge coincides with the minimum of the intensity distribution *only* for the case of a 180° phase angle. Moreover the pattern is not symmetrical for other phase angles. Thus the wavelength used is critical, and must be tuned to the particular phase plate used, a precaution which has not formed part of normal practice. Longer photographic exposures are now needed, to compensate for the reduced light intensity in monochromated light.

The Zero'th Order from a Knife Edge Pattern

A simple knife edge used as a diaphragm results in a 'shadow' pattern, with a set of Fresnel fringes (Fig. 5). Although actually of slightly smaller amplitude than those generated by a phase plate[6], the lower dynamic range of the image means they are frequently better registered.

The location of the zero'th order fringe can be computed by measurement of (say) the first and second order minima, and from the knowledge that their location (z) with respect to the zero'th order is given for order i by

$$z = \{4(i+1) - 0.5\}^{0.5} \quad (7)$$

In practice only the first two or three orders can be measured in a z scan (Fig. 6).

Zero'th Order Determined from the Fresnel Fringe Spacing

It is possible to use the values of the fringe spacings in the z direction to evaluate Δc *directly* instead of by computation of the location of the zero'th order. This is because of the defined relationship[8] between fringe spacing and the second derivative of the refraction (and hence concentration) gradient, from which it follows at once that

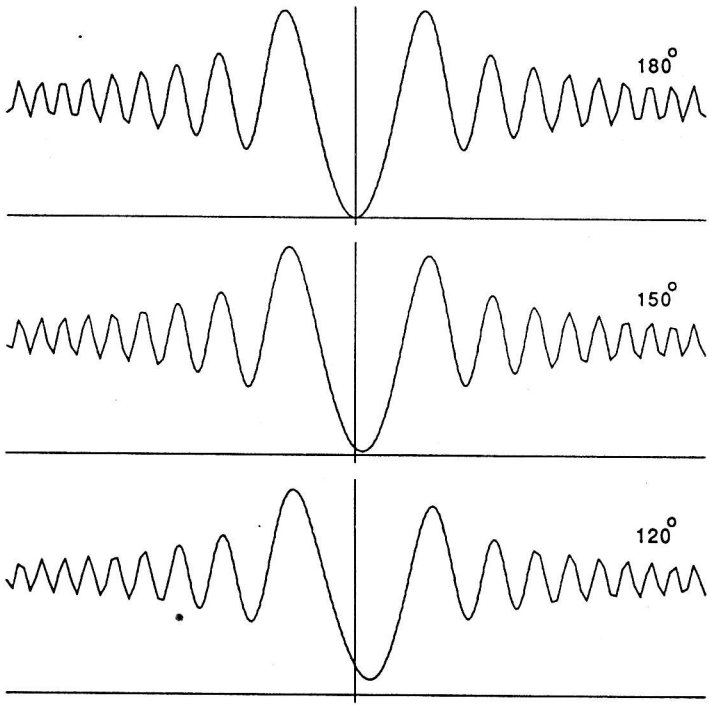


Figure 5. Computed intensity distribution of the Fresnel pattern yielded by a phase plate of phase angle 180°, 150° and 120°. The location of the true geometrical edge is shown by the vertical line in each case.

$$\Delta c = \int_{r_a}^{r_b} \int_{r_a}^{r_b} \left\{ \sum_{j=1}^{j=n-1} \frac{\left[\frac{z_{i+1} - z_i}{\{4(i+1) - 0.5\}^{0.5}} \right]}{\left[\frac{(4i-0.5)^{0.5}}{n-1} \right]} \right\} dr\, dr \qquad (8)$$

This equation defines the relationship between Fresnel fringe spacings and Rayleigh fringe shifts. The double integration is highly favourable with respect to error reduction in the data set, achieving at least an order of magnitude of diminution, greater if the summation can be effected by measurement of multiple fringes. A constant of integration is required for the first integration. This is in fact the zero'th order spacing, but as an independent estimate of this constant can be obtained from each radial scan, errors in its estimation are not serious.

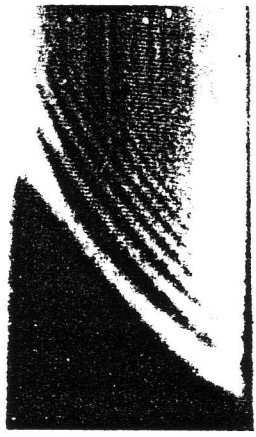
We have evaluated several procedures for determining the z spacings in a multiple fringe pattern. Direct fitting of the Fresnel function by a least squares algorithm has been implemented, but is not totally successful. This is primarily because the intensities in a Fresnel pattern, ranging from true zero upwards, cannot possibly be recorded photographically within the linear part of the gamma curve of the emulsion. The true pattern is thus convoluted with an envelope function, which as the Fresnel amplitude/intensity function is anharmonic, cannot readily be deconvoluted as with Rayleigh patterns[1]. Furthermore, as noted above, although up to ten or more Fresnel fringes can be discerned by eye, the intensity scans give only two or three clearly defined maxima/minima (Fig. 6).

Thus this approach, whilst in principle superior to the simple evaluation of the zero'th order, has yet to be developed to its full potential.

Data Capture from Chart Records

This is particularly simple. No method can equal the convenience and ease of use of a digitising table linked to a microcomputer. A spatial resolution of at least ten cycles per mm over the active area is required, which is readily attainable with most commercial tables. The use of a stylus *or* cursor with cross-hairs is largely a matter for individual taste. We have for many years used a BIT-PAD linked to an Apple II microcomputer - its current upgrade is an Apple Macintosh linked to a Wacom tablet. A problem for some users will be that no single format is standard: for those not feeling confident about writing their own system the simplest approach is to contact an existing analytical ultracentrifuge user owning a system mounted on a hardware platform matching the enquirer's.

(a)



(b)

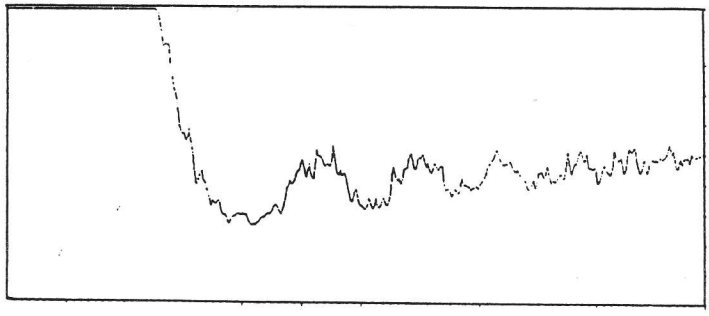


Figure 6. (a) A part of a Schlieren pattern recorded with a knife edge diaphragm from a solution of an enzyme (chloramphenicol acetyl transferase) at sedimentation equilibrium. Centrifugal direction is from left to right; (b) Vertical (z) scan across the above Fresnel fringes. The location of the centre of the peaks in the scans can be determined by established procedures[1]. The resulting precision is found to be of the order of 1% to 2% of the fringe spacing.

Particular points which need to be borne in mind by those writing their own software are:

For *velocity* runs, the programme must incorporate a check as to whether the original data is charted or photographic. This is because the time interval is *non-constant* in the former case, due to the differential time taken for the scanner to reach the boundary position.

For *equilibrium runs*, consideration must be given to the fact that intervals in the radial direction cannot be taken as wholly constant, due to the limitations of the hand-eye combination and possibly of the resolution of the table. It may be desirable to incorporate an algorithm transforming the data set to equal spacing in r (or usually better r^2).

In both cases, care should be taken that both raw and derived data are presented to the user *and* made available for further analysis and graphical presentation. The simplest way of achieving the latter aims is to store ASCII files which can be read into a commercial spreadsheet, unless the user wishes to undertake the (perfectly feasible but not particularly simple) task of writing dedicated presentation software.

4. CONCLUSIONS

Off-line evaluation offers a simple approach which is not instrument-dependent. For some time to come it will be used as the only rapid way to evaluate certain types of data, and the simple digitising table/microcomputer combination offers a simple and universal approach of an accuracy and precision acceptable for many purposes. Very high precision can however be obtained, where the original record is photographic. The evaluation of relative solute concentrations within an ultracentrifuge cell by Rayleigh interferometric fringe shifts can, for example, using optimal procedures and interpretative algorithms, yield a data set with a precision approaching f/500, where f is a single fringe shift[1]. The alternative Schlieren optical system can under identical experimental conditions (*i.e.* same solute concentration and optical path length) approach the Rayleigh fringe level of precision. In terms of Rayleigh fringe shifts, the direct evaluation of the zero'th order by a phase plate diaphragm attains a precision equivalent to f/300 in Rayleigh interference terms, though with some danger of systematic error, and by knife edge diaphragm attains f/150 to f/200. By the use of the fringe spacings and the transformation noted above (eq. (6)) a precision of close on f/300 is attainable in principle. This latter equation offers a formal proof of the transformation required to relate Rayleigh fringe increments to Fresnel fringe spacings.

These refractometric (Schlieren) optics have a number of advantages over Rayleigh interferometric optics. Their alignment is much simpler, and window

distortions are a much less serious problem. The widely held supposition that their precision is much inferior to Rayleigh optics lacks a theoretical basis and is now shown to be untrue in practice. Given more advanced two-dimensional analysis of the recorded fringe patterns to enable up to ten Fresnel fringe spacings to be analysed, the precision of refraction and hence concentration increment determinations should be essentially the same by either method, and it will be possible to choose the simpler refractometric system when experimental conditions so dictate.

ACKNOWLEDGEMENTS

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