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COLLECTING AND PROCESSING RECORDS FROM THE ULTRACENTRIFUGE IN "REAL-TIME" USING AN ON-LINE COMPUTER

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An analytical system consisting of an analytical centrifuge coupled 'on-line' to a computer was assembled and tested. Collection of records from up to 9 solutions was achieved through programmes which sum readings to reduce noise as well as controlling the positioning of the scanner. With this system it was found that the limit on accuracy for molecular weights at concentrations less than 0.01 g cm^{-3} was ± 3% estimated from sedimentation equilibrium experiments. The same system was used to collect records for similar concentrations from velocity experiments by employing a scanning schlieren. In this case the accuracy in estimating sedimentation coefficients was similar to those found when measuring photographs. Since the collection yields detailed information about the shape of the sedimenting boundary, the centroids of the boundary were routinely computed by second moment analysis rather than relying on the position of the maximum of the schlieren peak. In the same analysis estimates of diffusion coefficients were made routinely by calculating corrected height/area ratios for each scan. These calculations were made during the real-time of the experiment, so making available molecular parameters rather than records which must be evaluated some time after stopping the experiment.

1. Introduction

A significant milestone in physical biochemistry was reached when Svedberg and his colleagues [1] constructed an analytical ultracentrifuge and with it later showed that proteins were discrete molecules and not amorphous colloids. His centrifuge was possibly one of the most advanced instruments of that period and it provided an early bridge between biochemistry, chemistry, physics and engineering. Later work by Pickles and Beams (see Schachman [2] for a review on these early developments) ensured that the instrument was within the grasp of all biochemists and led to developments of ingenious systems for producing records of relative distributions of concentrations (c) of macromolecules or their dc/dr (with dc expressed as dn, the refractive index gradient) or absorbance, leading more directly to concentration. Classically these records were captured in photographs which were measured at the end of the experiment and the data processed by the most expedient, but not necessarily rigorous, procedure. As measuring techniques developed, so the demand for more precise and detailed analyses increased, and it is interesting to find that in addition to this paper there are two more systems [3,4] involving computers in this copy of Biophysical Chemistry, possibly reflecting the desire to provide instantaneous and accurate estimates of molecular parameters.

In early applications of the ultracentrifuge efforts were concentrated on detecting and measuring heterogeneity in preparations and photographic records were acceptable in accuracy and definition. Simpler and possibly more powerful techniques have replaced the ultracentrifuge as criteria of purity (for example, electrofocusing, gel electrophoresis and gel chromatography) and interests of physical biochemists have moved to using sedimentation to measure interaction between macromolecules as well as providing absolute measurements of molecular weights and hydrodynamic parameters. To achieve sensible precision in this modern work, the records must be precise and relatively large quantities of data must be measured in order to yield final estimates of parameters. Applying this criterion to the centrifuge has meant replacing the photographs by procedures which give instantaneous accurate records of both radii and concentration. Coupling a computer to the ultracentrifuge is an attractive combination for doing this.

It was surprising to find that 35 years after Svedberg

showed that proteins were discrete units, the precise molecular weight of bovine serum albumin was still in doubt [5], and it remained for Squire et al. [6] to provide a more definite estimate in 1968. This inability to provide concordant estimates of molecular weights is alarming and one prime objective of the present work has been to construct a system which gives routinely accurate estimates of molecular weights and allows the experimentalist time to consider the chemistry of the problem rather than having to make extensive and laborious measurements. Accuracy has always been high in estimating sedimentation coefficients and it must be a feature of any system that it should provide accuracy in these estimates at least equal with the estimates of molecular weights. Employing a computer means that it is no longer necessary to use approximate methods for calculating variables; instead theoretically sound algorithms can be employed. For example, it should be possible routinely to find the centroids of sedimenting boundaries by second moment calculations rather than measuring the radial dimensions of the maximum ordinate of the schlieren peak.

The work of Schachman and his co-workers [7] opened the way with their photoelectric scanner to constructing a computer controlled measuring system. The complexity of the original equipment advanced, making it possible to record measurements from multiple cells in one rotor and schlieren records can now be recorded by this equipment [8,9]. We expanded from this idea to give a computer controlled system which performed most of the analogue functions by digital and programmed algorithms [10]. There were many limitations on this original computer system arising mainly from lack of storage in the computer and restrictions on the arithmetical operations introduced through using a computer having a relatively short word. The system described in this communication is more elaborate and it is now possible to routinely collect accurate records for up to 9 solutions in one rotor as well as processing these records to give instantaneous estimates of molecular parameters. While testing the operation of this system, several previously unrecorded properties of bovine serum albumin were detected and some of these results have been used to illustrate the functioning of the system.

2. Experimental

The description of the system has been divided into two subdivisions, (1) Centrifuge and interface to computer hardware, and (2) Programs used to operate the system.

2.1. Centrifuge and interface to computer hardware

An analytical ultracentrifuge (Measuring and Scientific Equipment Ltd., Crawley, England) fitted with a photoelectric scanner and monochromator illuminated with a 150 watt xenon light was interfaced to a Nova 1220 computer (Data General Corp., Mass., USA) having 16 384 words of core store and an exchangeable disc. The mechanics of the scanner followed the principles described by Spragg et al. [11] and used an oscillating mirror to move the image of the centrifuge cell across the masked photomultiplier. In addition to the monochromatic light, the electronics also use signals from a secondary infrared beam of light which passes through a notch cut in the top of each sector of the centrifuge-cells and eventually falls on a photodiode. Signals from this infrared beam both trigger the sample and hold unit (fig. 1) and alert the program to the fact that a sector is aligned in the monochromatic light and ready for conversion from analogue to digital form. The pulses from two adjacent sectors are separated through gates by electronics supplied with the centrifuge. Transmission ratios are calculated in the computer from voltages collected from the photomultiplier so it is important to sample each pulse at the same angular position of the rotor. To allow for manufacturing tolerances in the sectors, two adjustable delays have been introduced between the photodiode and each sample and hold unit (fig. 1). The point where each pulse is sampled can be varied from one side to the other of the voltage pulses from the photomultiplier and the delays are tuned at the beginning of each experiment so that sampling occurs at the top of all pulses.

Two sample and hold units (settling times 1 μs and sampling bandwidth 350 kHz) are necessary, each following voltages from adjacent sectors. Because only 3 μs separate sectors at full speed of rotor, it would be impossible to convert from analogue to digital form (at the accuracy required) in 3 μs. The converter (12 bit) is fed through a multiplexer (requiring approx-

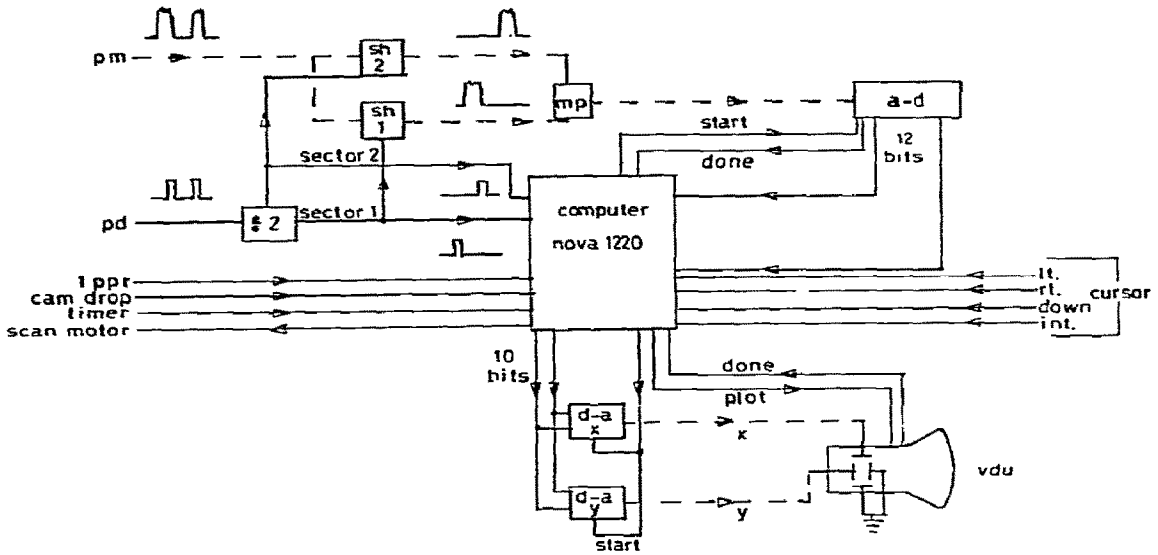

Fig. 1. Flow diagram of signals to and from the Nova 1220 computer. pm = photomultiplier; pd = photodiode (detecting sector); a–d = analogue to digital converter; d–a = digital to analogue converter; sh = sample and hold unit; ppr = pulse per revolution of centrifuge rotor; lt = left; rt = right; int = interrupt; vdu = visual display unit.

imately 8 μs to settle after switching) and the sampled voltages are clamped for 80 μs at their peak values before releasing the clamp, so allowing the sample and hold to follow the incoming voltage from the photomultiplier.

Three additional digital signals are supplied to the computer from the centrifuge: (1) a single pulse produced by each revolution of the rotor and fixed in its angular position with respect to the reference centrepiece. This position is required by the program in order to relate sequential readings collected from cells in a multi-hole rotor to the respective combinations of sectors for calculating the transmission ratios; (2) a change in level which indicates when the scanner reaches the drop in the cam so making it possible for the program to synchronise the search for the first reference hole; (3) a similar change in level from the clock in the centrifuge for collecting records at predefined intervals. Finally, the computer supplies pulses which drive the stepping motor attached to the scanner. This has a minimum stepping distance equal to 4×10^{-3} mm. Radial dimensions are measured as pulses supplied to this motor.

Having collected a set of records the computer stores the information on the exchangeable disc. These records are then edited before processing using a visual display. A 23″ display was constructed driven by two 10-bit digital to analogue converters (fig. 1). The editorial program displays the chosen record and a cursor is included within the displayed pattern which can be moved right, left, and down through three control switches. When this cursor is moved near a displayed point and a fourth switch is pressed, the index of the coordinate is returned to the program.

2.2. Programs

Once the centrifuge is operating and the necessary adjustments have been made to the electronics, the experimentalist must call a program to initialise various experimental parameters. These include defining the radial position for starting the collection, the time interval between scans, number of sectors in the rotor, their relative combinations and the signal to noise ratio to be reached during the averaging of noise on the incoming signals. As with our earlier system [8, 10] the scan of the cell-image is not continuous, but data is collected at discrete intervals along the cell. The in-

cremental distance for stepping the scanner must also be entered into the initialising program; it has a min imum value of half the width of the slit width on the photomultiplier (this width is always less than 0.1 mm). The radial distances are entered in linear dimensions (cm), so to convert these to machine variables the stepping motor must be calibrated in terms of pulses per centimetre. This calibration is also carried out by the initialising program at the wavelength of light to be used in the collection by counting the number of pulses required to move between the two reference holes. All these parameters are stored in a file available to the collection program.

Collecting a record starts by the operating system calling a second program. This can occur either through the teletype or the interval timer of the centrifuge. This program reads the initialised parameters from the stored file: it then moves the scanner to the first reference hole and records the number of pulses before moving to the starting position. This is the first datum of the record and, as with subsequent points, it receives and averages pulses from each sector and calculates the transmissions for the combination. The voltages from the photomultiplier are first accumulated to give $\bar{V}_i$ for n readings of the ith sector [eq. (1)]

$$\bar{V}_i = \sum_{j=1}^{n} V_{i,j} . \qquad (1)$$

From $\bar{V}_i$ is calculated the nth transmission, $T_{p,n}$, for each combination

$$T_{p,n} = \bar{V}_s / \bar{V}_i , \qquad (2)$$

where $s \neq i$ and is the reference voltage through the solvent. Since it is possible to have 9 solutions and one solvent in a six hole rotor, up to 45 combinations could be produced if all sectors were compared. Normally experiments involve only calculating $T_{p,n}$ for true transmissions rather than comparing all sectors, so p has a maximum of 9. Each $T_{p,n}$ is further averaged to provide a mean for calculating the ratio of signal to noise using eq. (3).

$$T_{p,K} = T_{p,K-1} + \frac{K-1}{K} T_{p,n} . \qquad (3)$$

Numerically $K = n$ but $T_{p,K}$ differs from $T_{p,n}$ since it is the mean of K transmissions while $T_{p,n}$ is the instantaneous transmission calculated from summed voltages. The noise is estimated as the variance from the residual sum of squares ($E_{p,K}$) calculated from eq. (4).

$$E_{p,K} = E_{p,K-1} + \frac{K-1}{K} (E_{p,K-1} - T_{p,K})^2 \qquad (4)$$

and a variance ($V_{p,K}$) is calculated as $E_{p,K}/[K(K-1)]$. The ratio of signal to noise is calculated as $T^2_{p,K}/V_{p,K}$ and this is compared with the expected ratio entered into the initialising program. Each combination is treated separately, and the program only moves the scanner to a new radial position when the ratios for all combinations are larger than the expected ratio. In schlieren experiments, $T_{p,n}$ [eq. (2)] is calculated from $(\bar{V}_s - \bar{V}_i)$ and the V's are calculated from means of the summed voltage.

Eventually the scan either reaches the end of the cell (defined by the lack of pulses from the photomultiplier) or the terminating position given to the initialising program. It then finds the bottom reference hole and records the number of counts. The series of averaged values for $T_{p,K}$ are stored away in a file labelled by the experiment and scan number, together with the relevant data from reference holes and starting radii.

This completes the collection of a record, leaving the results to be displayed and edited using the visual display unit. This process is carried out as a background job to collection and having minimum priority. The results of the editing can be processed during the experiment using the appropriate algorithms for the experiment.

Although experiments yield records from two basic types of experiment (equilibrium and velocity), processing these different records involves a common regression analysis. This is a conventional analysis in which the unweighted variables are fitted first to a linear and then a quadratic equation and the ratio between the residuals calculated. This ratio is then tested by an F-test following the calculation of the expected ratio for 95% probability of significance of the quadratic term using the number of degrees of freedom for the data. Should the test prove significant, the parameters and their standard errors for the quadratic equation are typed out; then, normally, the molecular parameters with standard errors are calculated from the linear equation. These statistical routines are combined with routines which transform records from either

equilibrium or velocity into linear equations, so producing two separate programs. Non-linear analyses can be carried out in the on-line system and several programs are used routinely for fitting non-linear equations to edited records. Time to complete the calculations is not a limiting factor in these analyses because the operating system of the computer suspends all programs when the interval timer calls from the centrifuge and continues them from the point of suspension when a collection is complete.

The absorbances were calculated by natural logarithms in the processing programs: hence, most of the numerical values for absorbance quoted in the figures are 2.303 times the conventional absorbances measured in spectrophotometers.

3. Chemicals and experimental conditions

Solutions were made in a phosphate buffer pH 7.0 ($\mu = 0.1$) containing 0.05 M sodium phosphate, 0.05 M NaCl, 10^{-3} M EDTA and 10^{-4} M NaN_3 (at 20°C, the density = 1.0045 g/m and viscosity = 0.010033 poise). The protein solutions were chemically equilibrated with this solvent either by passage down columns of Sephadex G100 (Pharmacia Ltd., UK), or dialysis. 40-mg samples of crystallised and lyophilised bovine serum albumin (Sigma Ltd., UK) were dissolved in the 10 cm^3 of buffer and this was purified by chromatography on a 30 X 6 cm column. Samples from the centres of peaks were used in the experiments. The samples were stored at 4°C for periods up to two weeks before use, and normally the absorbances were in the range 0.6 to 1.6. The experiments in the centrifuge were carried out at 20 ± 0.05°C using 20 mm double sector cells.

The value taken for the partial specific volume of bovine serum albumin was 0.734 [12] and $E^{1\%}_{1\,cm}$ of 6.61 [13] at 280 nm.

4. Results

Having assured ourselves that the programs and interface were functioning correctly, it was necessary to explore the accuracy and precision achieved in estimating molecular parameters for a well defined macromolecule. For this stage of the investigation the molecular weights of bovine serum albumin were estimated in the range of concentrations 0.4 mg cm^{-3} to 0.9 mg cm^{-3}. Approximately 2 mm columns were run at 12000 rev min^{-1} for greater than 20 h at 20°C in order to reach centrifugal equilibrium. The results from one run in which 7 solutions were simultaneously examined are given in table 1. A typical plot of ln c against r^2 is shown in fig. 2. Within the range of concentrations studied there was no obvious trend in molecular weights with concentration, and the mean molecular weight together with its standard deviation for this experiment was 66070 ± 1570. This value compares favourably with the accepted molecular weight for bovine serum albumin of 66500 [6, 14].

The dimer of bovine serum albumin was included in the experiment (table 1) in order to examine the range of molecular weights that can be accurately estimated using one speed. This knowledge is particularly important when estimating interaction constants between proteins where polymers accumulate towards the bottom of the centrifuge cells, and records from this region are generally less reliable than in the centre of the column of solution.

In the present example an average molecular weight of 120 290 ± 3700 was found for the two estimates of the dimer. The records from both solutions fitted a straight line relationship between ln c and r^2 better than the quadratic equation. This average molecular weight was lower than that expected for a pure dimer

Table 1
Molecular weights of bovine serum albumin and its dimer calculated from sedimentation equilibrium. The results are from 7 sectors in one experiment, each sector containing between 2 and 3 mm columns of solution. The results were calculated from 5 scans at equilibrium and standard deviations for these 5 scans are given with the means

Conc. (g/100)$cm^3 \times 10^2$	Mol. wt. ($\times 10^{-3}$)
9.59	66.23 ± 3.1
8.40	63.98 ± 5.77
6.75	67.24 ± 10.50
4.5	67.81 ± 9.06
3.52	65.07 ± 4.0
4.42 a)	116.56 ± 3.8
4.42 a)	124.03 ± 3.5

a) Dimer separated from the monomer on a column of Sephadex G100.

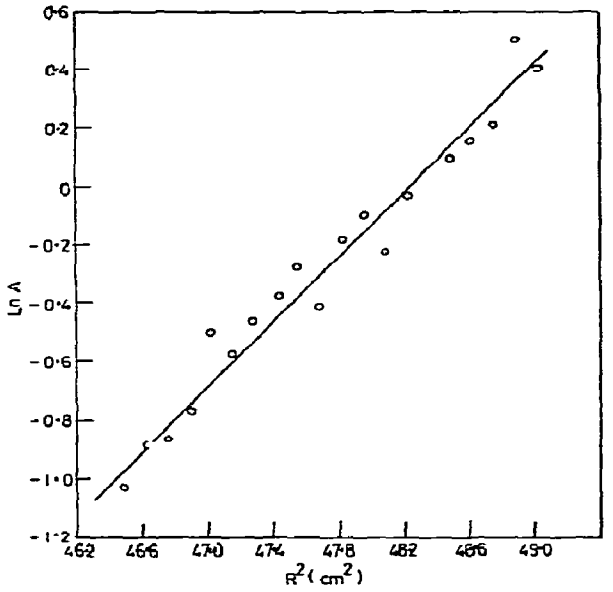

Fig. 2. Centrifuge equilibrium experiment on bovine serum albumin at a concentration of 0.5 mg cms^{-3}. The continuous line is from the regression equation $y = -26.173 + 0.543\,R^2$ and the apparent molecular weight was 63500 ± 2600. Speed of rotor was 12000 rev min^{-1}. ln A = natural logarithm of absorbance, measured at 280 nm.

of bovine serum albumin (133000) and would correspond to approximately 80 ± 7% dimer if monomer were the only contaminant in a mixture. This is not significantly different from estimates of the porportional contamination of dimer by monomer calculated from the elution pattern of the gel (approximately 90% dimer in the solution). This test showed that 10% monomer would not be detected under these experimental conditions at the significance level used in fitting the data to ln c versus r^2.

The coefficient of variation in the estimate of molecular weight for individual solutions was generally in the range 3—17% and could not be reduced despite increasing the signal to noise for averaging pulses during collection of data. As with our original simpler system [8, 10], the standard error on a measurement of absorbance could be reduced by increasing the signal to noise ratio until the error reached a relatively constant level of ± 0.014 at 280 nm (table 2) at a signal-to-noise of 180. Increasing the signal-to-noise ratio further did not significantly reduce this error. Inconsistencies across the windows are thought to be the source of this constant error, and using double sectors with the same window covering solvent and solution does not remove this error. The level of the error increased marginally to 0.015 at 260 nm (table 2). It has been assumed in the regression analysis that the main source of errors is from noise in the measurement of absorbance; however, it is important to consider systematic errors in the measurement of radial dimensions. In our system where a stepping-motor is pulsed and the count taken as a linear measure of the radius, there will be a quantal error of $\pm\frac{1}{2}$ pulse and equivalent to approximately $\pm 2.1 \times 10^{-4}$ cm. These counts are then converted to absolute measurements of the radii by counting the number of pulses between the two reference holes. The accuracy of this calibration depends on the error incurred in locating the centres of the two reference holes. One estimate of this error was collected by calculating the coefficient of variation for moving the scanner to the same position in the cell over many experiments (table 2). It amounts to 0.059% and is comparable with the estimated variation found for measuring the distance between reference lines on a schlieren photograph (0.057%, table 2). The coefficient of 0.059% corresponds to an absolute error of 3.9×10^{-3} cm in the radial dimensions. One expression of this uncertainty is the precision with which the radius of a meniscus can be measured and also in table 2 is compared the radial positions of a meniscus recorded both by scanner and measured from a schlieren photograph. The

Table 2
Statistics of the computer controlled recording of ordinates compared (where appropriate) with similar errors from schlieren records on photographs

	Photograph	Computer
Radius (coeff. of variation)	0.057%	0.059%
Location of meniscus (same run)	6.515 cm	6.510 cm [a]
Location of a position in the cell (s.e.)	–	± 0.0039 cm
s.e. of mean of absorbance [b]		
280 nm	–	0.014
260 nm		0.015

[a] Measured with a pre-set radial interval of 0.01 cm for scanner.
[b] Mean for 8 sectors containing water or absorbing solution in each sector.

difference of 5×10^{-3} cm between the two measurements is within the interval set for stepping the scanner down the cell and similar to the uncertainty in locating the centres of the reference holes. Though these are small errors they contribute to the uncertainty in estimating the molecular weights, and an attempt has been made to include these errors in the analysis by averaging the estimates for several scans which have been individually processed. It would seem from the present results that noise from absorbance limits accuracy to between 3% and 17% for individual sectors and the overall accuracy becomes approximately ± 3% when several records are averaged.

One main objective in constructing the system was to study the dissociation of proteins in dilute solutions. It was, therefore, of importance to know how the error and precision of the estimates varied with absorbance. Table 3 shows results from an experiment designed to study this variation in which scans of equilibrated solutions were collected at three wavelengths, giving a total of 8 absorbances. As expected, the precision of the molecular weight was greater for the higher absorbances; however, the standard errors remained relatively constant for solutions having absorbances greater than 0.054 but increased markedly for absorbances below this value. A record was also made at 340 nm where bovine serum albumin shows negligible absorption and surprisingly a definite pattern was recorded from which a molecular weight for the highest concentration (0.9 mg/ml) was estimated to be 69500 ± 20030 (fig. 3). The median absorbance for this solution at 340 nm was 0.02. The slope from the straight line relationship between ln c and r^2 became progressively less significant as the concentration decreased; however, the median absorbance was relatively constant at 0.02 for all solutions. These unusual results may be caused by surface denaturation of the protein on windows during the experiment. The rates of denaturation and accumulation were slower than the time required to reach centrifugal equilibrium and the absorbance was mainly caused by scattering of the light from the film of denatured protein on the windows. If this is the explanation, then it means that base-lines cannot be accurately assessed after the experiment but must be made before introducing solutions of proteins. In general it was found that the base-lines gave an absorbance of approximately ± 0.01 in most cells, and this has been ignored in the present analyses.

While assessing the performance of our system it was found that bovine serum albumin was not stable in dilute solutions. Solutions having absorbances of approximately 0.7 at 280 nm (1 cm cuvette) were used routinely in the early stages of the work and low molecular weights were consistently recorded. For example, the molecular weight for a solution stored for 5 days at 4°C after chromatography was estimated to be

Table 3
Estimates of molecular weights of bovine serum albumin at different absorbances. The estimates were from selected sectors scanned at three wavelengths, 280 nm, 290 nm and 300 nm. The absorbances were recorded by a spectrophotometer in 1 cm cuvettes

λ (nm)	Absorbance	Mol. wt. ($\times 10^{-3}$)
280	0.748	64.785 ± 2.85
280	0.434	64.48 ± 2.66
290	0.389	60.61 ± 1.77
280	0.383	59.96 ± 1.20
290	0.195	61.42 ± 2.63
290	0.054	80.74 ± 7.05
300	0.044	76.16 ± 11.97
300	0.025	66.81 ± 11.98

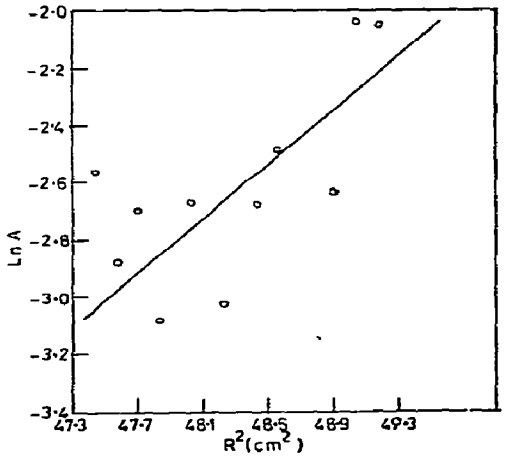

Fig. 3. Centrifuge equilibrium experiment on bovine serum albumin at a concentration of 0.5 mg cm^{-1}, and the record was measured at 340 nm. The continuous line is from the regression equation $y = -30.988 + 0.589\ R^2$ giving an apparent molecular weight of 69500 ± 20300. ln A = natural logarithm of the absorbance.

36760 ± 4450 for the recordings taken 1 mm from the meniscus and 66460 ± 12130 over the last 1 mm (total column height 2.3 mm). This solution originally gave an average molecular weight of 66230 ± 3100 (table 1, first value) when run immediately after chromatography. The cause of the instability in the protein is not known, but it may be relevant to the explanation that the molecular weight after standing was approximately half the expected value for bovine serum albumin.

Accuracy in estimates of molecular weights from records of sedimentation equilibrium is limited mainly by errors in measurement of concentration at defined radial positions and uncertainty in the measurement of these positions only introduces small systematic errors. In velocity experiments, errors introduced from both absorbance and radial dimensions become major factors in the systematic and random errors on estimates of sedimentation coefficients. It is, however, important in the analysis of macromolecular behaviour in solutions that the system should provide accurate analyses of records from velocity experiments. There are two stages in this analysis; first the centroid of the sedimenting boundary must be located at fixed time intervals, and then these locations are combined to provide an estimate of the sedimentation coefficient. Uncertainties in locating the reference holes introduce errors in the absolute radial dimensions for each scan; however, as shown in table 2, these errors are similar to those accepted for measurements from photographic records. This gave confidence that estimates from the scanning schlieren should be at least as precise as those accepted from photographic records. Introducing a computer to collect this data does produce a valuable bonus in that it is now possible to calculate routinely the centroid of the boundary by second moment analysis. A further important aspect of the scanning schlieren is that it is approximately five times more sensitive than the photographic procedure, making it possible to record more information from low concentrations (< 1 mg cm^{-3}) of the macromolecule.

Records from either the absorbance or refractive index gradient at each radial ordinate can be collected with the system. The absorbance is measured using the algorithms given in eqs. (1)–(4) and processed by either a probit approximation [15] or non-linear analysis [16]. The only change required in the optical system is to introduce a knife-edge in the focal plane of the collimating lens making the optics resemble the normal schlieren without the cylindrical lens [8,9]. Thus, when light is deflected from its normal path and the edge is rotated to an angle to this path, some of the deflected beam is cut off by the edge while the undeflected beam continues undiminished. Both beams (separated in time) are focused to a spot on the cathode ofthe photomultiplier and their relative intensities will be determined by the amount passing the knife-edge. The operation of both centrifuge and computer is not changed when collecting schlieren-records and the optimised data make allowance for deflections caused by the solvent. Reisner and Rowe [9] have summarised the alignment of this type of system for the Model E centrifuge, and their comments apply equally to the MSE centrifuge. It should be mentioned, however, that since a deflection is measured as the integral light-intensity in a radial plane for the beam transmitted through each sector, the reference intensity from the solvent does not allow for lack of uniform illumination in the circumferential axis of each sector, but only for variations in the radial direction. Systematic errors from this source will have negligible effect on the precision of the record if the circumferential width of the beam of light illuminating the sector is larger than the width of the sector at all radii. Visible light (in the range 400 nm to 550 nm) was used for work with scanning schlieren making it possible to operate the photomultiplier at considerably lower voltages than for UV with a corresponding decrease in the time required to average the pulses. A further advantage in measuring relative deflections at discrete positions across the boundary is that the algorithms employed in processing the records can include estimates of diffusion coefficients from the ratios of area/maximum height [17]. Briefly, our procedure for editing has been to select from a complete record the menisci, beginning and end of the schlieren peak for each scan. From these edited data are then calculated the centroids of each frame by second moments leading to estimates of the sedimentation coefficient by regression analysis. Then the ratios between the height of the maximum ordinates and the areas of the peaks are computed, leading to an estimate of the variance of the boundary (the ratio height/area). These estimates are corrected for sectorial dilution and centrifugal field by the methods suggested by Trautman [18].

Table 4
Estimates of $s_{20,w}$ and $D_{20,w}$ for bovine serum albumin from records collected by the scanning schlieren procedure

Conc. (g/100 cm^3)	$s_{20,w}$ ($s \times 10^{13}$)	$D_{20,w}$ (cm^2/s $\times 10^7$)
0.15	4.53 ± 0.35	5.45 ± 0.59
0.14	4.76 ± 0.21	5.53 ± 0.5
0.039	4.51 ± 0.48	5.56 ± 0.39

This set of values is fitted to a regression equation using time as the abscissae. If a straight line is significant, then the diffusion coefficient is calculated from the slope. If the spreading of the boundary is not linear with time, then the calculation of the diffusion coefficient is abandoned, but the regression coefficients are given.

Having the facility to calculate both sedimentation and diffusion coefficients makes it possible to study routinely the detail of sedimenting boundaries in crucial experiments designed to study heterogenity in macromolecules. We have found this particularly useful in work with bovine serum albumin where it was suspected that storage of dilute solutions was accompanied by decomposition into a half molecule. Results of velocity experiments on three concentrations are summarised in table 4. The estimates of weight average sedimentation coefficients were consistently higher than expected for bovine serum albumin at these concentrations (approximately 4.43×10^{-13} s [6, 14]) while the diffusion coefficients were lower (this parameter is relatively insensitive to changes in concentration [19, 20] and generally accepted to be 6.1×10^{-7} cms^2 s^{-1}). Measurements of diffusion coefficients by fluctuation spectroscopy [21] for a concentrated sample of a similar purified solution (a concentration of 2.0 mg cm^{-3} was required for spectroscopy) gave a result of $5.78 \pm 0.07 \times 10^{-7}$ cm^2 s^{-1} and confirmed our estimate from the centrifuge, It was disturbing to find, however, that neither method detected the presence of the oligomer by curvature in the regression analysis. Having detailed data on the profile of the boundary made it possible to compare the experimental record with a simulated profile for a molecule of $s = 4.3 \times 10^{-13}$ s and $D = 5.92 \times 10^{-6}$ cm^2 s^{-1}. This was computed from the Fujita–MacCosham [21] solution to the Lamm differential equation, so it includes the approximation of no concentration dependence for both sedimentation and diffusion coefficients. Examples of these two profiles are shown in fig. 4 where it can be seen that the oligomer caused distortion of the leading edge of the peak. Similar detailed measurements could have been made from photographs, but it is doubtful whether this would be made routinely in the analysis of velocity runs. In this experiment the dimer would have moved to 6.37 cm, so it seemed that dimer formed during storage for one week at 4°C.

It should be noted that the estimates and comparisons were made during the experiment, so giving the experimentalist the opportunity to adjust experimental conditions if so desired. Analyses made on concen-

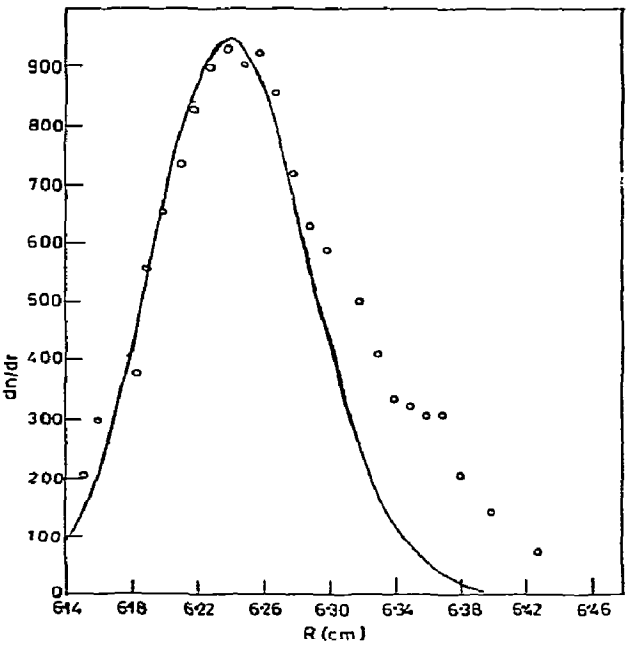

Fig. 4. Comparison between a computed schlieren profile (continuous line) and a bovine serum albumin (concentration 0.6 mg cms^{-3}) after 1940 s from reaching 37000 rev min^{-1} (the continuous speed of the rotor was 49950 rev. min^{-1}). The profile was computed from the Fujita–MacCosham relationship for a molecule having $s = 4.3 \times 10^{-13}$, $D = 5.93 \times 10^{-7}$ cm^2 s^{-1} and meniscus at 6.1 cm. The scale of dn/dc is arbitrary but proportional to volts measured at the photomultiplier.

trations of protein less than 0.010 g/100 cm^3 by centrifuging myoglobin and using absorbance at 410 nm, showed that disturbance of the boundary caused by convection increased the spreading of the boundary and reduced the estimates of sedimentation coefficients. Creation of a density gradient down the cell helps to reduce convection [16, 23] but makes quantitative interpretation of the results more difficult, so these experiments have been omitted from this discussion.

The errors on the sedimentation coefficients were relatively high but not significantly greater than those normally found for measurements from photographic records at these concentrations of macromolecule. In order to assess the proportion of errors introduced in editing and regression analysis, a series of profiles was generated using the Fujita–MacCosham [22] relationship and analysed by the complete procedure. The parameters used in generation were $s = 4.5 \times 10^{-13}$ s and $D = 6.3708 \times 10^{-7}$ cm^2 s^{-1} and the estimated values were $4.477 \pm 0.002 \times 10^{-13}$ s and $6.461 \pm 0.155 \times 10^{-7}$ cm^2 s^{-1} respectively. This exercise showed that the analysis produced a small systematic error in the estimate of sedimentation coefficients but the random error was negligible for this parameter. The estimated diffusion coefficient was higher, but because of the relatively large random error in the estimate it was not significantly different from the generating value. The errors found when analysing simulated records arise in part from rounding in the arithmetic of both the simulation and analysis, but possibly the major source was from approximations in formulating the linear algorithms. Nevertheless, the example with bovine serum albumin showed that applying a combination of routine linear analysis with simulation provided a useful semi-quantitative analytical procedure for examining the shapes of profiles in sedimentation experiments.

5. Discussion

The combination of a MSE analytical ultracentrifuge with a relatively powerful small computer provides an efficient and accurate system for studying the hydrodynamic properties of macromolecules. Having available large numbers of relatively precise measurements of known standard deviations [10] not only relieves the experimentalist of the tedium of measuring these from photographic records, but also provides immediate estimates of molecular parameters during the experiment. Later, it is possible to evaluate the significance of experiments in relation to an appropriate statistical procedure. The results given in this communication show that the error on estimates of molecular weights for dilute solutions is approximately ± 3% provided estimates from more than one solution are averaged. Fortunately it is as easy to make measurements and estimates on 9 solutions as with one; the limits are volumes of solutions (0.1 cm^3 per sector for 1.5 mm column) and concentrations (ideally absorbances should be greater than 0.1).

Having a large backing store and an efficient operating system for the computer is necessary if records are to be processed in the time-scale of the experiment. This has proved important when examining interacting systems composed from proteins which are both labile and expensive to prepare. Our greatest problem was gaining sufficient confidence to accept the results produced by the system. Even after extensive testing, unexpected results were considered an annoyance caused by a malfunctioning of part of the system. It was only when we continued to get the same results that we realised we could accept the system as a faithful recorder of experiments. Once this stage was reached, we were able to forget the recording of experimental data and to concentrate on interpreting the data.

Possibly the greatest advantage gained from incorporating 'on-line' computers is always having available a complete set of coordinates across a sedimenting boundary. These can be analysed by rigorous analytical procedures during the experiment. For example, the approximate procedure of measuring the movement of the maximum of a schlieren peak can be replaced by estimating the centroids of these boundaries and calculating true weight average molecular parameters. This procedure is particularly useful in velocity experiments when investigating possible interacting systems and when low levels of heterogeneity are suspected.

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