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UV LASER SCANNING AND FLUORESCENCE MONITORING OF ANALYTICAL ULTRACENTRIFUGATION WITH AN ON-LINE COMPUTER SYSTEM *

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A new optical system for the analytical ultracentrifuge is described, which permits sedimentation to be monitored by fluorescence. The optical system is based on a laser light source, which is focused to a narrow (50 micron) beam. The radical scanning of the beam provides information on the distribution of fluorescing material with distance in the centrifuge cell. Data collection and processing are performed in conjunction with an on-line computer system which sorts incoming fluorescence pulses according to rotor hole and cell sector, averages families of pulses to improve signal to noise ratios and fits the data (in the experiments reported here) to equations to determine sedimentation coefficients. Initial experiments with the system have been performed with bovine serum albumin and indicate that sedimentation can be readily monitored by fluorescence with solutions at concentrations as low as 20 micrograms per ml, with excitation at 257 nm. At these concentrations, the optical density is only in the 0.01 range, too low for experiments with absorption-scanner optical system. Even lower concentrations can be used when fluorescent labels are used with excitation in the visible region of the spectrum. The preliminary studies indicate that fluorescence monitoring of sedimentation will substantially enhance the range of experimental possibilities in ultracentrifugation by improving both the sensitivity of measurements and the discrimination between sedimenting species on the basis of their fluorescence characteristics.

1. Introduction

Use of the analytical ultracentrifuge with a photoelectric scanner and on-line computer has brought about a substantial enhancement in the convenience and precision of sedimentation measurements. For example, in recent experiments on hemoglobin, the tetramer–dimer equilibrium constants determined with a scanner–computer system [1, 2] have been used as a probe of conformational state under a wide variety of conditions [3]. These measurements were permitted by an ease of data acquisition resulting from the capacity to study up to 15 solutions simultaneously in multichannel cells and multicell rotors, with rapid data collection and on-line processing. The experiments with hemoglobin were also aided by the high absorbance of the protein in the visible region of the spectrum, since experiments could be performed with solutions in the micromolar concentration range, where subunit dissociation is appreciable.

For many other proteins, which absorb only in the UV region of the spectrum, the opportunity to monitor dissociation processes is limited by the inability to work with sufficiently dilute solutions. This limitation can be substantially overcome by the construction of a fluorescence monitoring optical system for the ultracentrifuge. Such a system is described here, based on the scanning of a thin laser beam (about 50 microns in diameter) radially along the centrifuge cell while monitoring end-on fluorescence. The fluorescence signal is integrated electronically and then delivered to the computer where it is averaged and processed. Initial experiments with the system have been performed by monitoring the intrinsic fluorescence of bovine serum albumin and human globin. The range of possible experiments can be expanded by the use of fluorescent probes, with excitation by visible laser lines. Work with a rodamine labelled α-bungarotoxin has permitted studies on the properties of the acetylcholine receptor in very dilute solutions.

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2. Methods

2.1. Computer system

A laser scanner, using either absorption or fluorescence for monitoring sample concentrations, requires the versatility inherent in a computer collection system to make full use of the potentials of the method. We present first a brief description of the computer system currently in use in this laboratory with special attention to the most recent modifications. The reader is referred to our earlier papers for a more detailed description [1,2].

Fig. 1 is a block diagram illustrating the basic components of the system. A photomultiplier is the starting point for measurement of concentration using either the conventional scanner or the scanning laser system with absorption or fluorescence. In the case of absorption the photomultiplier current is proportional to the transmitted light intensity and in the case of fluorescence the current is proportional to the fluorescence emission from the sample or reference. The preamplifier stage design was complicated by the requirement for low noise and fast response time and the amplifier that was chosen consists of a combination of a transistor and an operational amplifier used in an inverting feedback configuration to insure linearity. The 2N1309 input transistor is in the grounded emitter configuration for large current gain and the operational amplifier is a Fairchild 715. The input current is connected directly to the base of the transistor and the feedback resistor to this point is 50,000 ohms. The use of a very high gain feedback circuit is necessary to provide linearity in measuring high optical densities (OD). Reliance on the preamplifier in the Beckman scanner (no feedback control) for linearity is unwise because this circuit is nonlinear as illustrated by the conventional calibration stairsteps being unequal between 1 and 2 OD. The result this will have is that the measured OD will be significantly smaller than the actual OD at OD's above 1.

A second stream of information from the centrifuge consists of the Schmitt 1 and Schmitt 2 pulses available from the multiplexer test points. These pulses are generated by the detectors present in the rotor chamber from the coded rotor collar. These information streams are used in two ways. First, the interface generated coded signals are passed to the Data General Nova computer simultaneously with the voltage values from the A/D converter and serve to identify the rotor hole as well as whether the signal is from sample or reference, or the dark or baseline voltages. The second way in which the multiplexer information is used is to control the integrator window to insure that the

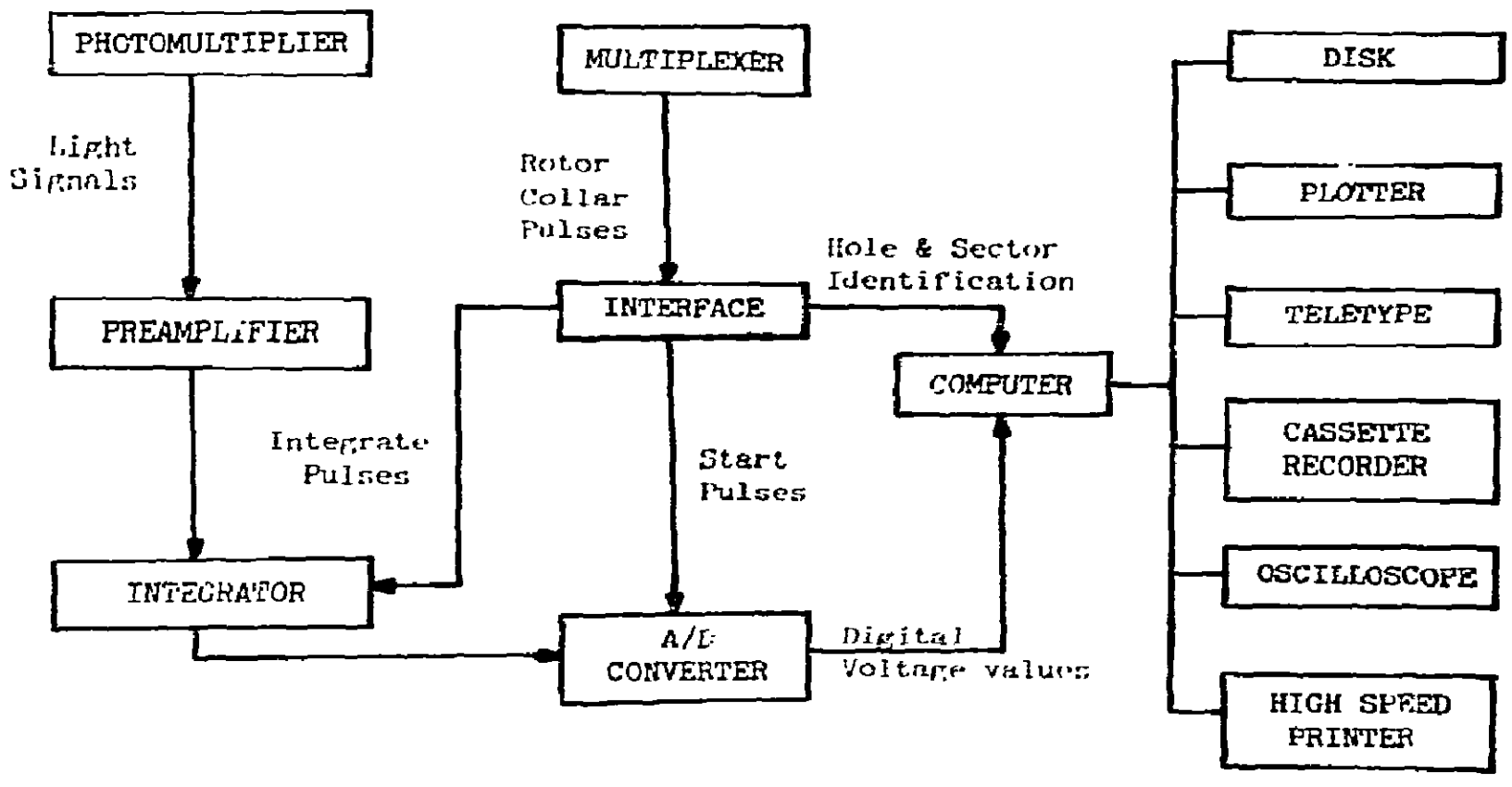

Fig. 1. A block diagram of the apparatus used to collect, analyze, store and output data from a Beckman Model E ultracentrifuge.

correct light pulse is being integrated and to start the A/D conversion of the voltage proportional to this light pulse when the integration is complete. The rotor collar is oriented so that the rising edges of the Schmitt 1 pulses occur within 10 degrees of the sample light pulses. Since the edges of the black paint on the rotor collar are not uniform a correction must be introduced and to do this the interface provides a separate variable time delay for each rotor hole. This adjustment is quite simple and consists of triggering an oscilloscope from the variable collar pulse and superimposing the photomultiplier light pulses from all holes in the rotor by adjusting the delay for each hole in turn. This operation is performed once the rotor is at speed and is stable for the duration of the experiment. In the absence of rotor precession this adjustment is easily made to within 0.2 degrees.

The availability of a logic pulse which occurs before the light signal arrives at the photomultiplier allows integration of the total light passing through the sample or reference. This is a valuable improvement because most A/D converters use a very fast sample and hold or follow and hold circuit and hence are sensitive to high frequency noise which may be present in a signal. To reduce this noise a circuit was built which integrates the total light signal from sample or reference and passes this integral to the A/D converter. The basic circuit is an operational amplifier with an input resistance and a feedback capacitance. The amplifier is gated by logic pulses to integrate for a time set to include the sample or reference light signals and then hold that integral for a time during which the A/D converter performs its conversion. The operational amplifier selected for this circuit is the four channel Harris Semiconductor HA-2405 PRAM. This device has four inputs which are selectible by logic signals and was used as follows. When the integrate mode is selected the voltage of the preamplifier is passed through an input resistance to a selectable feedback capacitance where it is integrated. At the end of the integration mode the PRAM is switched to a hold mode where any change in the output is fed through a capacitor to the input where it is cancelled and the output remains constant while the A/D conversion is performed. After the A/D has sampled the integral, the logic switches the PRAM to a follow circuit to erase the integral and prepare for the next light signal. The integrator has proven to be of value in reducing the

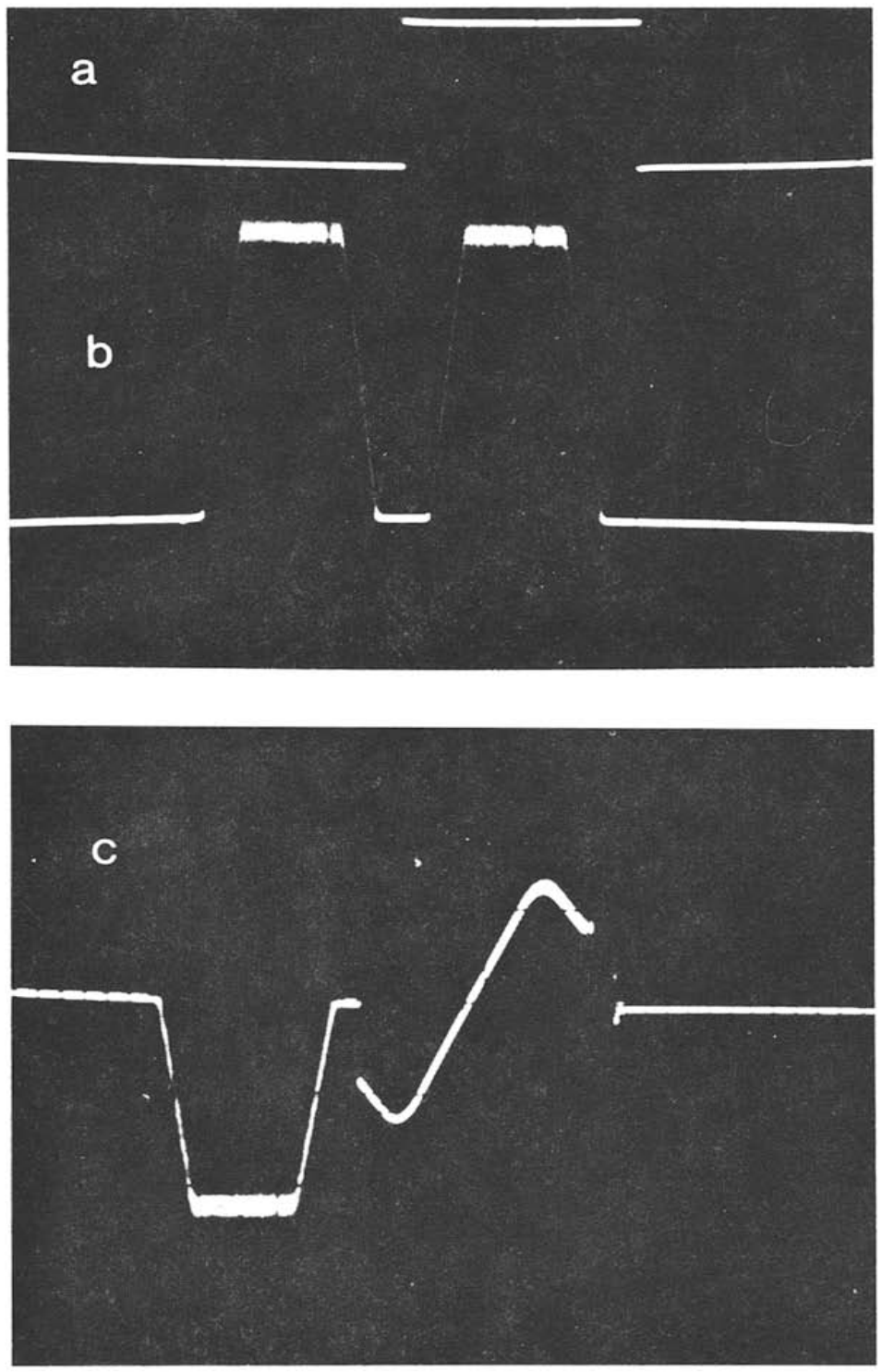

Fig. 2. Oscilloscope trace showing (a) the logic pulse defining the area of the reference pulse to be integrated, (b) the photomultiplier light pulses corresponding to the absorption optical system with the scanner at the outer counterbalance, and (c) the output of the integrator performing the integral shown in parts (a) and (b). Note for the sample pulse the integrator follows the negative of the pulse voltage. Since the signal voltage is positive for the maximum light signal and negative for the baseline due to the design of the preamplifier, the integral decreases, increases, and again decreases. The hold portion of the integral is a very short horizontal section just following completion of the integral. The baseline is integrated in the absence of light and subtracted by the computer from the sample and reference integrals to eliminate any assumptions concerning the absolute DC voltage level.

signal to noise level in low light applications and is essential for fluorescence experiments because of the

large amount of shot noise present in the light signals. Fig. 2 shows oscilloscope traces of the three signals mentioned above: (a) the logic pulse describing the window for the integration of the reference voltage; (b) the basic photomultiplier light signals; and (c) the resulting integral from parts (a) and (b). Note in the integral the output follows the inverted input for the sample and performs an integral for the reference pulse. The use of an integrator provides a significant increase in the signal to noise ratio. An integration is also performed for the dark area or baseline. The A/D converter passes the digitized voltages to the computer where after averaging generally for 100 revolutions the resulting averaged voltages for sample minus baseline and reference minus baseline are stored on the disk for later conversion to OD or fluorescent emission (F).

A real time clock in the computer provides a linear measure of the progress of the scan and, in connection with the known positions of the counterbalance edges.

Table 1
Existing computer programs

1. Calculate radius, optical density or fluorescence and standard deviation for standard absorption, laser absorption and laser fluorescence
2. Collect automatic velocity scans and perform runtime calculation of sedimentation coefficient
3. Output data via printer, plottor or oscilloscope
4. Calculate molecular weight of a pure species
5. Calculate apparent molecular weight as a function of concentration
6. Calculate dissociation constant in monomer-dimer reaction
7. Calculate sedimentation coefficient by inflection point or transport method
8. Fit to a general equation e.g. determine binding stoichiometry of HP to Hb dimer in the reaction:
   $Hb_4HP + 3HP \rightleftharpoons 2Hb_2HP_2$

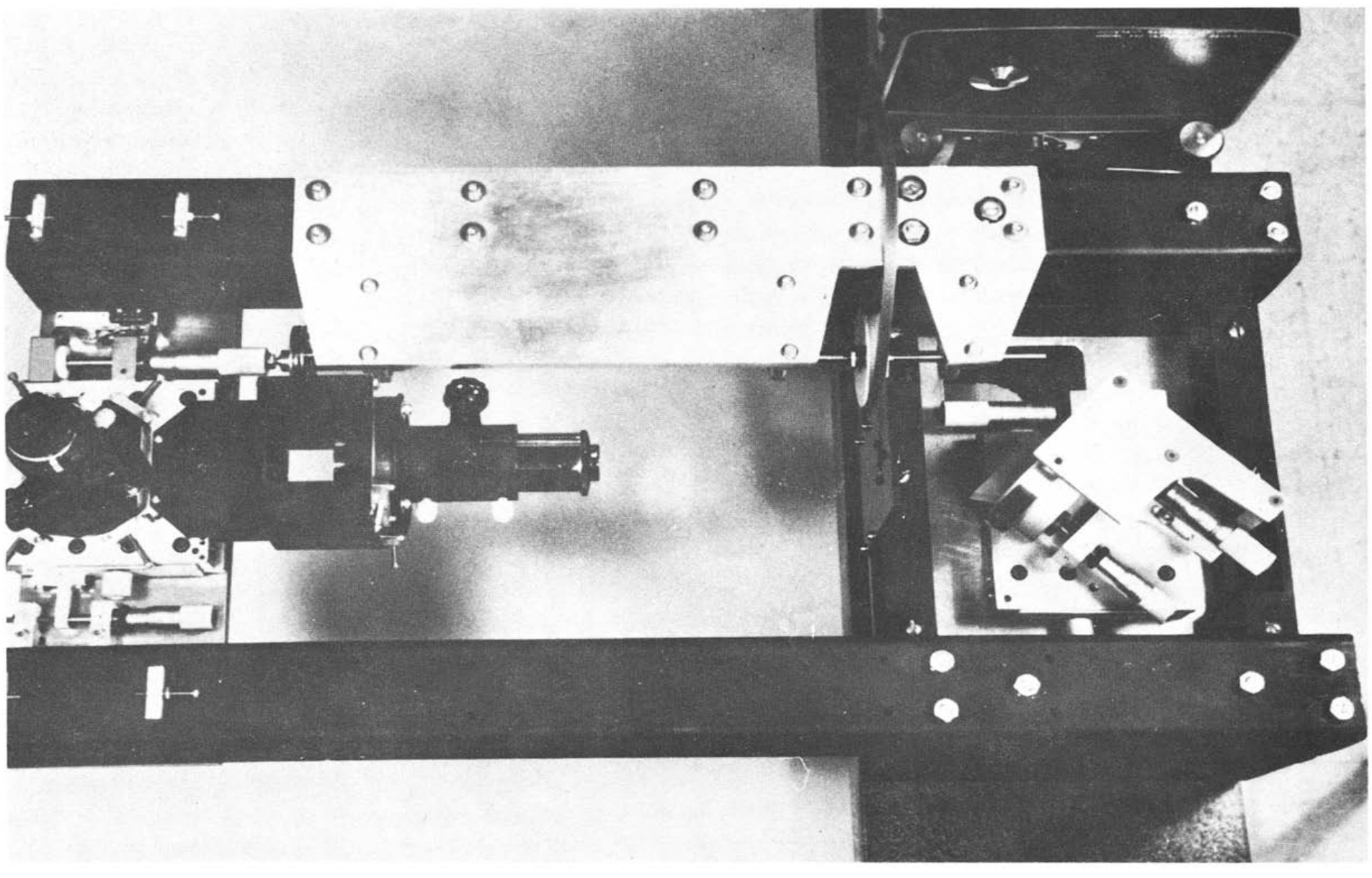

Fig. 3. Top view of laser scanner before installation beneath the Model E chamber.

is used to give an accurate determination of the radius for each point collected. After the data is stored in permanent form on the disk it is immediately available to one or more of the various fitting or plotting programs that have been written for the computer. Table 1 is a list of some of the available computer programs for data collection and analysis. Most of the fitting and plotting programs are written in Fortran which is available with the Nova computers. The availability of Fortran enables users to write their own fitting programs or to modify existing fitting programs with a minimum of detailed computer knowledge or effort.

2.2. *Laser scanner*

In order to use a focused laser beam as a light source with the Model E ultracentrifuge, it was necessary to construct the mechanism necessary for moving the focused spot across the centrifuge cell and for collecting the light on the other side of the rotor. Fig. 3 is a top view of the scanning platform as it was designed and in fig. 4 the complete system is shown in position below the chamber of the Model E. The 5 watt argon laser (CR-5) and UV generator were obtained from Coherent Radiation. For most protein fluorescence work without a dye present, the 514 nm, 2 watt argon laser line is passed through the UV generator and the resulting 257 nm line is filtered and used to scan the sample. The UV generator consists of a crystal of am-

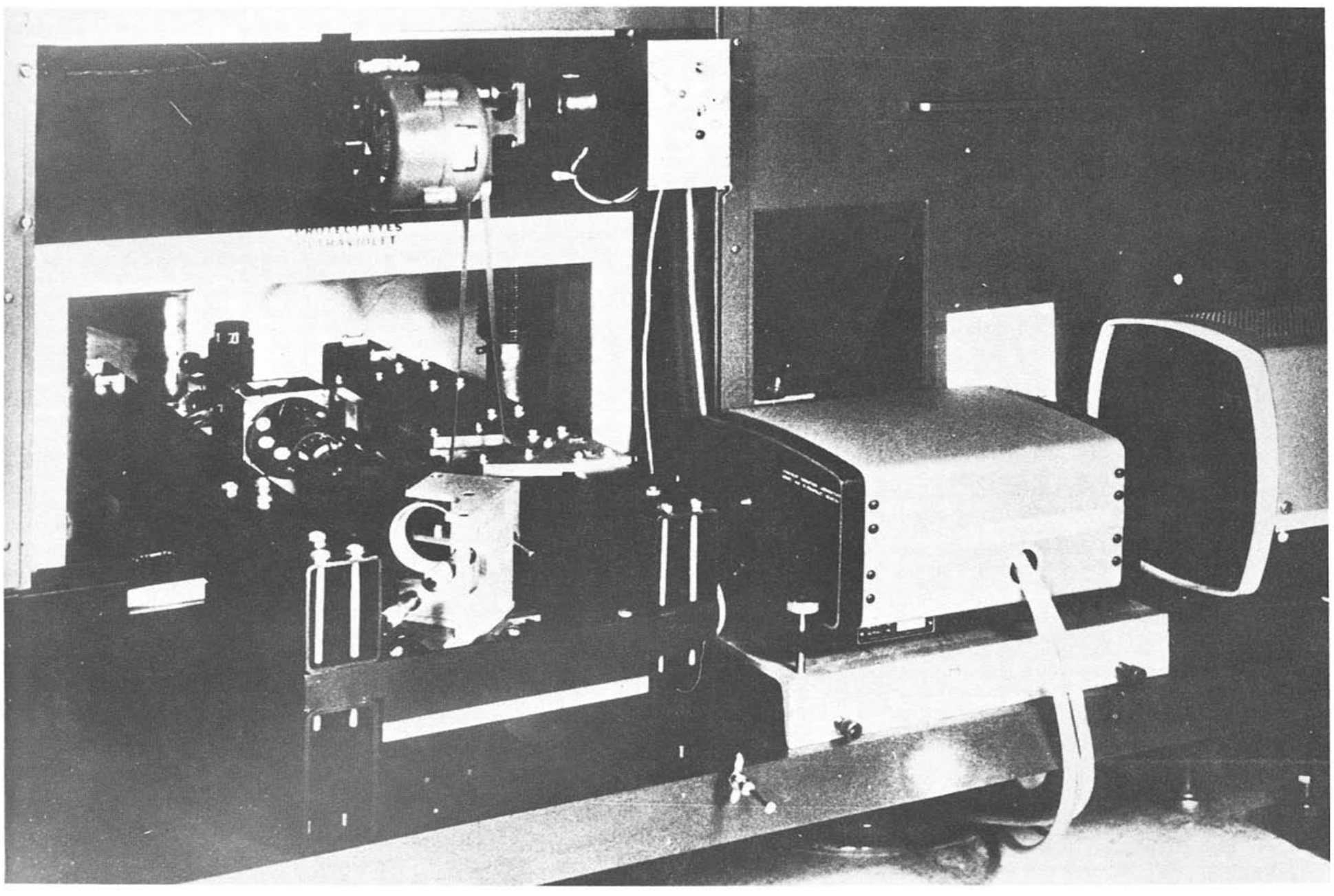

Fig. 4. The laser scanner in position. The low profile allows the chamber to be lowered without interference.

monium dihydrogen phosphate in a temperature controlled environment and associated lenses to focus the incoming green light and collimate the UV light generated by the crystal.

A constant deviation (Pellin Broca) prism is used to bend the desired UV light through 90° and send it through an iris diaphragm. The main green laser line is bent through a different angle and hence blocked by the iris. The prism is mounted on an adjustable mount so that other wavelengths available at the laser may be easily used without adjusting the iris or laser position. As shown in fig. 5 the light beam then passes through a planoconvex lens, a front surfaced 45° prism to reflect the light vertically along the standard schlieren optical path, and a second convex lens. These optical components are mounted on a motor driven stage that provides the radial scan of the centrifuge cell. The motor is connected to a gear reduction box which provides for 10 selectable speeds for the scanning stage. These scan times range from 20 seconds to two hours. The drive belt is connected to a gear which, by a shaft connected to a micrometer screw, drives the stage containing the focusing components.

The placement of two lenses on the moveable stage allows the focal point of the laser to be maintained at a constant vertical position in the cell throughout the scan, and the two lenses provide adjustment of the focal position for any of the several wavelengths available from the laser in addition to the UV from the UV generator. The output of a laser is a diffraction limited gaussian beam that may be focused to a very small spot. Due to the 1.2 cm thickness of the conventional centrifuge cell, it is necessary to not only focus the beam to a point but also maximize the depth of focus distance, d. The relation between the minimum spot radius, r, and the depth of focus is given by $d = 2\pi r^2/\lambda$, where λ is the wavelength used. For $\lambda = 257$ nm and a standard cell, the average spot diameter is about 50 microns. Calculations indicate that this resolution will easily provide sufficiently accurate radius and concentration measurements. The design of the optical equipment provides room for the standard absorption optical system to be used without interference from the laser system.

The windows of the sample chamber are flat quartz windows and hence do not affect the focus or scanning of the laser beam. Fig. 5 details the chamber layout for a fluorescence monitoring experiment. The

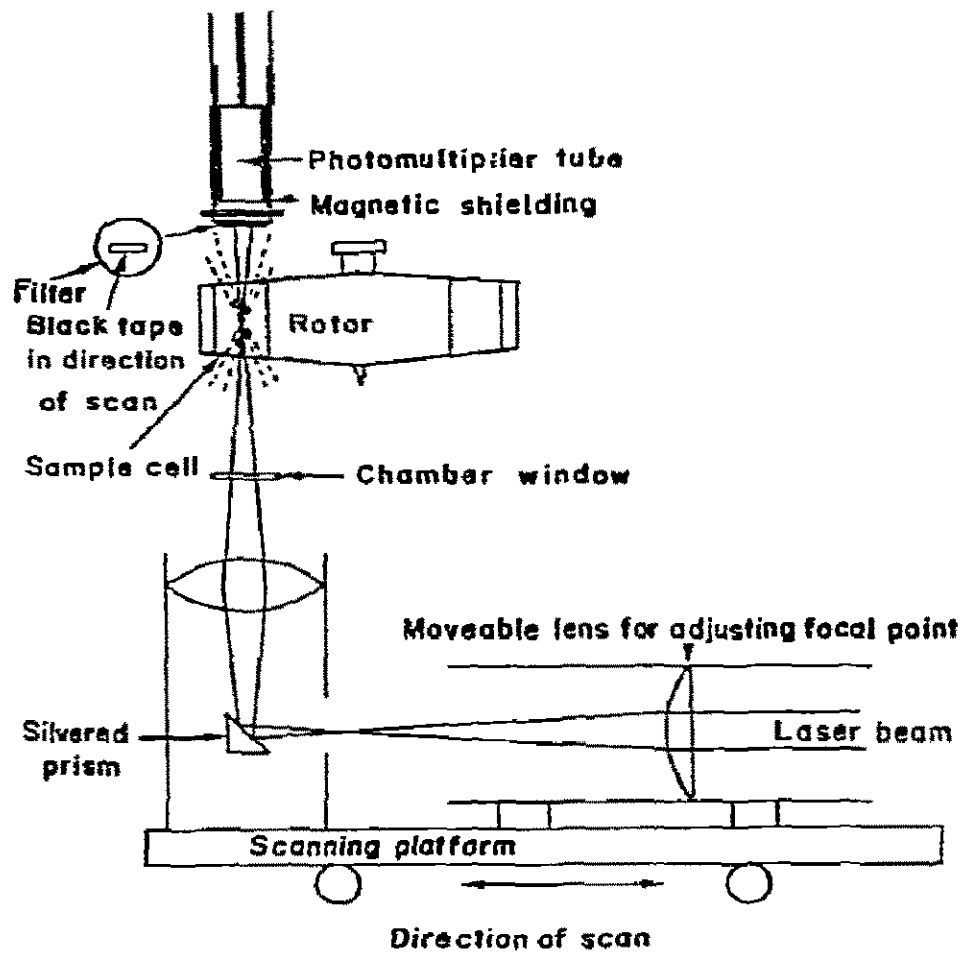

Fig. 5. Schematic of the layout of the various components involved in a fluorescence scan. The laser beam is not to scale for clarity.

incoming focused beam excites the fluorescence of the sample which is radiated in all directions from the point of excitation as indicated by the dashed lines. It is this radiation in all directions that necessitates excitation with a focused beam, for if the total sample were illuminated, then to achieve radius resolution or depth of focus through the cell, most of the fluorescent light would have to be thrown out or "stopped down". Then the very low light intensity remaining would make measurements of low concentration difficult or impossible. The exciting laser line must be blocked completely so that it does not interfere with the longer wavelength fluorescence emission. This is accomplished by using a narrow piece of black tape on a flat glass filter. The purpose of the tape is to stop the still focused laser beam with a minimum of reflection, and the flat glass with a cutoff around 300 nm will completely block the small amount of scattered laser light which remains. If we do not use the tape to block the laser beam and instead rely completely on the glass filter, the beam is still blocked but the small amount of fluorescence and phosphorescence from the

glass contributes unwanted noise to the system. When the laser scanner is positioned at the counterbalance holes the laser light is not blocked by this mask and the fluorescence of the filter allows detection of the counterbalance edges necessary for radius calculation. The fluorescence of the sample is of longer wavelength and is passed by the glass plate and focused on the photomultiplier tube by a lens located directly above the chamber window. In this way a maximum amount of light is collected by the photomultiplier tube. The tube itself is shielded from magnetic fields present near the drive motor of the centrifuge to insure low noise operation. In fluorescence an effort must be made to collect as much light as possible and hence the laser is operated at maximum output of 2 watts (514 nm) resulting in 16 mW (257 nm) and the photomultiplier is positioned to receive a maximum amount of light. With a fluorescent dye present on the sample allowing visible excitation we must turn down the power of the laser and replace the filter with a more suitable one for blocking the main laser beam. For an absorption experiment using the laser scanner [4] it is only necessary to remove the tape and filter and pass the incident light directly to the photomultiplier. For absorption at any wavelength the laser delivers too much light and some filtering must be done to reduce the main laser beam to an intensity where the photomultiplier response is linear.

Because of the low light levels involved in fluorescence detection some unexpected sources of stray light had to be eliminated. The most obvious source of stray light is the room lights and they must be turned off or blocked with black paper from entering the sample chamber from the bottom. A second source of light in the chamber is the bulbs used to illuminate the rotor collar for the multiplexer. Stray light from these bulbs contributes noise to the system which can be eliminated by painting the rotor with a flat black paint near the collar and placing a mask to block the reflected light from entering the detector view without significantly affecting the fluorescence signal. Further reduction of this stray light was accomplished by modifying the electronics of the multiplexer detectors to make them more sensitive and then decreasing the bulb intensity by a large amount. A third source of stray light was discovered to be the heater coil in the bottom of the rotor chamber. Since we are not using a long wavelength cutoff filter, the photomultiplier tube is sensitive to the red light emitted by this coil. The solution for the data collected in this paper was to turn the heater control off for the duration of each scan. Since this was only a short time, there was little or no loss of temperature control. The more permanent solution is to filter out wavelengths longer than say 400 nm since almost all of the fluorescent intensity is between 300 and 400 nm.

3. Theory

The highly monochromatic light available with the laser light source eliminates problems with Beer's law commonly encountered with the standard light source and monochromator. Because the light from the monochromator is used to illuminate the whole cell, and a slit at the photomultiplier tube selects only the light at the radius to be studied, there is always the problem of not having as much light as is desired. Thus the monochromator slit is opened wider and the light is no longer monochromatic and at high concentrations OD is no longer linear with the concentration. The laser eliminates the problem of not having enough light in the case of absorption by using the total light output of the laser to illuminate one radius of the cell and collecting all the light transmitted. With fluorescence low light levels are always a problem and averaging of many revolutions is sometimes necessary to get an accurate measure of the concentration.

Fluorescence monitoring is not subject to the Beer's law problem but it is subject to non-linearity in the fluorescent emission. The intensity of emission is given by

$$F = I_0\phi(1-10^{-\epsilon cd}),$$

where I_0 is the incident light intensity, ϕ is the quantum efficiency of the fluorescence, ϵ is the extinction coefficient, c is the concentration and d is the path length of the cell. The exponent in this equation may be expanded in powers of OD = ϵcd and if we ignore terms higher than the first power we obtain $F = 2.3I_0\phi$OD. This equation is only accurate for low values of OD but that is where the fluorescent scanning is most useful. For OD equal to 0.01 the error in assuming a linear dependence of F on concentration is 0.5%. For the experiments reported in this paper the maximum error is approximately 0.25%. The assumption

of linearity was made for ease in programming the computer and in the case of experiments where it is desired to work with higher concentrations the collection program could be revised to calculate the concentration from the exact equation rather than from the approximation. The computer collection program must use a formula for calculating the fluorescence from the observed voltages of the form

$$F = V_{samp} - V_{ref}/V_{ref} .$$

Here V_{samp} and V_{ref} are the voltages from the sample and reference sides of the cell after the baseline has been subtracted. The subtraction has the effect of removing stray fluorescence due to cell windows and buffer effects while the division is a normalization to correct for changes in the laser intensity during the scan and more importantly changes in the response of the photomultiplier due to the progress of the scanner.

One possible problem in focusing a laser beam on a sample is that of damage to the protein at high light intensities. In the case of a fluorescence scan where the maximum UV intensity is used we may do a comparison with the mercury–xenon light source. Although the laser is focused to a 50 micron diameter spot it only remains in one position for 0.4 seconds during a two minute scan of the sample. The mercury light source on the other hand illuminates the whole cell for the duration of the scan and the average amount of light striking the sample during the scan can be shown to be approximately equal for the two systems. Thus damage to the sample will be no more with the laser than with the mercury–xenon lamp. During the course of a velocity run, if the lamp remains on between scans while the laser beam waits in the counterbalence position, the effective exposure would be less for the laser system. Calculations of the number of photons absorbed and the number of molecules present show that during each two minute scan (the time taken for each scan of the data presented later) each molecule absorbs one photon. Using an average figure of 250 for the number of photons per molecule required to produce 50% inactivation [5] we see that 250 two-minute scans of the sample would produce 50% inactivation. Since far fewer scans are normally used for most substances the laser scan would produce little or no damage to the sample.

4. Results and discussion

4.1. Comparison of oscilloscope traces

Fig. 6 shows the photomultiplier traces obtained from (a) the conventional scanner, (b) a helium–neon (633 nm) laser absorption scan [4] and (c) a fluores-

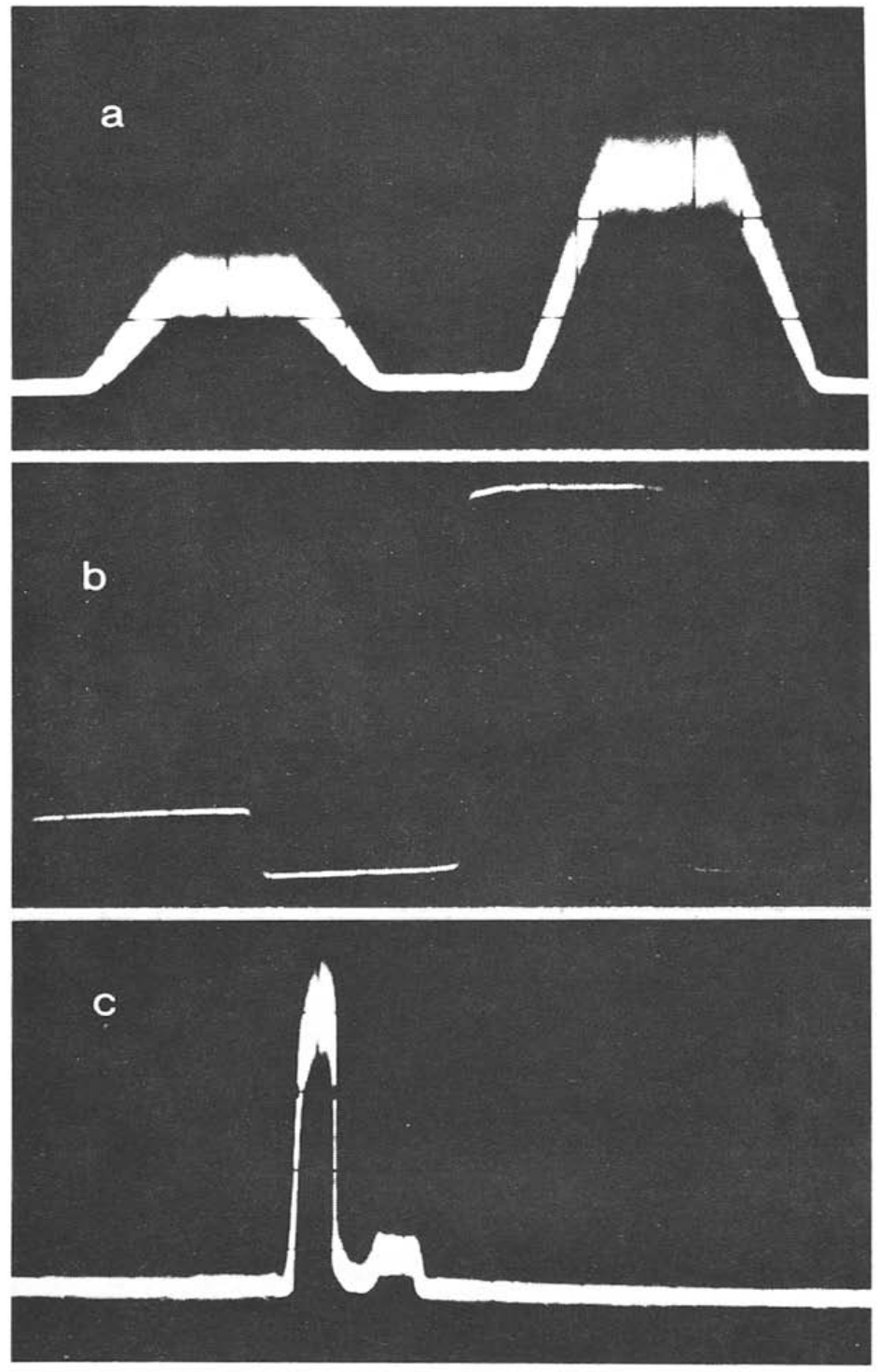

Fig. 6. Oscilloscope trace for comparison of (a) the photomultiplier output corresponding to a low intensity light signal from the conventional scanner; (b) the photomultiplier output from a laser absorption experiment using the red 633 nm line of a helium–neon laser; and (c) the photomultiplier output from a fluorescence experiment with BSA excited at 257 nm.

cence signal of bovine serum albumin (BSA). Trace (a) illustrates the type of noise obtained when the light intensity is low. This trace was taken with a fairly narrow slit at a wavelength of 633 nm in order to allow comparison with the laser trace at the same wavelength. At this wavelength the response of the photomultiplier tube is rather poor and the shct noise of the light can clearly be seen in the flat regions of the signal. Also notice the sloping sides of the trace as the slit of the photomultiplier enters and leaves the image of the cell. By comparison, trace (b) shows a much stronger light signal because the total light output of the laser is used and the shot noise is eliminated. The sharp rise and fall of these sample and reference pulses is due to the focused property of the laser. Trace (c) shows a much lower light intensity than the previous picture and a corresponding larger shot noise. The characteristic that identifies this picture as being due to the fluorescence is that the sample voltage (on the left) is larger than the reference (on the right). The source of the fluorescence in the reference is background from the windows, lenses and buffer.

4.2. Velocity experiments with BSA

In order to demonstrate the capacities of the fluorescence collection system, sedimentation velocity experiments were performed using different concentrations of BSA with the standard absorption system to determine the lower limits for good data. The concentration was then lowered by a factor of two and data was collected with the laser scanner and fluorescence monitoring. These results are shown in figs. 7–10. For all the BSA experiments the buffer was pH 5.4, 0.01 M acetate with 0.1 M NaCl. The temperature was 20°C and the rotor speed was 60,000 rpm. Fig. 7

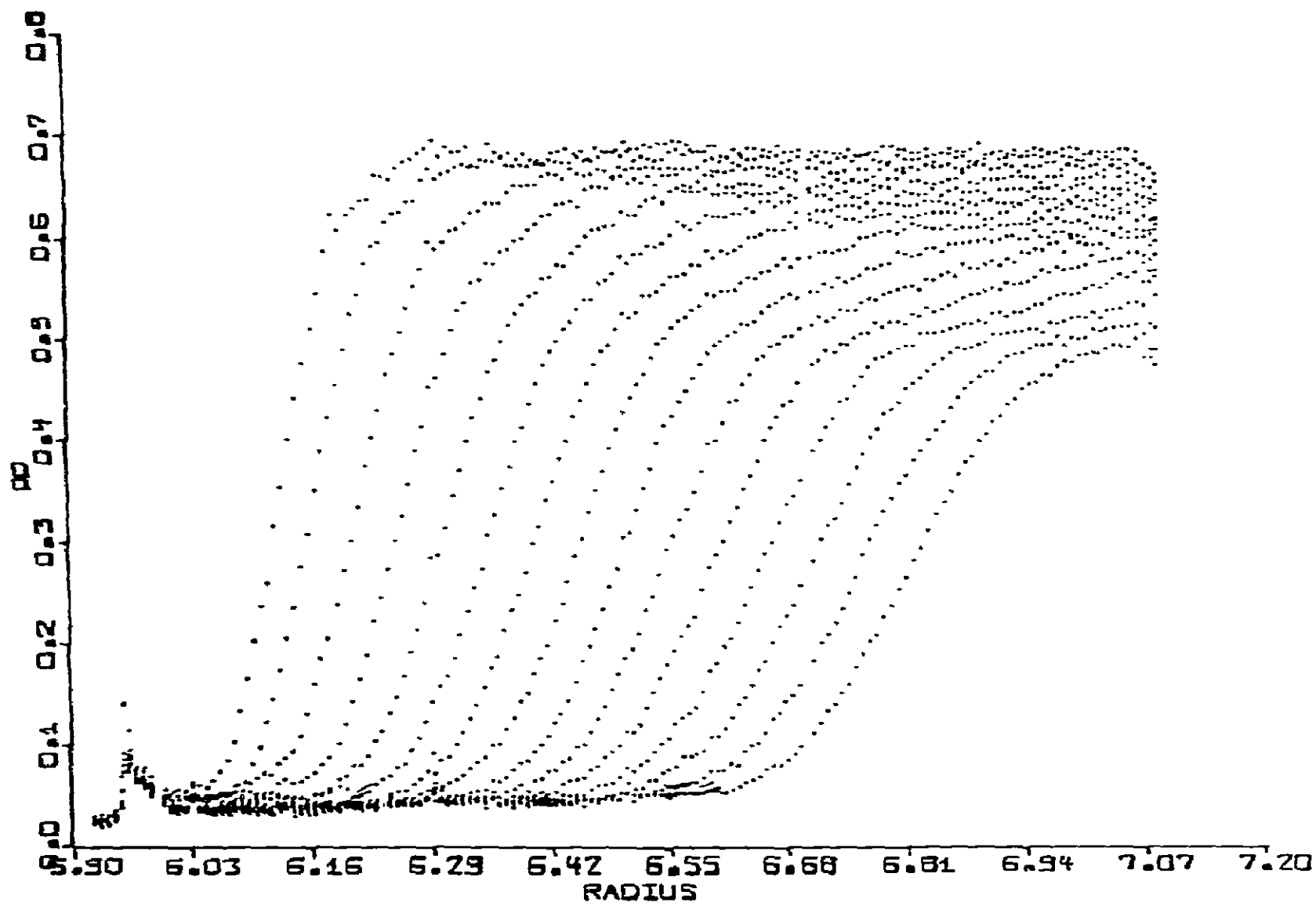

Fig. 7. Computer generated plot of consecutive scans taken from a BSA sedimentation velocity experiment at 1 mg/ml BSA. The data was collected using the conventional scanner computer system.

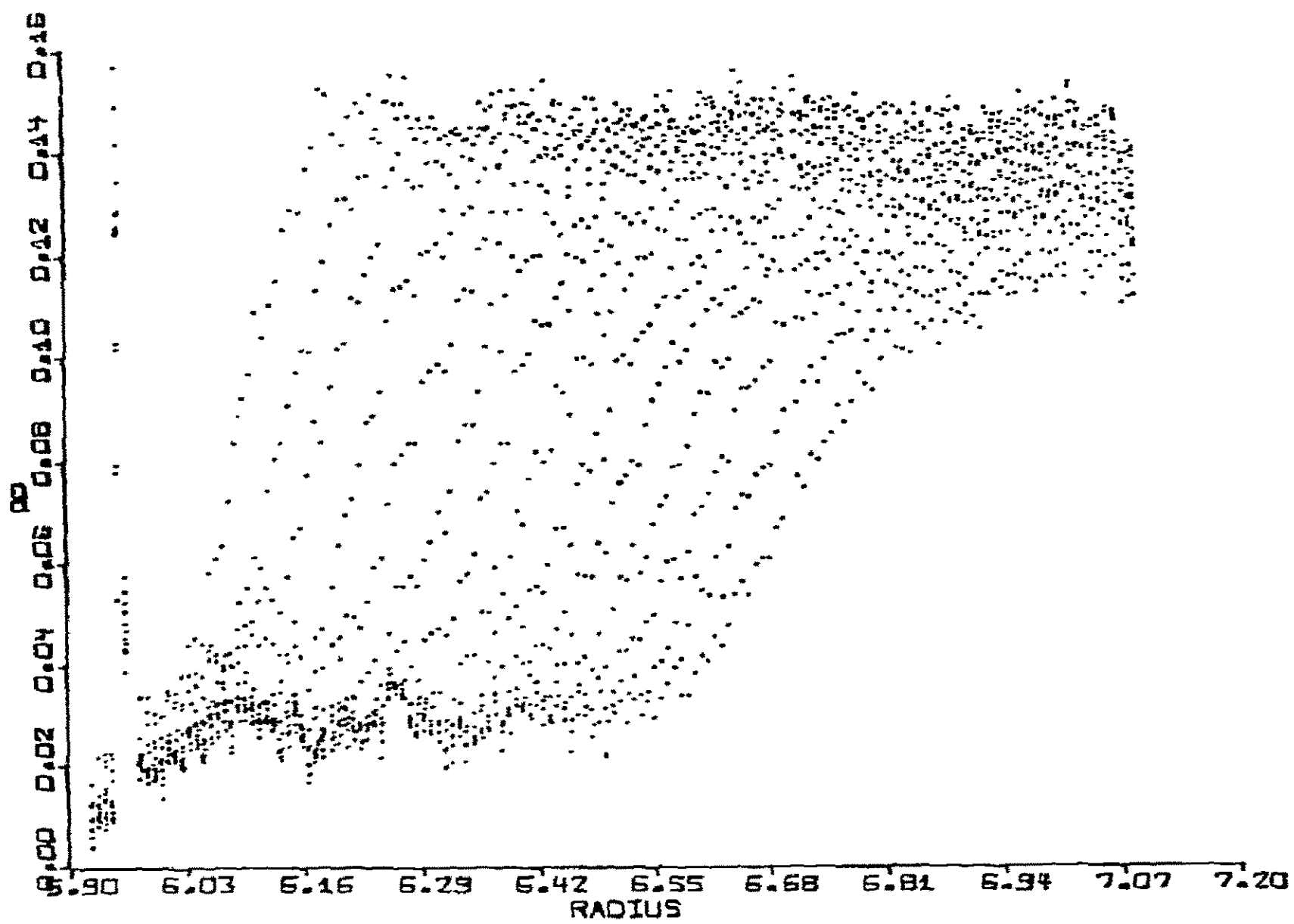

Fig. 8. Same as fig. 7 but with 0.2 mg/ml BSA.

shows the scanner under optimal conditions with the initial concentration of 1 mg/ml corresponding to an OD of 0.7 for a 1.2 cm path length. Fig. 8 is data collected for an initial concentration of 0.2 mg/ml and shows correspondingly more noise. Fig. 9 is clearly the limit of the scanner for this application and corresponds to a concentration of 40 μg/ml or an OD of 0.025. The concentration was lowered to 20 μg/ml and fluorescence was used to collect the data in fig. 10. In order to compare these results it must be kept in mind that the uniform appearance of the scanner traces is the result of the automatic scanning feature of the model E which was used to take these scans every 8 minutes and that this feature is not yet implemented with the laser scanner. Thus the operator had to start by hand each of the fluorescent scans and no attempt was made to be precise in the timing of each scan. The real time clock of the computer recorded the times and so no assumption has been made concerning the uniformity of the scans. The time taken for each scan was approximately 2 minutes. The laser data presented here represents the first experiments done with the laser scanner and the increase in sensitivity of at least a factor of ten demonstrates the potential of the method.

The boundary position was calculated from the half height of the curves and a plot was made of $\log_e$ (radius) vs. time as shown in fig. 11. Since the lowest concentration scanner trace did not give sufficiently accurate data only the two higher concentrations were plotted. Two of the three fluorescence experiments were also plotted. That the curves do not superpose indicates that the initial meniscus positions were not the same for the samples; however, they are clearly parallel. A least squares fit of the data was performed and the results are given in table 2. Since the

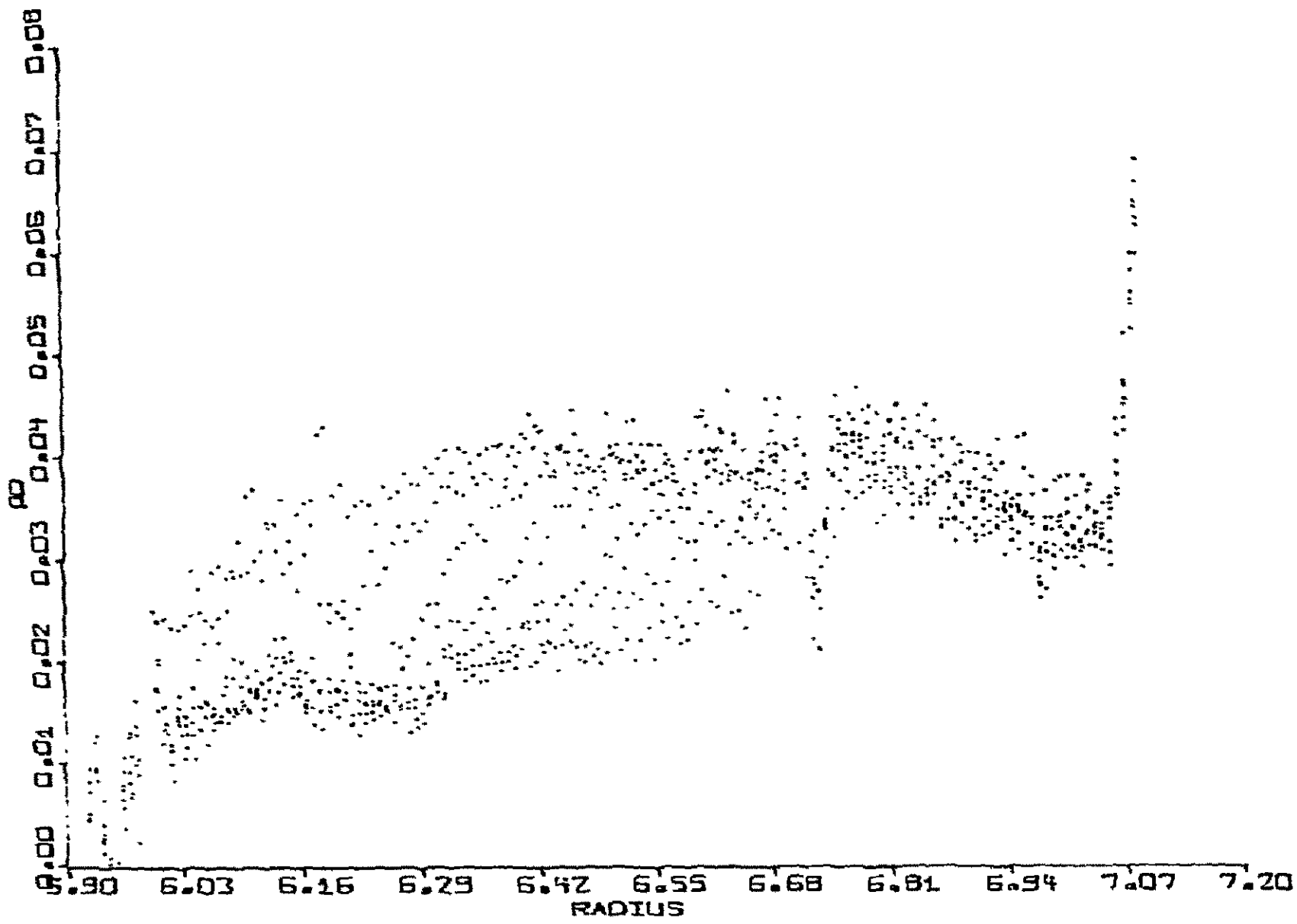

Fig. 9. Same as fig. 7 but with 40 μg/ml BSA.

Table 2
Observed S values for BSA

Optical system	Concentration	S value	Standard *deviation*
UV fluorescence	20 μg/ml	4.04 S	0.06 S
	20 μg/ml	3.96 S	0.08 S
	20 μg/ml	4.07 S	0.06 S
UV scanner	1 mg/ml	4.04 S	0.11 S
	0.2 mg/ml	4.00 S	0.07 S

BSA samples were not purified in any way the absolute values of S_{20} are not to be taken as definitive but the agreement between the standard scanner data and the laser fluorescence data cannot be mistaken.

4.3. General comments

The studies presented here indicate that it is now possible to use fluorescence to monitor the sedimentation of macromolecules in the ultracentrifuge. The use of fluorescence is made feasible by the illumination of the spinning centrifuge cell with an intense UV laser beam focused to a small spot (about 50 microns in diameter). The spot is scanned across the cell and thus different regions of the cell fluoresce as the scan proceeds. The increased sensitivity of fluorescence methods over absorbance now permits measurements to be performed with solutions at concentrations at least an order of magnitude lower when the intrinsic fluorescence of the protein is used. Preliminary experiments have also been carried out using extrinsic fluorescence, as in studies on the sedimentation of the acetylcholine receptor in complex with a rodamine-

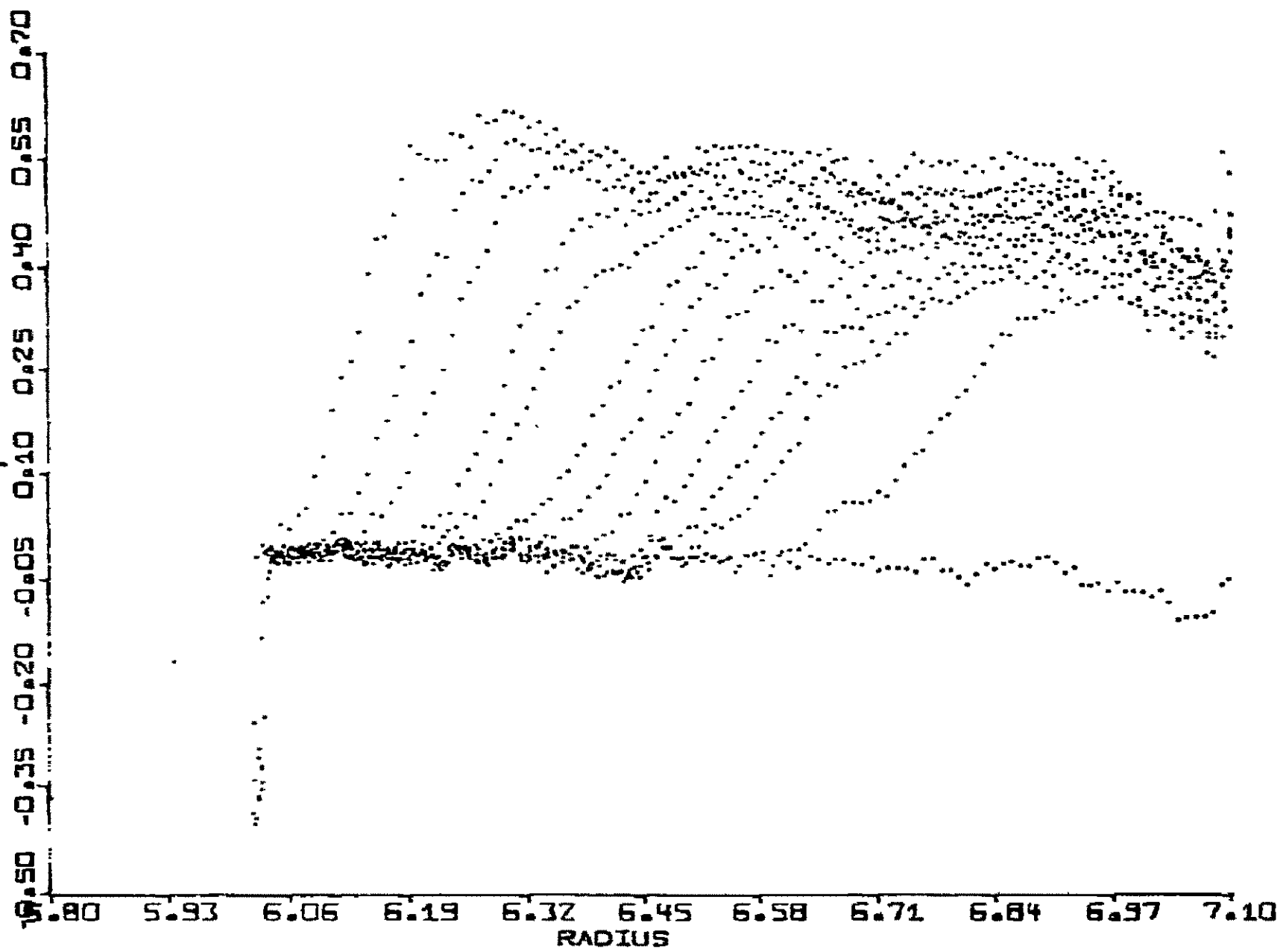

Fig. 10. Computer generated plot of consecutive scans taken from a BSA sedimentation velocity experiment at 20 μg/ml BSA. This data was collected using the fluorescence monitoring system.

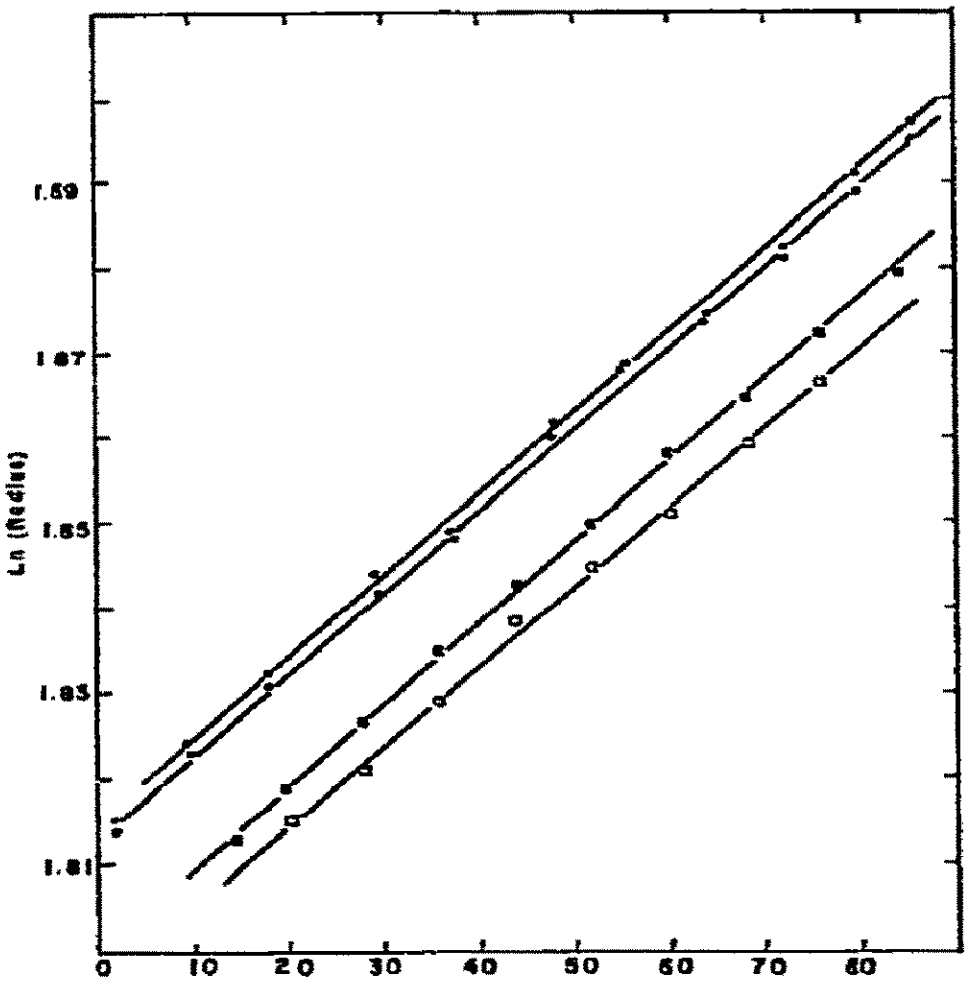

Fig. 11. Plot of $\log_e$ (radius of the boundary) vs. time for sedimentation velocity experiments. Conventional absorption experiment with 1 mg/ml BSA (○), conventional experiment with 0.2 mg/ml BSA (●), and fluorescence experiments with 20 μg/ml BSA (■ and □).

labelled α-bungarotoxin. In this case excitation is at 528 nm and conditions permit fluorescence measurements with solutions two orders of magnitude lower than the minimum concentration possible with absorbance scanning. The ability to select excitation wavelengths where only the material of interest will fluorescence makes it possible to follow the sedimentation of such material in mixtures. Thus the incorporation of a fluorescence monitoring device provides new capabilities both in the sensitivity and discrimination possible in sedimentation studies.

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