

STUDIES OF THE CELL CYCLE AND REGULATORY PROCESSES  
IN THE MOUSE LEUKEMIA CELL L5178Y

by

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Dec 10, 79  
Date

This thesis is dedicated to my husband, Scot, for his unending patience, love and respect.

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## ABSTRACT

The structural changes during the life cycle of populations of the mouse leukemia cell line L5178Y have been described by polarized light scattering measurements for the first time. Exponentially growing cells were synchronized by an automatic excess thymidine-colcemid treatment technique. At intervals throughout the life cycle samples were removed from the suspension culture and fixed with glutaraldehyde. The effect these cell samples had in changing right-hand circularly polarized light to  $45^{\circ}$  linearly polarized light was measured at angles  $6-160^{\circ}$  to the incident beam. The reproducibility of the light scattering signals for each time interval was statistically evaluated and found to have good inter-trial correlation for each time period in the angular range  $6-60^{\circ}$  to the incident beam. Statistically significant changes were seen between cell samples during the synchronous life cycle. Therefore, the system developed would seem to have application as an extremely sensitive measure of cellular structural changes resulting from low level chemical, physical or biological agents.



## INTRODUCTION

Fundamental to the study of cell biology is an understanding of cell structure and changes that occur in that structure during a cell's life cycle. Myriads of experiments have been performed to elucidate events in the cell cycle (38, 41, 50). Some have analyzed physiologic changes (2, 3, 19, 21, 23, 24, 40, 51, 52, 55) and some structural changes (4, 5, 14, 17, 18, 30, 35, 37). Common techniques employed have been electron microscopy (20, 30), nuclear fractionation (35), nuclease digestion (34), cell membrane component analysis (37), autoradiography (9, 10, 25), pulse labeling (14), x ray diffraction (15, 39) and optical studies measuring circular dichroism (28) or birefringence (27). These studies commonly rely on techniques destructive to the cells being studied.

The following is a review of the development of the techniques employed in this research and of cell cycle regulatory events as pertain to the cell line under analysis.

### L5178Y and its Synchronization

L5178Y, first described as a spontaneously arising murine tumor (16), is a lymphoblastic continuous cell line. These spherical cells replicate in vitro without surface attachment. When cultured in Fischer's medium (16), they maintain their strain-specific tumorigenicity and characteristic biochemical properties for prolonged periods of time (16).

The first studies of the L5178Y cell cycle were by Watanabe and Okada (54). Measuring the cell number, mitotic index, eosin uptake and percent tritiated thymidine uptake of an exponentially growing population, they determined the duration of the four cell cycle phases at different incubation temperatures. The optimum temperature for replication was found to be 37°C. The G1 phase, the interval after mitosis and before DNA synthesis, was calculated to be 1.8 hours at 37°C. The S phase, the DNA synthesis period, was 7.3 hours. The G2 phase, the interval after DNA synthesis and before mitosis, was 1.2 hours. Mitosis was 0.55 hours. The population doubling time (average time for a population in exponential growth to double in number) at 37°C was 10.8 hours.

Doida and Okada (12) attempted to use the double thymidine block and chilling techniques to synchronize the L5178Y cells with only limited success. However, they were successful when they arrested exponentially growing cells in or at the beginning of S phase with 0.0025 M thymidine. Then the thymidine was removed after 5 hours and  $10^{-6}$  M deoxycytidine and 0.025 µg/ml colcemid were added for another 5 hours. The deoxycytidine counteracted any toxic effects of the thymidine. The colcemid stopped the cells in the metaphase of mitosis. The cells were again washed, fresh medium added and the cells allowed to progress through the cell cycle.

Despite a slight lag period in the cell cycle and a slightly shorter population doubling time of 9 hours, their successive excess thymidine and colcemid treatment method had numerous advantages.

These included: 1) a short time to achieve synchrony (one doubling rather than two), 2) a high percent of synchrony (89-99%), 3) a suitability for cell lines with short total population doubling times and long S periods, 4) the capability to synchronize entire cell populations, particularly those in suspension and 5) minimal cytotoxic or cytopathic effects as experienced in other systems (8).

In 1974 Okada and Shinohara (36) reported the development of an automated synchronizer for L5178Y suspension cultures. Their procedure called for the automatic addition of 0.625 ml of 0.1 M thymidine to a 50 ml culture (1 to  $1.5 \times 10^5$  cells per ml) ten hours before the desired completion time. The thymidine is distributed by magnetic stirring for 30 seconds. Five hours later, 0.5 ml of  $10^{-2}$  M deoxycytidine and 0.25 ml colcemid (5  $\mu\text{g/ml}$ ) were added and the culture agitated. At completion time, the cells were centrifuged and the supernatant media removed. Fresh, pre-warmed Fischer's medium with 10% horse serum was added to allow the cells to progress synchronously through the next cell cycle. They reported an 82-87% synchrony with the automated system.

As with Doida and Okada's manual method, a lag period and shortened population doubling time were observed. The advantages of Doida and Okada's method still prevailed in the automated system with the additional advantage that cells could be synchronized overnight without human attendance. The electronics and equipment necessary to set up the automatic cell synchronizer were relatively inexpensive and easy to assemble.

### Polarization Effects in Light Scattering

In any optical signal only three things can be measured: the wavelength, the total intensity and the states of polarization (47). Although there are sixteen possible combinations in the Mueller matrix for small particle scattering (32), most optical systems have been capable of characterizing a maximum of two scattering elements (53).

In 1973, Hunt and Huffman (26) developed an instrument capable of determining all sixteen scattering matrix elements as a function of scattering angle. A monochromatic light was passed through the preparatory optics equipment to select exactly which polarization state will pass on to the sample. The light may be horizontally polarized, right-hand circularly polarized or polarized  $45^{\circ}$  to horizontal. As the light passes through the suspension of scattering particles, these states of polarization are mixed. The particle suspension for light scattering analysis must have a concentration so that single particle scattering is in effect. This allows the effect of thousands of particles in the suspension to be averaged for an overall measurement. At  $2^{\circ}$  intervals from the incident beam, the scattered light is selected by the analytic optics for measurement of a desired polarization state. Because each incoming polarization state may remain unchanged or be converted to either of the other two polarization states as a result of the scattering particles, nine possible measurements may be made. In addition, total intensity may

be measured. This means ten of the scattering matrix elements may be measured by this instrument. The remaining six may be calculated from the data obtained.

Realizing most optical systems applied to biological problems measured only forward direction polarization changes or angular intensity alteration, Bickel et al. (7) applied the Hunt and Huffman instrumentation to a study of bacterial spores. Kilksen et al. (29) followed with erythrocyte studies. In both cases, the instrumentation proved to be a sensitive indicator of structural changes in the cells examined, particularly the  $S_{34}^*$  matrix element. This element relates the efficiency of the scattering particle in converting the right-hand circularly polarized light to  $45^\circ$  linearly polarized light as measured at angles to the incident beam. An absolute number can be given this efficiency because the electronics normalizes the curves.

#### Statement of the Problem

The research reported in this thesis presents the first optical system for studying structural changes during the life cycle of a synchronous population of the lymphoblastic, continuous cell line designated L5178Y. The study consisted of two aspects: first, the development of the biological conditions and techniques by which the instrumentation of Hunt and Huffman (26) measuring the polarization states in scattered light could be applied to studying mammalian cell structure and growth regulation; second, a study of the time sequence

of events in the life cycle of the L5178Y cells. Special emphasis was devoted to events occurring in the nucleus, for which this technique is exceptionally well suited.

## MATERIALS AND METHODS

### Cell Line

The L5178Y cells were obtained from Drs. Nakamura and Okada of the University of Tokyo (Department of Radiation Biophysics, Faculty of Medicine). The cells were subcultured in Fischer's medium (GIBCO, Grand Island, New York) for murine leukemia cells with 10% horse serum (Flow Labs, Inglewood, California) designated FM<sub>10</sub>. Penicillin and streptomycin were added at 100 units/ml and 100 µg/ml concentrations respectively, as was 5 µg/ml fungizone. All media constituents, including the horse serum, were filtered through 0.2 µm pore size membrane filters (Gelman Instruments, Ann Arbor, Michigan) to insure sterility and remove particulates which interfere with consequent light scattering analysis. The cells were subcultured at a ratio of  $2.5 \times 10^4$  or  $5 \times 10^4$  cells per 5 ml fresh media. They were incubated at 37°C in Falcon plastic tubes or Brockway prescription bottles.

The L5178Y cells were frozen in regular growth medium supplemented with 10% dimethyl sulfoxide (DMSO). Either acetone:dry ice or a Revco -60°C freezer were used to slow freeze the cells at the rate of approximately one degree per minute. The cells were stored at -170°C in liquid nitrogen for future use.

### Total and Viable Cell Counts

All cell counts were performed manually with the use of a hemacytometer. Total cell counts were calculated per standard hemacytometer procedures (31). Viable cell counts were determined by the trypan blue exclusion test (31).

### Synchronization of L5178Y

The automatic synchronizer of Okada and Shinohara (36) has been adapted for use in this research (Figure 1). On the evening preceding the desired date for use of the synchronized cells,  $2 \times 10^7$  cells in logarithmic growth phase were centrifuged at 1000 rpm for 5 minutes. The cells were resuspended in 100 ml of fresh, cold FM<sub>10</sub> to obtain the optimum concentration of  $2 \times 10^5$  cells/ml. This 100 ml cell suspension was placed in a sterile 250 ml flask with an air-tight sealing cap. A sterile two-inch magnetic stir bar was added to the flask, and the flask mounted upon a magnetic stirrer in a 37°C incubator. After checking the timers and electronics, the external magnetic stir bars were properly aligned against opposite sides of the flask. Sterile centrifuge tubes, each containing a sterile magnetic stir bar, were aseptically placed inside the flask opposite an external magnet. To cup #1, 1.2 ml of sterile 0.1 M thymidine (Sigma, St. Louis, Missouri) was aseptically added. To cup #2, 1 ml of sterile  $10^{-2}$  M deoxycytidine (Sigma) and 0.5 ml sterile colcemid (5 µg/ml) (Sigma) were added. The flask was tightly sealed. Timer #1 was set to 10 hours before the desired completion time. Timer #2



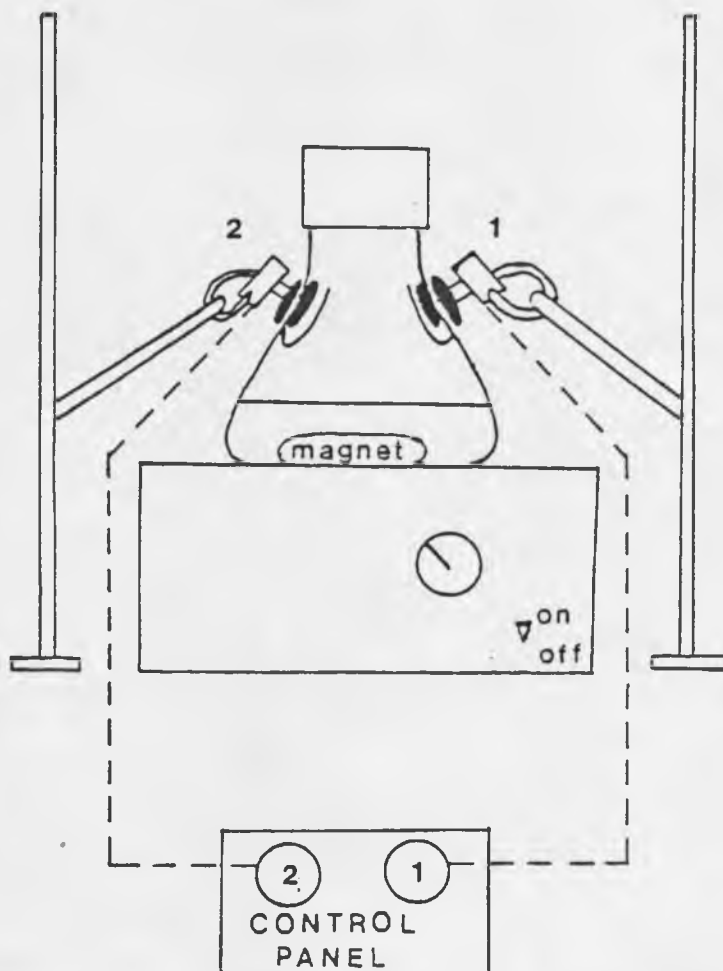


Figure 1. Apparatus for Synchronization of L5178Y Suspension Cultures by the Automatic Excess Thymidine-Colcemid Treatment Technique of Okada and Shinohara (36). -- Under direction of the preset control panel, magnetically held centrifuge cups automatically add thymidine (#1) and deoxycytidine and colcemid (#2) to the culture.

was set to 5 hours before the desired completion time. At completion time, the cells were centrifuged in pre-warmed centrifuge cups and holders at 1600 rpm for 3 minutes. The used media was removed and 200 ml of fresh pre-warmed FM<sub>10</sub> was added. The cells were placed in an airtight suspension culture flask at 37°C to proceed through the next cell cycle in synchrony.

#### Sample Preparation for Light Scattering Analysis

Several solutions appropriate for suspending the L5178Y cells were tested for their inherent light scattering properties. These included normal saline, Earle's Balanced Salt Solution (BSS), FM<sub>10</sub> and Hank's BSS with phenol red.

Several methods were examined for fixing the cells. Cells suspended in normal saline were treated with formalin for three hours at room temperature. Formalin:cell suspension ratios of 1:1, 2:1, 3:1, 1:5 and 1:10 were used. Methanol fixing of the cells in saline and FM<sub>10</sub> was also examined. In addition, the cells were fixed in suspension by addition of an equal volume of membrane filtered 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 90 minutes at 22°C (1).

Cell sample concentrations of  $1.4 \times 10^4$  to  $2.4 \times 10^5$  per ml were tested for variation in light scattering signal.

Duration of the signal of a given sample was tested immediately following the fixing procedure to one week after the fixing procedure.

### Measurement of Polarization States in Light Scattering

The samples were analyzed by the instrumentation of Hunt and Huffman (26) as modified by Bickel for biological studies (6). Light 4416 nm in wavelength was right-hand circularly polarized by the preparatory optics. Outgoing 45° linearly polarized light was selected for by the analytical optics and was detected by a photomultiplier tube. The intensity of this  $S_{34}^*$  matrix element was digitally recorded for computer analysis on an IMSAI 8080 and graphically plotted on recorder paper (Figure 2). If none of the incoming right-hand circularly polarized light was converted to 45° linearly polarized light, a value of zero was obtained. If all was converted to positive 45° linear polarization, a positive 100% value was obtained. If all was converted to negative 45° linear polarization, a negative 100% value was obtained.

### Computer Statistical Analysis

Digital intensity data were transmitted from the IMSAI 8080 to the University of Arizona Computer Center DEC-10. An analysis of variance using the factorial anova program of Sokal and Rohlf (49) was performed using the Control Data Corporation Cyber 175 computer at the same computer center. Bivariate analyses using the product moment correlation coefficient program of Sokal and Rohlf (49) were also performed on the Cyber 175. Bivariate means and confidence ellipses were plotted manually. The 95% confidence limits of the

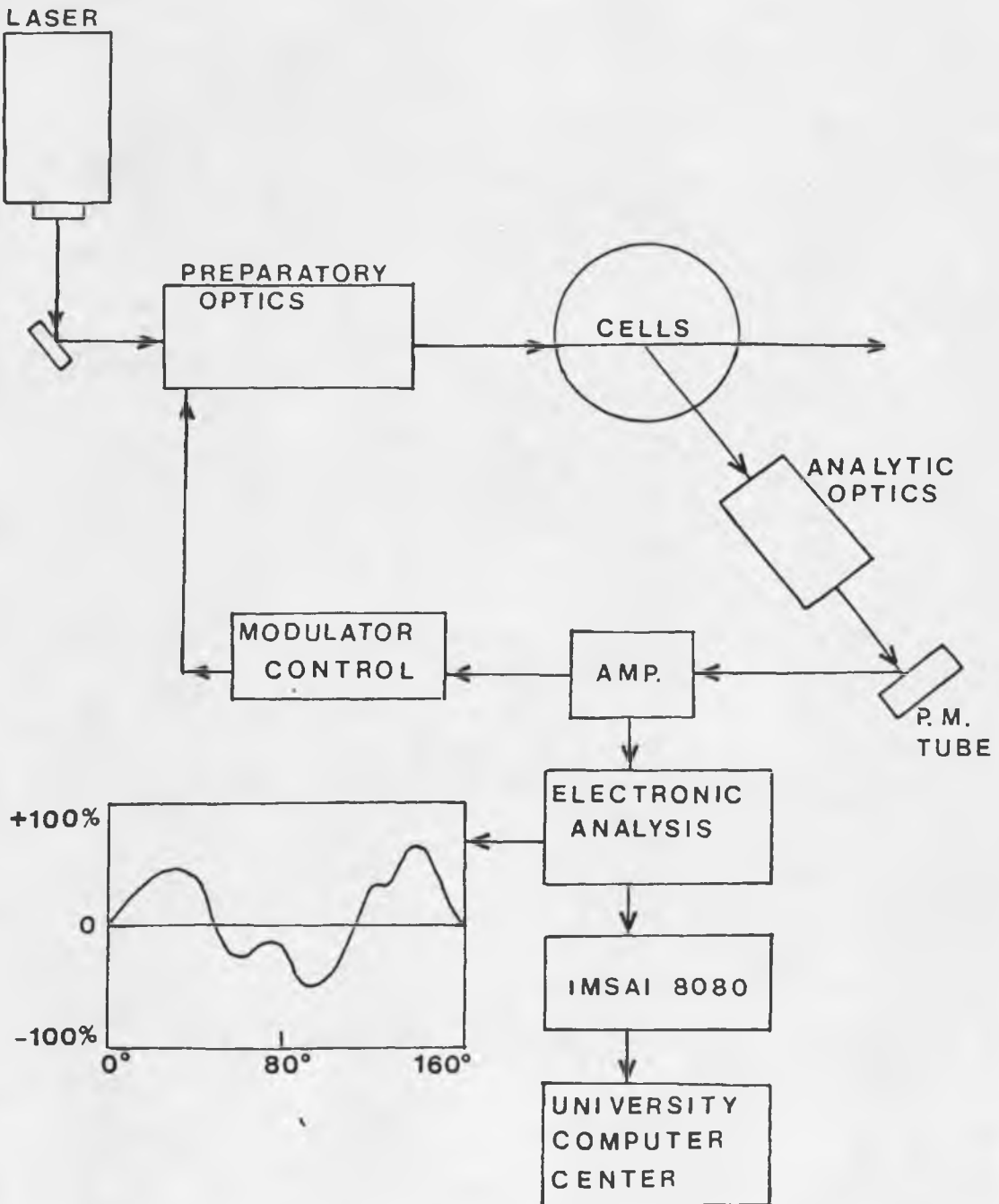


Figure 2. Schematic Diagram of the Instrumentation Used to Measure the Polarization States of Light Scattered from Hourly Samples of Synchronized L5178Y Cells at Angles to the Incident Beam.

means of measurements at specified culture ages over all angles considered in two trials were calculated by the following formula:

$$C.L.95\% = \bar{X} \pm t_{270, \alpha 0.5} \sqrt{\frac{EMS}{n}}$$

where  $\bar{X}$  is the mean average for trial 1 and trial 2 at a given hour,  $t_{270, \alpha 0.5}$  is the 95% confidence level t-value for 270 degrees of freedom (42), EMS is the error mean square of the 3-way interaction (the best available estimate of the random variable) in the analysis of variance between trials 1 and 2 for the angles 6-60° and N is the number of values included in the calculation of  $\bar{X}$  (in this case, n = 56). The 95% confidence limits of the means of measurements at these same culture ages over all angles considered in each trial separately were calculated by a similar formula where  $\bar{X}$  is the mean for trial 1 or trial 2 at a given hour and n = 28.

### Cell Sizing

After light scattering analysis, the cell suspensions were analyzed for particle size distribution by a Coulter Model Z<sub>BI</sub> and Coulter Channelyzer (Coulter Electronics, Hialeah, Florida). The Channelyzer counted particles until one channel had accumulated 400 cells. The results were plotted by an X-Y Recorder II (Coulter Electronics).

The particle sizes were calculated in microns by the following formulas:

a)  $V = K I A Ch$

b)  $V = 0.523 D^3$

where  $V$  = cell volume,  $K$  = a constant 7.06,  $I$  = current,  $A$  = amplification,  $Ch$  = channel number on a full scale of 0-100 (or 0.5 the channel number on a 0-50 scaled plot) and  $D$  = cell diameter. These calculations were done for the peak channel or channel with the most cells. It was also done for the channels on each side of the peak channel which had 50% of the maximum counts. These are referred to as the maximum and minimum sizes. Any second size peaks observed were also calculated and referred to as a "seconds" peak or range.

#### Staining and Photography

Unfixed cells from each hourly sample were washed twice in membrane filtered Earle's BSS. The pellet was resuspended in a drop or two of Earle's BSS. A drop of this cell suspension was placed on a clean glass slide and air dried. The slides were fixed with Spray-cyte (Clay Adams, Parsippany, New Jersey) spray fixative according to manufacturer's directions. The slides were then dipped in filtered May-Greenwald stain (Allied Chemical, Morristown, New Jersey), dipped in freshly prepared and filtered Giemsa (Allied Chemical) (1:10 in distilled water), rinsed with distilled water and air dried.

Photography of the stained cells was done on a Reichert microscope using an oil immersion objective, 7.5X ampliplan and a 5.4 amp light source (Hacker Instruments, West Caldwell, New Jersey).

An Ihagee Exacta Model VX 35 mm camera containing Kodak Panatomic-X  
FX 135 film was used to photograph the stained cells.

## RESULTS

### Sample Preparation for Light Scattering Analysis

For analysis by the Hunt and Huffman equipment (26), cells must be suspended in solution as a group of independently scattering particles. Selection of the solution is crucial because any inherent scattering by the solution could mask or alter the scattering signal of the cells. Of the solutions tested, unfiltered saline, unfiltered Earle's BSS, unfiltered and filtered FM<sub>10</sub> and Hank's BSS with phenol red produced unacceptable signals. Filtered Earle's BSS and Fischer's medium without horse serum produced negligible signals.

L5178Y cells have been found to lyse within minutes in balanced salt solutions without horse serum (33). Since the presence of horse serum in any solution produced undesirable light scattering, fixation of the cells became mandatory. All formalin and methanol fixing attempts resulted in clumping of the cells. However, glutaraldehyde fixing caused no gross or microscopically detectable clumping of the cells.

For a given sample, cell concentrations of  $0.5 \times 10^5$  to  $2 \times 10^5$  per ml produced no discernible differences in the light scattering curve obtained. The curves obtained when the samples were analyzed immediately after fixing were reproducible for 48 hours. After 48 hours, degeneration of the curves was observed.



The method adapted for sample preparation was as follows; a sample of  $2 \times 10^6$  cells was removed from the synchronized suspension culture. During the first two hours after release from the colcemid block, 20 ml of the culture yielded this required cell number ( $2 \times 10^6$ ). For succeeding time periods, after mitosis had occurred, a 10 ml sample was sufficient to yield the necessary cell number. The cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 90 minutes at room temperature. The samples were centrifuged at 1000 rpm for 5 minutes. The supernatant portion was removed and the pellet similarly washed three times in 10 ml of membrane filtered Earle's BSS. The cells were suspended finally in 20 ml of the filtered Earle's BSS. A cell count was performed and filtered Earle's BSS added as necessary to make all samples of a run the same concentration.

#### Life Cycle Studies Using Biological Parameters

The life cycle position and degree of synchrony of the L5178Y population was monitored by three parameters. First, cell counts throughout the cell cycle indicated when mitosis had occurred as the cell number increased from approximately  $2 \times 10^5$  to  $3.4 \times 10^5$  per ml (Figure 3). The degree of synchrony obtained was 80-88%. Cell sizing data yielded additional information showing the cells were largest before division, smallest immediately after division and intermediate during DNA synthesis (Figure 3). The appearance and homogeneity of the cell population during the four life cycle phases are illustrated in Figure 4.

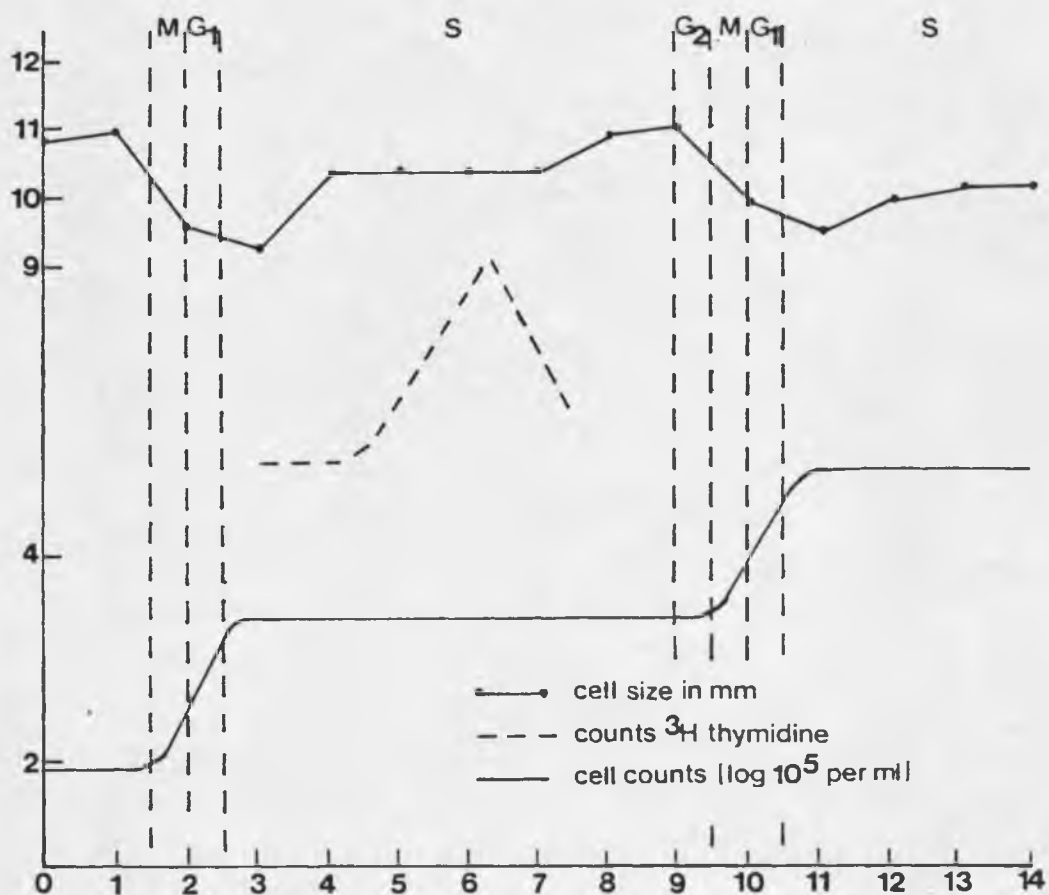


Figure 3. Evaluation of the Degree of Synchronization of L5178Y Cells by Hemacytometer Cell Counts and Electronic Cell Sizing. -- These data are compared to  $^3\text{H}$ -thymidine uptake data as observed by Okada and Shinohara (36).

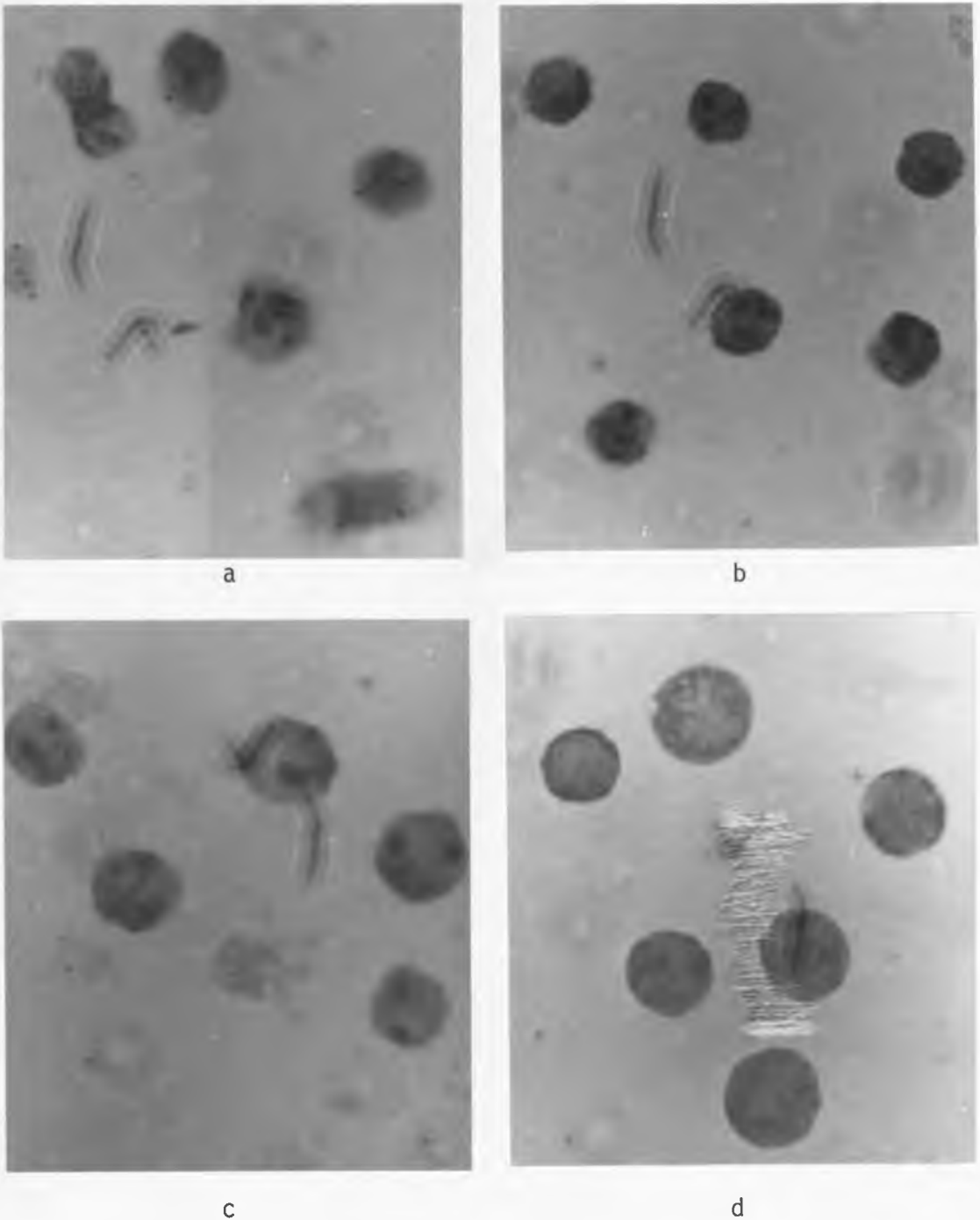


Figure 4. Appearance of May Greenwald-Giemsa Stained Synchronized L5178Y Cells. -- Of particular interest are the changes of the cell size and internal organization during the M phase (a), G1 phase (b), S phase (c) and G2 phase (d) of the life cycle.

Life Cycle Studies  
Using Light Scattering

Light scattering curves for two synchronized life cycle trials are represented in Figure 5. For each hour after release from the colcemid block, the  $S_{34}^*$  matrix element was measured from  $6^\circ$  to  $160^\circ$  from the incident light beam. In all trials the curves had similar properties: the curves were first negative, turned positive in the middle of the angular range and oscillated in the latter half of the curve.

A preliminary attempt to quantitate changes in the curves during the life cycle was done by measuring the lengths of each curve (Figure 6). For the two trials represented in Figure 5, the curves had a maximum length in early S phase with all other times having only slight variation in curve length.

Further quantitation of variation between the curves of Figure 5 was attempted by a statistical analysis of variance using the model I factorial procedure (49). Relative to the 3-way interaction, 2-way interactions were found to be significant (Table 1). Because 2-way interactions override any independent factors, no indication of inter-trial correlation could be determined from these data.

To further probe the sources of variation between hourly samples, model II bivariate analyses (49) were performed. The first bivariate analysis compared the hourly samples of both trials over the entire  $6^\circ$  to  $160^\circ$  angular range. Then bivariate analyses were

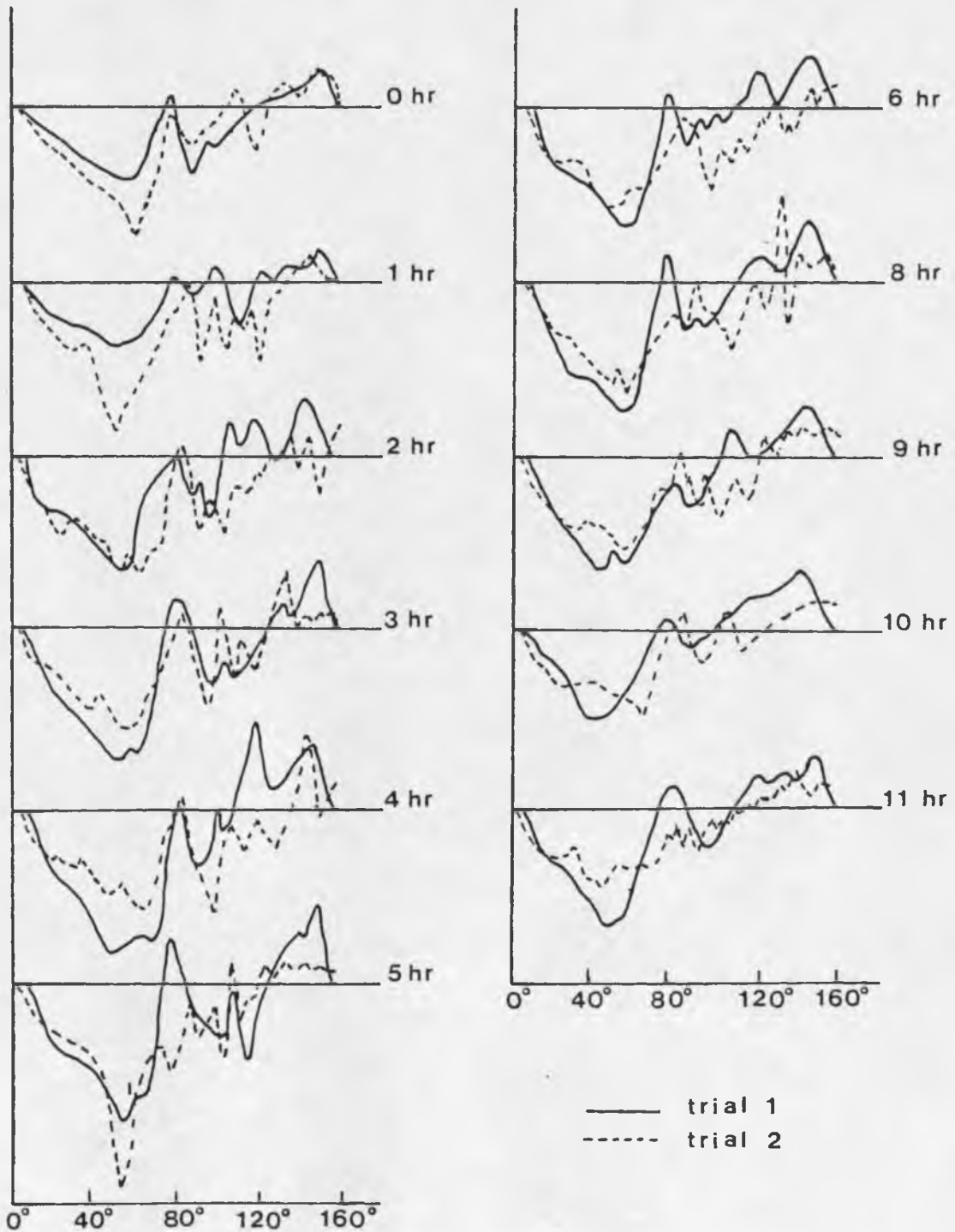


Figure 5. Light Scattering Signals ( $S_{34}^*$ ) of Synchronized L5178Y Cells. -- Samples were taken and signals recorded at hourly intervals following the removal of the excess thymidine and colcemid used to synchronize the cell population.

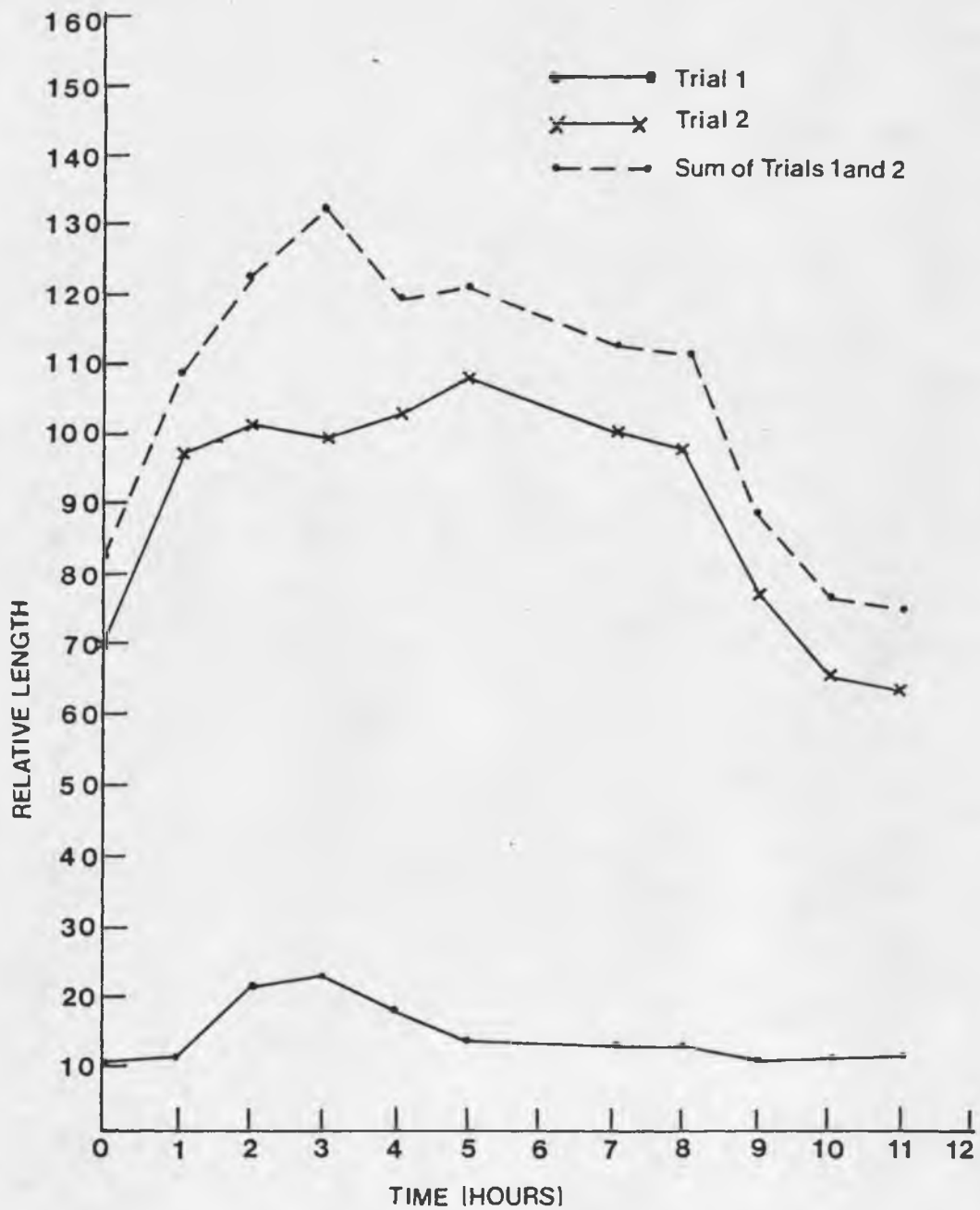


Figure 6. Relative Curve Lengths of Polarized Light Scattering Signals of Synchronized L5178Y Cells.

Table 1. Summary of Analysis of Variance of Polarized Light Scattering Measurements of Synchronized L5178Y Cells for the Angles 6-160° to the Incident Beam.

SOURCE	SS	DF	MS	F RATIO
ABC	10.6879	770	.0139	--
AB	12.6026	770	.0164	1.18
AC	.5081	10	.0508	3.65*
A	.7796	10	.0780	5.61*
BC	2.5636	77	.0333	2.39*
B	64.6898	77	.8401	60.44*
C	.0146	1	.0146	1.06

\* = significant ratio at 0.05 confidence level

A = number of hours

B = number of angles

C = number of trials

SS = sum of squares

DF = degrees of freedom

MS = mean square

performed for the 11 hourly samples examining the angular ranges  $6^{\circ}$ - $60^{\circ}$ ,  $62^{\circ}$ - $110^{\circ}$  and  $112^{\circ}$ - $160^{\circ}$ . The correlation coefficients of each bivariate analysis are plotted in Figure 7 along with the critical value (42) for each angular sample size. Correlation was generally good over all angles, but was best for measurements at  $6^{\circ}$ - $60^{\circ}$ . Angles  $62^{\circ}$ - $160^{\circ}$  all have at least one time period in which the curves are not statistically reproducible.

In addition to the correlation coefficients, the 95% confidence limit ellipses for the means were calculated and plotted for each bivariate analysis (Figure 8a, b, c, d). Good correlation between trials is indicated by a narrow ellipse, the major axis of which has a slope of approximately 1.0. As correlation decreases, the ellipse widens and lengthens until a spherical configuration is approached indicating poor correlation between trials. During the life cycle analyses, correlation was best between trials in the  $6^{\circ}$ - $60^{\circ}$  angle range of the light scattering data and erratic at later angles.

A second analysis of variance using the model I factorial procedure (49) was performed on the data for  $6^{\circ}$ - $60^{\circ}$ . No significant interaction of time and angle can be distinguished from these data (Table 2). However, it can be determined that over all angles significant changes occur with time. Conversely, over all times, significant changes occur between angles. A plot of the 95% confidence limits of the means averaged for both trials at each hour (Figure 9) best identifies the interaction of time and angle in the  $6^{\circ}$ - $60^{\circ}$  range.



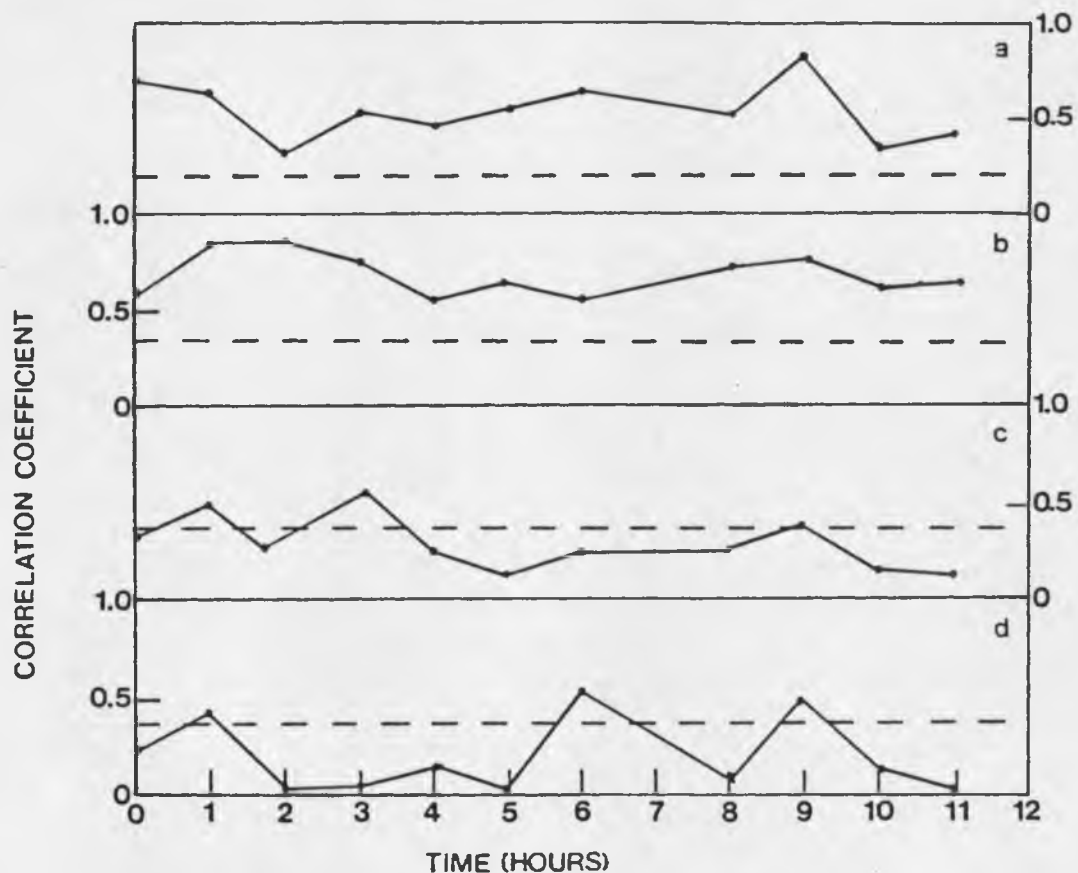


Figure 7. Correlation Coefficients of Bivariate Analyses to Determine Precision of Polarized Light Scattering Analysis of Synchronized L5178Y Cells. -- Analysis of the angular ranges 5-160° (a), 6-60° (b), 62-110° (c) and 112-160° (d) are given. The critical value for the correlation coefficient for each sample size (42) is indicated as -----.

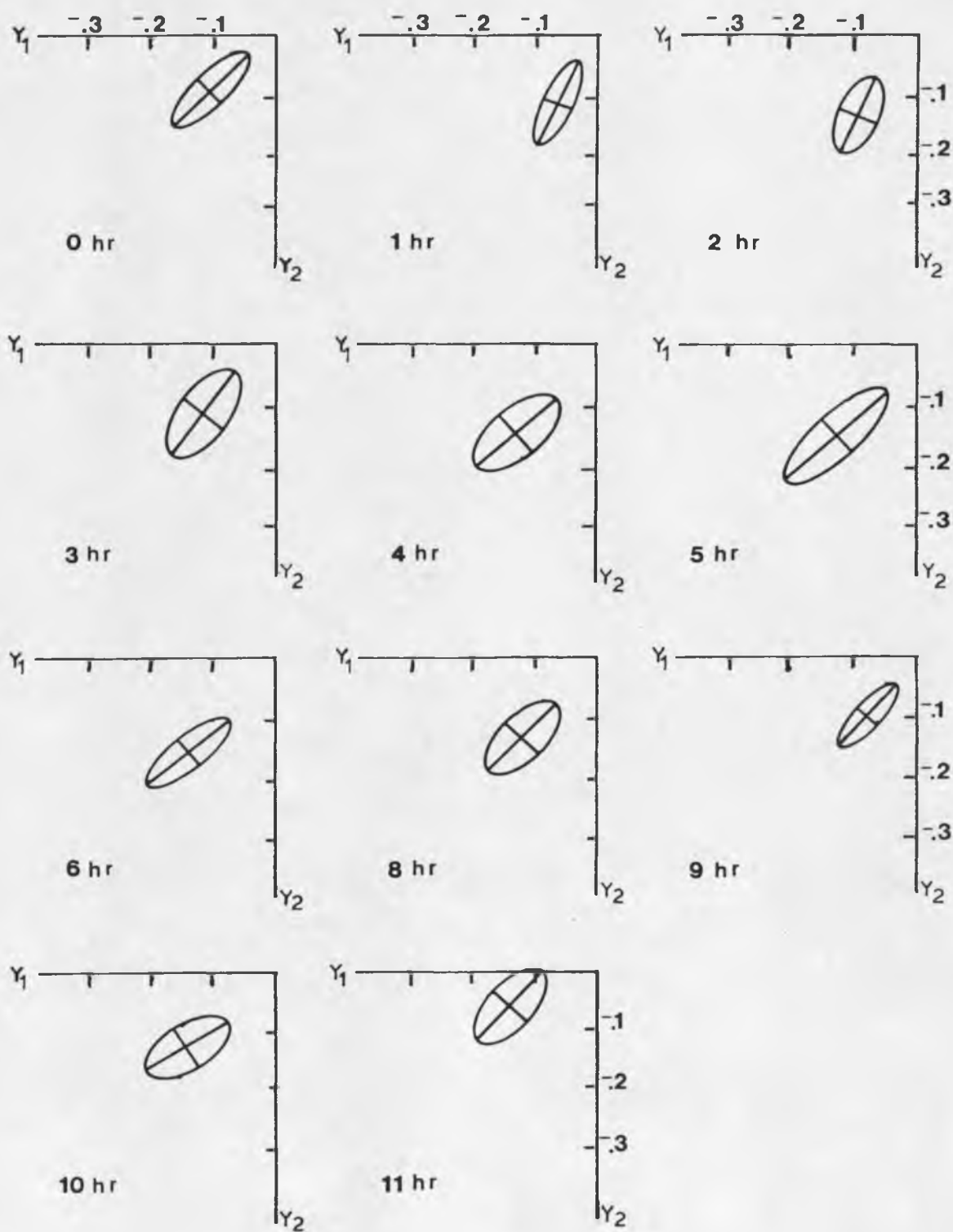


Figure 8. Bivariate Confidence Ellipses for Hourly Polarized Light Scattering Measurements of Synchronized L5178Y Cells. -- a.  $6-160^\circ$  to the incident beam.

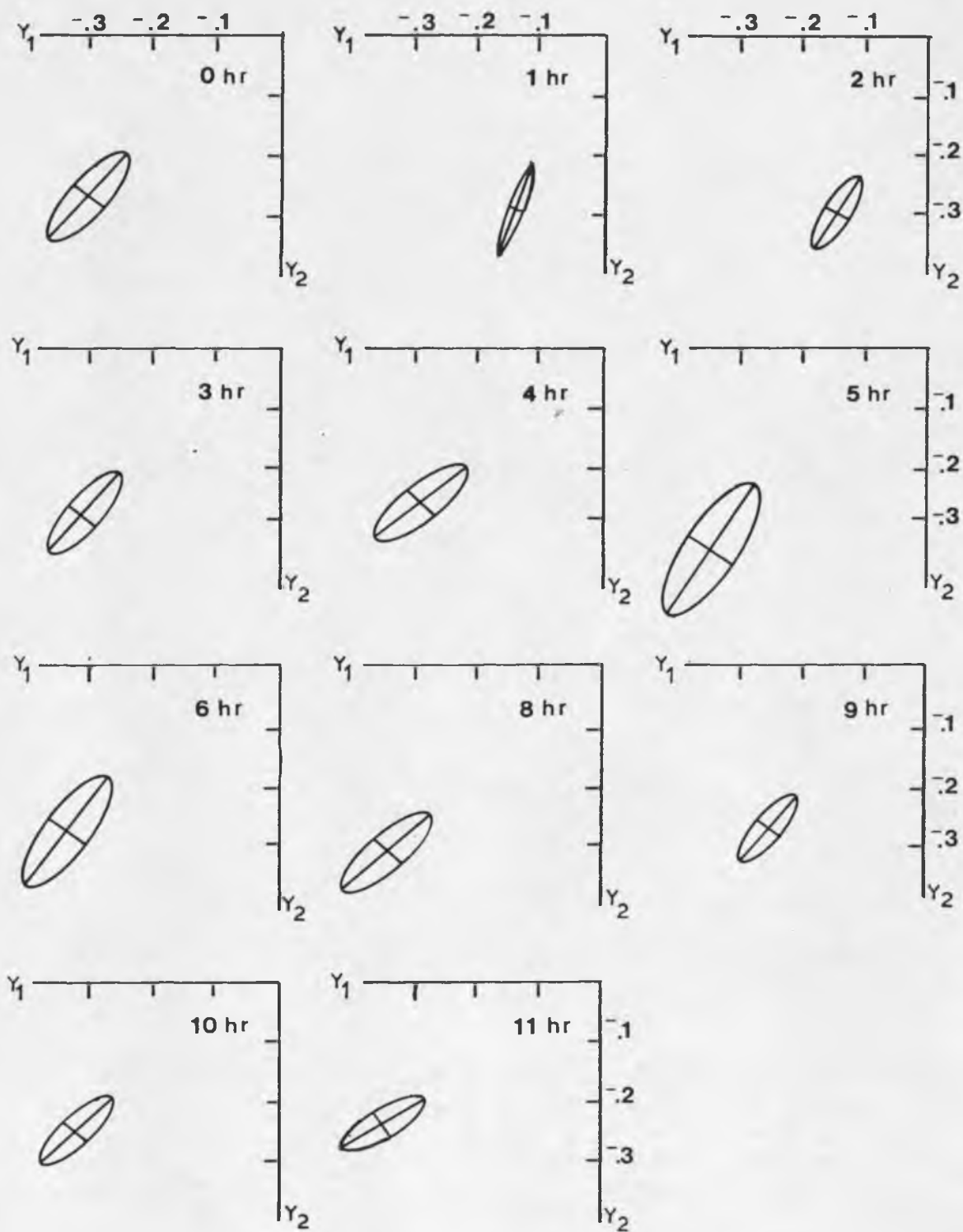


Figure 8. (Continued) Bivariate Confidence Ellipses. --  
 b.  $6-60^\circ$  to the incident beam.

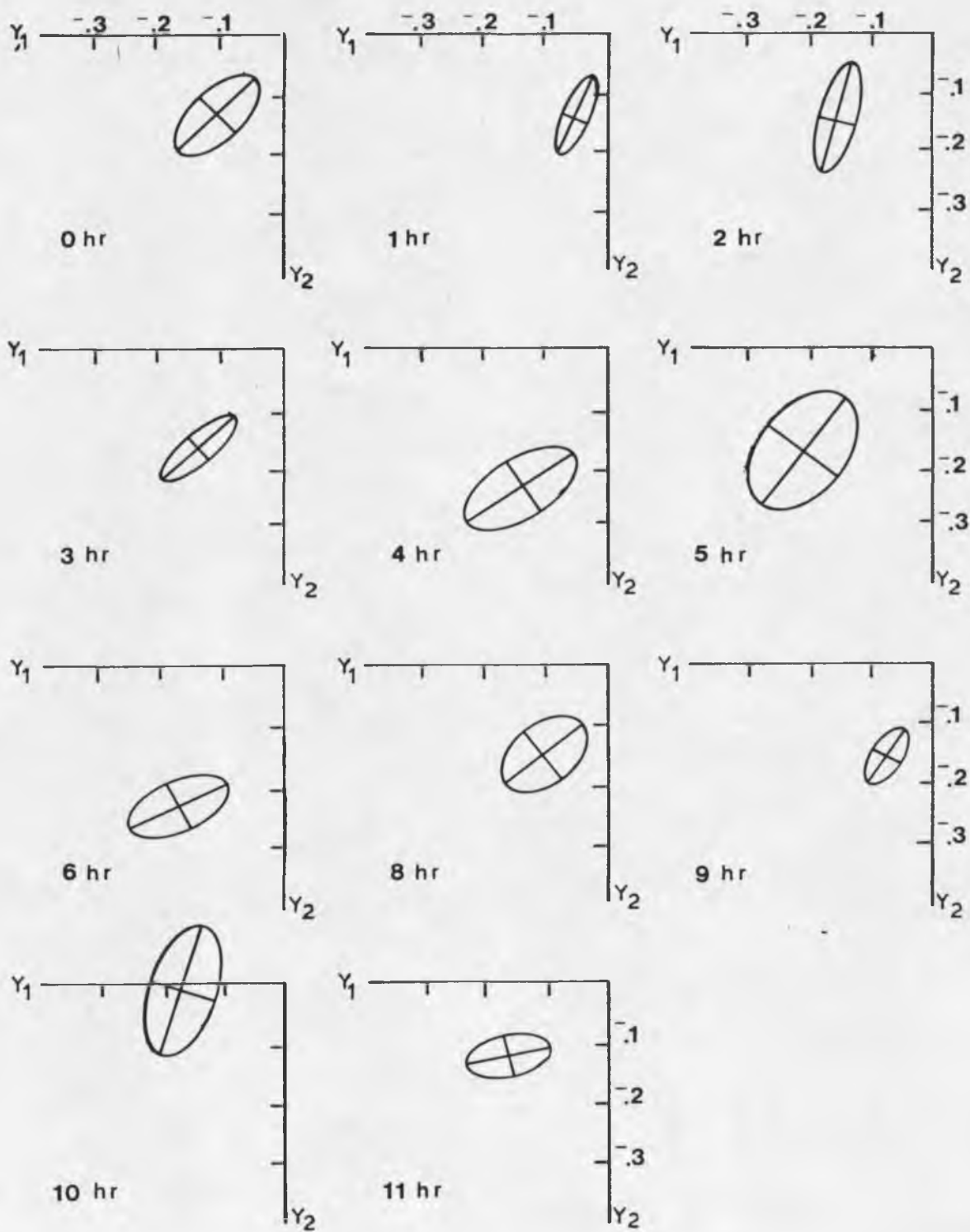


Figure 8. (Continued) Bivariate Confidence Ellipses. --  
c.  $62-110^\circ$  to the incident beam.

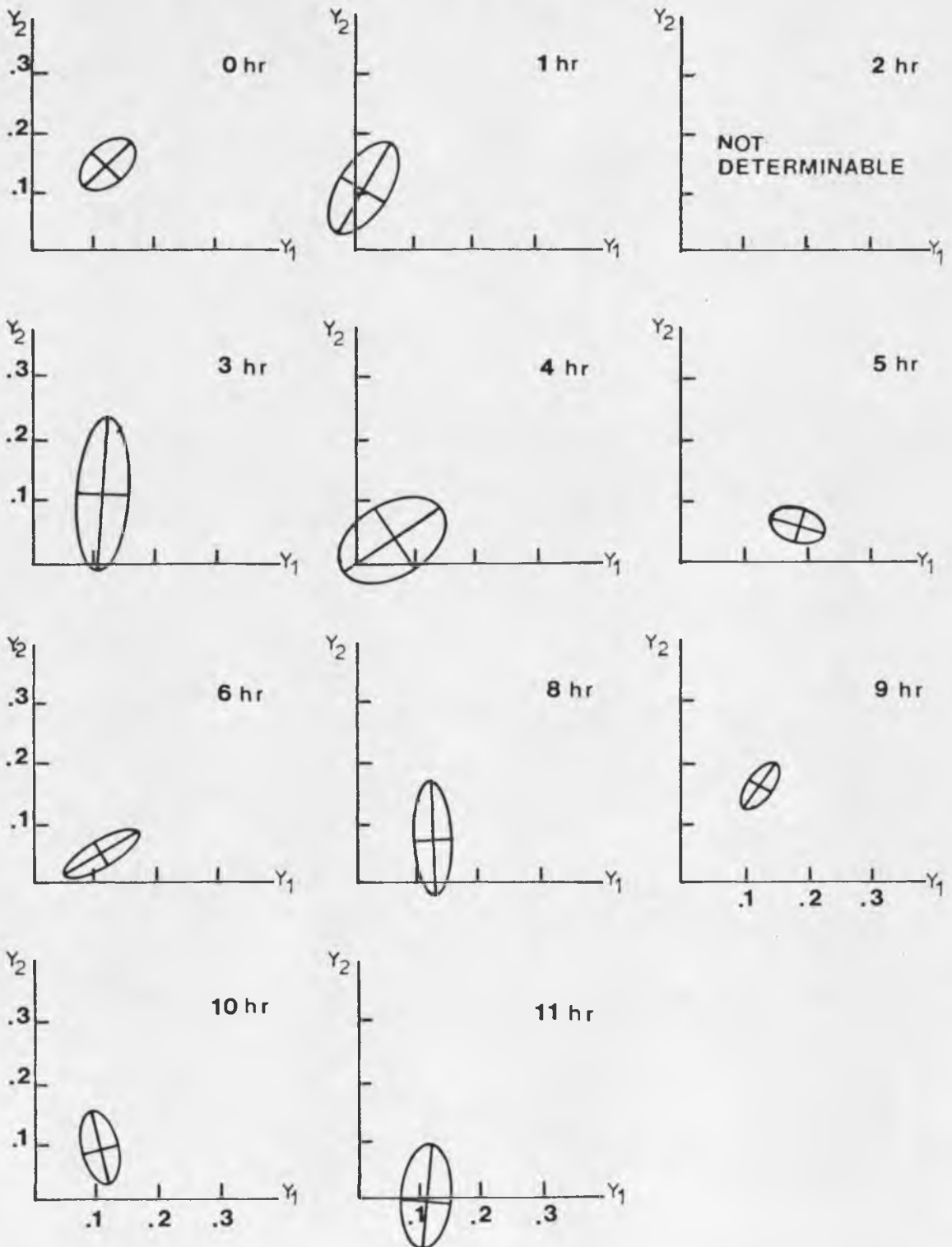


Figure 8. (Continued) Bivariate Confidence Ellipses. —  
d.  $112-160^\circ$  to the incident beam.

Table 2. Summary of Analysis of Variance between 2 Trials of Polarized Light Scattering Measurements of Synchronized L5178Y Cells at 6-60° to the Incident Beam.

SOURCE	SS	DF	MS	F RATIO
ABC	1.8417	270	.0068	--
AB	2.0984	270	.0078	1.15
AC	1.0865	10	.1087	15.99*
A	.8400	10	.0840	12.35*
BC	.1869	27	.0069	1.01
B	14.2632	27	.5283	77.69*
C	.0282	1	.0282	4.15*

\* = significant ratio at 0.05 confidence level

A = number of hours

B = number of angles

C = number of trials

SS = sum of squares

DF = degrees of freedom

MS = mean square

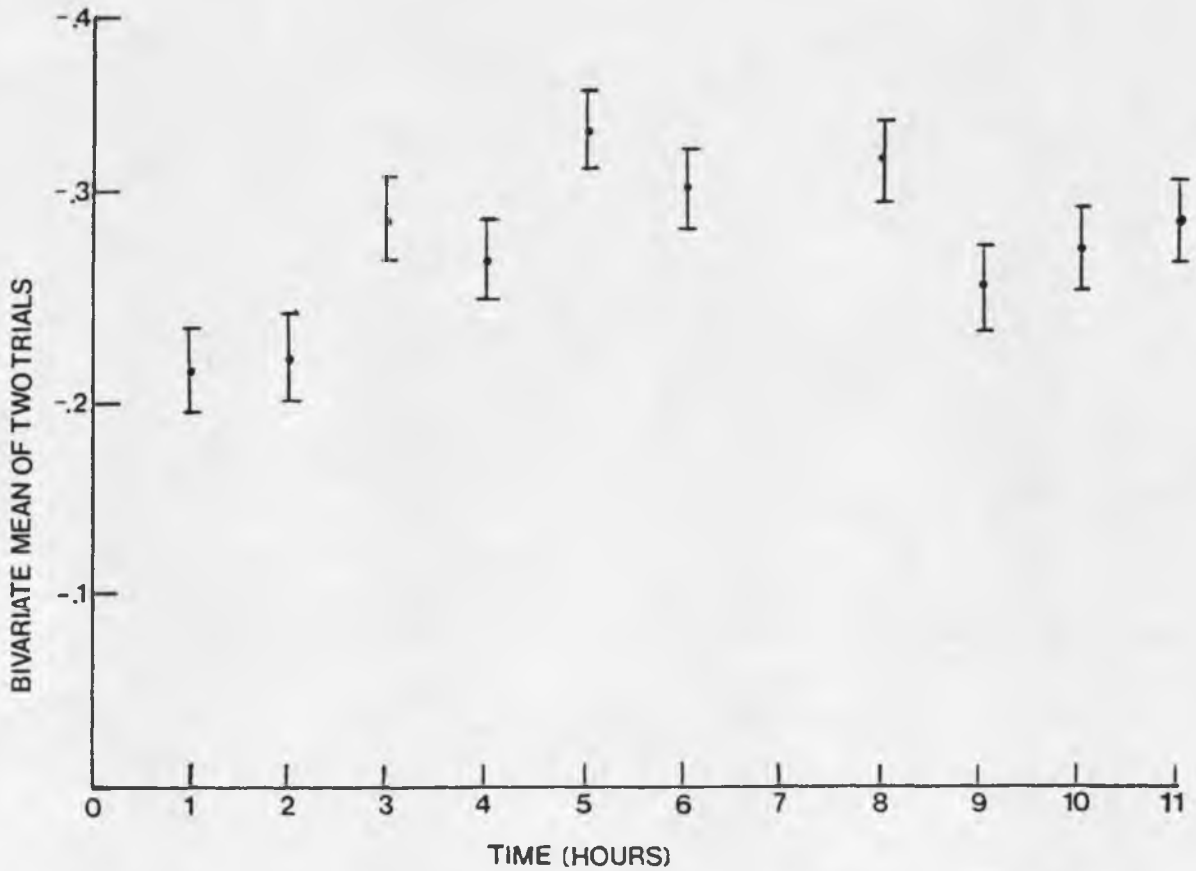


Figure 9. Ninety-five Percent Confidence Limits of the Mean of Trials 1 and 2 of Polarized Light Scattering Measurements Made Hourly at Angles  $6-60^\circ$  to the Incident Beam During the Synchronous Life Cycle of L5178Y Cells.

Hours having no overlap of their mean within the  $\pm$  95% confidence limits are statistically different from one another. A plot of the 95% confidence limits of the mean for each trial at each hour (Figure 10) illustrates the significant trial to trial interaction which has been found.



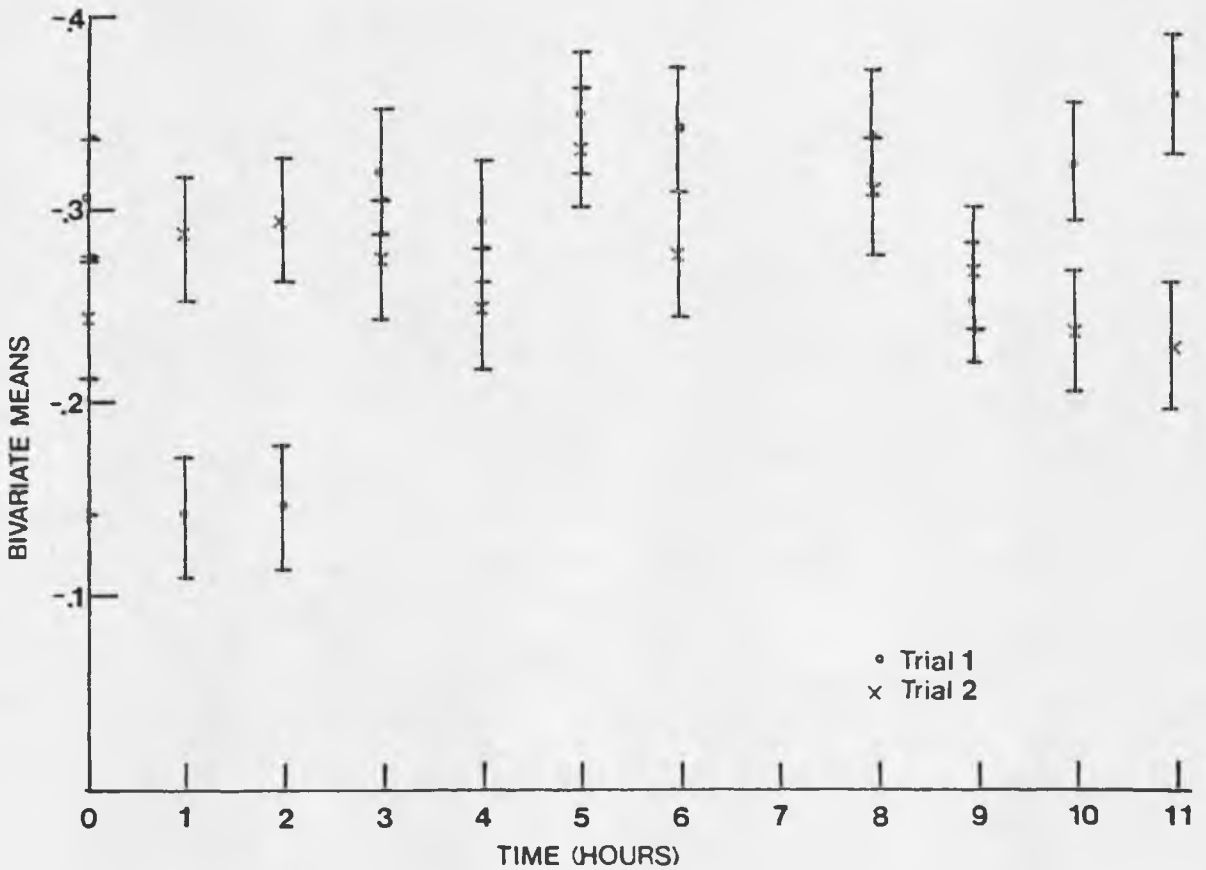


Figure 10. Ninety-five Percent Confidence Limits of the Hourly Means of Trials 1 and 2 of Polarized Light Scattering Measurements Made at Angles  $6-60^{\circ}$  to the Incident Beam During the Synchronous Life Cycle of L5178Y Cells.

## DISCUSSION

Cell structure research has relied heavily upon optical observations. Progressively, naked eye observations were improved by magnification and a variety of wavelength-dependent phenomena. In 1973, the instrumentation of Hunt and Huffman (26) became the most advanced optical measurement system available. With this new system, it became possible to measure the complete set of characteristics of scattered light, including intensity and all states of polarization as a function of the wavelength and scattering angle.

Because optical studies in cell biology have been a mainstay, it was only natural that this instrumentation be used to study biological systems. The nature of the biological system must, however, be conducive to analysis by the instrumentation. The objects to be analyzed must be uniform in size, shape and structure. They must be in suspension for analysis. They must also be considerably larger than the wavelength of the light used.

The system developed in this research was designed to fit those qualifications. The L5178Y cells are spherical and uniform

in size (Figures 3 and 4). They can easily be synchronized in their life cycle phase to a high degree (36) to further increase the homogeneity of the population. Finally, they remain in suspension for periods of time sufficient for light scattering analysis.

The methods developed to synchronize and prepare the cells for light scattering have been chosen to minimize artifactual structural changes. Minimal structural changes were detected as a result of synchronization (12, 36). Glutaraldehyde fixation, the method of choice to preserve intact cellular structure (1) was employed. In addition, all precautions were taken to eliminate, as much as possible, artifactual changes in light scattering signals due to extraneous particulate matter. These precautions included using clean glassware and membrane filtering every solution contacting the cells.

The applicability and reliability of this system for studying mammalian cell life cycle events has been examined from two standpoints: by examination of the light scattering curves relative to structural changes known to occur in mammalian cells during the four life cycle phases, and by quantitation of the reproducibility of curve changes at any given time in the life cycle.

In this in vitro system, the first life cycle phase encountered is mitosis. The cells are blocked in early M by the colcemid (36). After washing the cells and resuspending them in fresh FM<sub>10</sub>, their progress through mitosis can be detected by the increase in cell number and decrease in average cell size (Figure 3). As these

small daughter cells progress from M to G<sub>1</sub>, they reassemble their polysomes and nucleoli and synthesize proteins using mRNAs from previous cell cycles without significant RNA synthesis (11). In the G<sub>1</sub> period syntheses of new mRNA and proteins essential for entry to the S phase (13) occurs. As seen in Figure 3, early S causes a slight increase in cell size. Measured by the uptake of <sup>3</sup>H-thymidine (36), S phase in L5178Y cells peaks about six hours after release from the colcemid block. The cell size remains unchanged in mid-S (Figure 3) as nuclear and mitochondrial DNA is synthesized (50). After the climax of DNA synthesis at six hours, the cells again start protein synthesis (48) and increase their size in preparation for another mitosis. In late S and G<sub>2</sub> are spindle fiber protein, cellular lipid and phospholipid and RNA synthesis (50).

Changes in the light scattering curves reflect both the volume changes of the L5178Y cells and the intracellular structural changes. The exact contribution of each is difficult to determine, although cell size has been attributed by Brogan, Scott and Collins (22) as the most important factor in low angle ( $2^{\circ}$ - $5^{\circ}$ ) light scattering intensity changes. Visually (Figure 5) one can see the signals become more negative in value in the first portion of the curve through the fifth hour. This peak returns to approximate the 1 and 2 hour mitotic curves during the second mitosis which occurs at approximately the tenth hour. Consistent variation of the light scattering curve in the latter portions is difficult to discern visually.

Quantitation of preliminary visual observations of changes in the light scattering curves throughout the L5178Y cell cycle was approached from a statistical standpoint. Two questions were addressed: 1) are the signals for hourly samples reproducible?, and 2) if so, are the changes between light scattering signals of the hourly samples significant?

The results of this research indicate the answer to the first question is yes. The correlation coefficients determined in the bivariate analyses show good correlation (variation solely due to randomness of trials of the same population) when data for all angles of each hourly sample are considered. Best correlation is obtained between trials in the first  $60^{\circ}$  of the light scattering curve. Unacceptable correlation between trials (variation due to unexplained causes) was obtained from  $62^{\circ}$  to  $160^{\circ}$  of the curves for most of the hourly periods. The unreliable nature of the curves in this latter portion of the curve may have two sources. Despite all efforts to eliminate extraneous particulate matter, unknown particles which scatter at these angles may have entered the system. Alternatively, internal scattering of the instrumentation may be the cause of erratic signals from  $62^{\circ}$  to  $160^{\circ}$  in this particular system.

The answer to the second question, "Are the changes between the light scattering signals of the samples significant?" is also positive. Although the analysis of variance for the reproducible portion of the light scattering curves was incapable of discerning any significant interaction between time and angles (Table 2),

reflecting the parallel nature of the changes occurring with time at  $6^{\circ}$ - $60^{\circ}$ , it does show that the effects of the independent factors are highly significant at some times and some angles. Identification of those times and angles is, however, impossible from the analysis of variance data. A plot of the means for the angles  $6^{\circ}$ - $60^{\circ}$  does show the effect of time over all angles considered (Figure 9). Certain periods are statistically different than others (no overlapping values in the 95% confidence limit areas). The cells of mitosis (1 and 2 hour) are significantly different than cells from any other life cycle phase, although not significantly different from each other. This was seen microscopically and reflects the fact that mitosis is over one-half hour in duration and the cell population is not entirely synchronous in its entry and exit of the 0.55 hr M phase (54). Consequently, the light scattering curves could be used to identify certain time periods. Were the mean over all angles,  $6^{\circ}$ - $60^{\circ}$ , above 0.32, the population could be placed somewhere in S phase (hour 5 through 8). Means 0.25 to 0.30 would indicate G1, early S, G2 or second mitosis. It is interesting to note that although correlation between trials just before and at the second division (9 and 10 hour) appeared similar to that in the first mitosis, the means are significantly different. Early M (1 and 9 hour) have no overlapping values. Similarly mid-M (2 and 10 hour) have no overlapping values. One would expect them to be similar. This discrepancy can be attributed to the loss of synchrony of the population as the cells progress through the life cycle.

This inability to distinguish each hourly sample does not, however, rule out a comparison of these data to signals obtained from synchronized L5178Y cells treated by some chemical or biological agent. When doing that, one must be cognizant of the trial-to-trial variation which occurs (Figure 10). The sources of this variation are difficult to pinpoint. In complex mammalian cell systems, otherwise undetectable cellular changes in metabolism or structure may occur. Perhaps the level of precision of the hemacytometer cell counts at different intervals in the cell cycle causes cell concentration changes which are reflected in the light scattering signals more at some life cycle stages than others. Whatever the cause, all precautions must be taken to minimize trial-to-trial variation. A single synchronous culture, split into equal portions for both control and treatment studies, seems imperative for valid comparisons of treated and non-treated cells.

It would appear, then, that this could be a sensitive and reliable system for probing the "still highly speculative" (38) mammalian cell cycle and regulatory processes. These treatments may include low level doses of carcinogens or virus, as well as the use of temperature sensitive L5178Y mutants (43, 44, 45, 46).

## SUMMARY

A system has been developed by which the life cycle of the lymphoblastic cell line L5178Y can be studied optically using the equipment of Hunt and Huffman (26). Preparatory procedures have been developed to minimize artifactual structural changes throughout the L5178Y life cycle and maximize clarity of the light scattering data. The uniqueness of this system lies in its capability of making any optical measurements desired and the sensitivity of the  $S_{34}^*$  matrix element to detect minute cellular structural changes. The data obtained have been shown to be reasonably reproducible in the  $6^{\circ}$ - $60^{\circ}$  angular range of the polarized light scattering curve. Changes seen in the light scattering curves have been associated with events previously shown to occur in L5178Y cells at given intervals of its life cycle. Therefore, this in vitro system can be recommended for further studies into structural cellular changes resulting from treatment of the cells by various chemical, physical or biological agents.



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