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Permeance of Novikoff Hepatoma Gap Junctions: Quantitative Video Analysis of Dye Transfer

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Summary. Fluorescent dyes are commonly used to study permeable (gap) junctions, but only rarely have quantitative values for junctional dye permeability been determined. In the present study, junctional permeance (*PA*, i.e., the product of the junctional permeability coefficient, *P*, times the junctional area, *A*) to Lucifer Yellow CH (LY) has been obtained for pairs of Novikoff hepatoma cells. Dye was microinjected into one cell and the subsequent transfer monitored by a SIT camera and recorded on video tape. The intensities of fluorescence in the injected and "recipient" cell were measured using a Digisector (Microworks) digitizing board and an Apple II Plus computer to analyze the video records. These changes in intensity, along with an estimate of volume of the spherical cells, were used to calculate the junctional permeance (*PA*) of cell pairs according to Fick's diffusion equation. Junctional permeances show considerable variation ranging from 0.08×10^{-11} to 27.0×10^{-11} cm³/sec. Using the mean *PA* and a previous estimate of the mean number of junctional channels per interface in the Novikoff cultures, a value for diffusion coefficient of LY through gap junctions is calculated to be about 1.4×10^{-6} cm²/sec. There is a general proportionality between mean *PA* and cell volume for hepatoma cell pairs of a certain size range. Such a relationship between cell volume and junctional capacity suggests one source of variation of *PA*. Other possible sources, e.g., related to position in the cell cycle, are discussed.

Key words gap junctions · dye permeability · cultured cells · video analysis · Lucifer Yellow

Introduction

Gap junctions are cell surface membrane specializations which allow direct cell-to-cell exchange of ions and other small substances (Loewenstein, 1981; Peracchia, 1980). The role of these junctions in the coordination of cardiac and some types of smooth muscle contraction is well appreciated. Other roles of junctional "communication" have been suggested, such as in the control of growth and differentiation, in the responsiveness of cell populations to hormones, and in the general integration of cellular activities within most tissues and organs, but these suggestions have remained largely unproven (*see* Sheridan & Atkinson, 1985, for a recent review). Besides questions related to physiological functions of gap junctions, there are also many outstanding questions related to the regulation of both channel diameter and channel number within a single gap junction (Loewenstein, 1981; Bennett & Spray, 1985), as well as the overall junctional area between cells (Sheridan, 1973).

Novikoff hepatoma cells (N1S1-67), isolated originally from a rat solid tumor and adapted to growth in suspension culture (Plagemann & Swim, 1966), have been utilized in our laboratory for many years as a model system in which to study cell-cell communication via gap junctions. We have been particularly interested in correlating junctional permeability with the underlying structure (Johnson & Sheridan, 1971; Sheridan et al., 1978) and in delineating the stages of junction formation between dissociated and reaggregated cells (Johnson et al., 1974; Pederson, Sheridan & Johnson, 1980; Preus et al., 1981*a*,*b*). Whereas, in our earlier work, we reported on the correlation of structural components with ionic junctional conductance, measured electrically, more recently we have developed a method to measure the permeance of the junctions to fluorescent dyes. For small tissue culture cells, a single brief microinjection of dye is a relatively easy and highly successful technique and should result in less cell damage than the three or four microelectrode impalement technique required to measure junctional ionic conductance (Socolar & Loewenstein, 1979).

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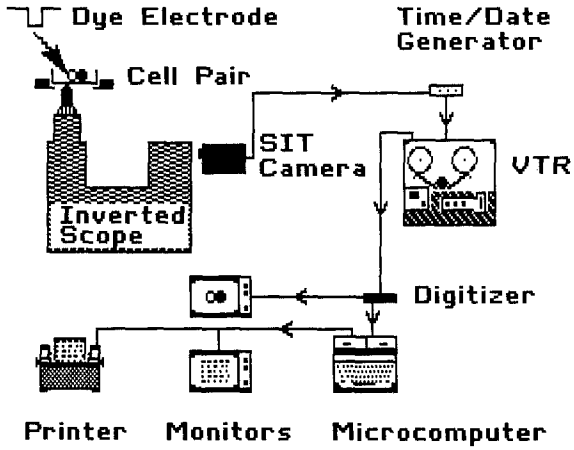

Fig. 1. Diagram of equipment set-up for dye injection experiments. This computer-generated cartoon depicts a Novikoff cell pair on the stage of the inverted microscope just prior to dye injection. The dye is ejected from the microelectrode using negative-going pulses. The dye transfer experiment is videotaped (*VTR*) through a SIT camera and simultaneously observed through a TV monitor. The tape is replayed for analysis using an Apple II-Plus computer equipped with a Digisector board

Some of these findings have been presented earlier in preliminary form (Liu, Kam & Sheridan, 1982; Biegon, Atkinson & Sheridan, 1986).

Materials and Methods

Culture of Cells

As described previously (Johnson et al., 1974; Preus et al., 1981*a*), Novikoff hepatoma cells (N1S1-67) were grown in suspension culture in S210 medium (Gibco) containing 10% calf serum (Biologos) and supplemented with 10 mM glutamine. Culture flasks were tightly capped and shaken at 200 rpm in a shaker incubator (New Brunswick Scientific, New Brunswick, NJ), maintained at 37°C. Cell density was maintained between 5×10^4 and 2×10^6 cells/ml by daily subculture. Doubling time of the cells was ten to twelve hours. For the experiments described below, cells were taken from log-growth cultures (2×10^5 to 8×10^5 cells/ml).

Dye Injections and Video System

Dye injection was carried out with micropipettes fabricated on an Industrial Science Associates puller (Ridgewood, N.Y.) from Omega-dot capillary tubing (Frederick Haer, Brunswick, ME) and filled with a 4% aqueous solution of Lucifer Yellow CH (Sigma) (Stewart, 1978). When filled with 3 M KCl, such electrodes had resistances between 25 and 40 MΩ. Dye was ejected from the pipettes by negative-going current pulses or, more commonly, by briefly overcompensating the electrode capacitance ("ringing the amplifier"), using the negative capacitance of the WPI 701 amplifier (WPI, New Haven, CT). The injections were made under direct visual control on the stage of a Zeiss IM35 microscope equipped with phase-contrast and, for fluorescence, epi-illumination optics with a quartz-iodide source and FITC interference filter assembly (Zeiss, Oberkochen, West Germany). The cells also were viewed through a silicon intensified target (SIT) camera (Model 65, Dage-MTI, Michigan City, IN) and video monitor (Audiotronics, Spring Lake Park, MN). A Panasonic VTR (NV-8030) and RCA time-date generator provided permanent records which were analyzed, as described further below, using a Digisector board (DS-65 Videodigitizer, Microworks, Del Mar, CA) and Apple II Plus microcomputer. A diagram of the set-up is shown (Fig. 1). It should be noted that we chose to use LY because it is a highly fluorescent dye of suitable mol wt (457), which does not cross nonjunctional membranes and which can be visualized conveniently with FITC filters (Stewart, 1978). However, the method is applicable to any fluorescent dye that is a suitable tracer.

Dye Injection Protocol

An aliquot of the log-growth cell suspension (2 to 3 ml) containing single cells, cell pairs, and small clumps of cells was transferred to a 60-mm culture dish. The dish was placed on the microscope stage, and maintained at room temperature (ca. 25°C) and ambient CO_2 for the duration of the experiment (no longer than 90 min). A pair of cells of equal diameter was positioned in the center of the microscope field and the dye-filled micropipette positioned over one of the cells. Dye was ejected as the pipette was lowered. When the cell was penetrated it became rapidly and uniformly fluorescent. The pipette was removed from the cell after 1 to 5 sec of dye injection and moved out of the field of view after which dye ejection was stopped. The injection and subsequent transfer of dye was recorded on the VTR for later analysis. During the time of transfer, usually 2 to 3 min, the exciting light and microscope focus were not changed and both the SIT camera and videorecorder were operated in manual mode, thus keeping the gain constant irrespective of fluorescence intensity.

Digitizing Fluorescence Intensity

The intensity of cellular fluorescence was digitized using the Apple II Plus microcomputer equipped with a Digisector board (Microworks, Del Mar, CA). This board and supporting software converts the analog electrical signal from the video recorder (or TV camera directly) into digital values corresponding to a grey-scale, ranging from 0 to 63 relative integer units. The program directs the computer to take the average of 20 readings from each of three coordinates on the monitor screen, in practice over the center of the injected cell and the recipient cell and over a cell-free adjacent region of the culture dish (designated as background). These three points are preset for each cell pair by positioning a software-generated cursor over the appropriate area and fixing the *x-y* coordinates. The samples are taken in the sequence—injected, recipient, background—over a time span less than 3 sec.

In order to carry out the analysis it was necessary to ensure that the dye LY neither was bleached nor leaked out of the Novikoff cell and that the digitized values were linearly related to fluorescence intensity. Therefore, we injected single, isolated Novikoff cells with LY and determined the digitized intensities as a function of time and of emitted (fluorescence) light intensities, which we varied directly by placing calibrated neutral den-

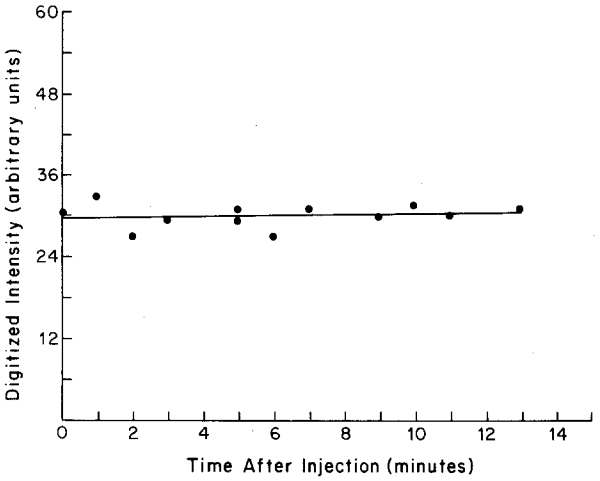

Fig. 2. Retention of LY in a single Novikoff cell. This figure is a graph of the digitized dye intensity values of LY over the center of a single Novikoff cell during the first 15 min after microinjection. The value is quite steady, indicating the lack of fading and/or loss of LY from the cell across nonjunctional cell membrane over a time period greatly exceeding the period usually required for re-equilibration of dye between two coupled cells

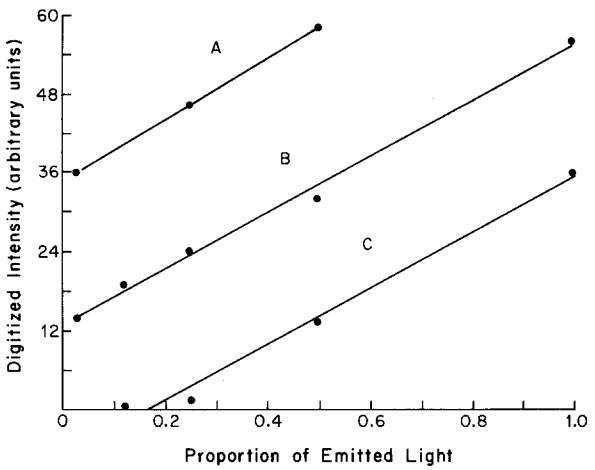

Fig. 3. Linearity of recording system. The data points generated for this figure were obtained by microinjecting LY into a single Novikoff cell and then reducing the emitted fluorescent light intensity with neutral density filters. The three separate lines represent three different gain settings of the SIT camera and the VTR. When "background" is properly adjusted to read just above zero, the full range of digital values, 0 to 63, is available to monitor the dye transferred (as in line *B*). Routinely, this adjustment is made on the VTR (video gain) with the SIT camera at maximum gain

sity filters in the light path. The results indicate that there is no fading of fluorescence over times greatly exceeding the dye transfer times (Fig. 2). Thus, neither bleaching nor dye leakage are significant under our experimental conditions. The experiments with neutral density filters indicate that the digitized values are linearly related to fluorescence over most of the grey-scale range (Fig. 3). Problems occur only when the fluorescent intensity exceeds the saturation level for the system (i.e., giving digital values near 63). Otherwise, when the background is arbitrarily set just above zero by adjusting the gain of the VTR, the digital values 0 to 63 are proportional to fluorescence intensity as indicated indirectly by the close fit of the experimental data to the theoretical relationship described below [Eq. (1)] (subsequent studies have confirmed this relationship directly; M.M. Atkinson, *data not shown*).

Calculation of Junctional Permeance (*PA*)

Provided fluorescence intensity is proportional to dye concentration and the two cells have equal volume, V, the passive diffusion of dye from the injected cell to the recipient cell can be expressed mathematically as follows:

$$\ln (I_1 - I_2) = \frac{-2PA}{V} t + \ln I_o \qquad (1)$$

where I_1 and I_2 are the digitized intensity values for cell 1 (injected) and cell 2 (recipient), respectively, I_o is the initial intensity of fluorescence in cell 1, i.e., at $t = 0$, P is the permeability coefficient for the dye moving through the junction and A is the area across which the permeation of dye is occurring (Atkinson & Sheridan, 1985). The computer takes the sets of intensity values and corresponding times and solves Eq. (1) by least squares analysis to give the slope, $-2PA/V$, of the linear relation between $\ln(I_1 - I_2)$ and t, The value for V is estimated from the diameters measured on the video monitor (the cells in any pair typically had equal diameters within ± 0.5 mm on the screen; the effective magnification on the screen was set for 1000× and therefore 0.5 mm = 0.5 μm and was ca. 5% or less of the diameter). *PA* is then calculated from the slope and the estimated volume, *V*.

Results

Video Analysis of Dye Transfer in Pairs of Cells

In confirmation of earlier reports from this lab (Johnson & Sheridan, 1971), isolated pairs of Novikoff hepatoma cells in culture media are interconnected by permeable junctions; these junctions allow LY injected into one cell to transfer to the adjacent cell. Redistribution of dye is typically complete within 2 to 3 min. In many cases, dye re-equilibrates in 60 sec (Fig. 4). Although there is a wide range in dye permeance values (*see below*), very rapid, almost immediate (within 10 sec) equilibration is rarely seen, and this result probably indicates that only a small percentage of cells in pairs are still connected by cytoplasmic bridges. At the other extreme, less than 10% of the cell pairs fail to show detectable transfer by 3 min, a result similar to that reported earlier for electrical coupling in these cultures (Sheridan et al., 1978).

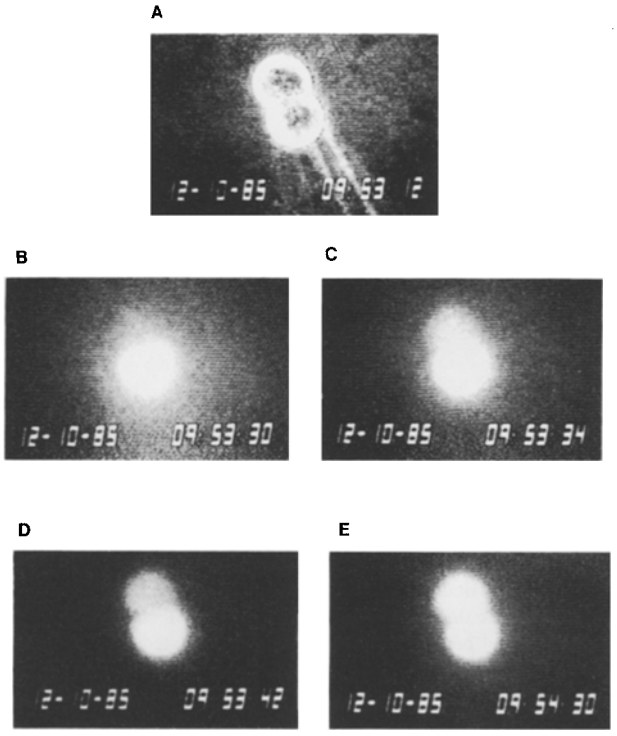

Fig. 4. Illustration of dye transfer experiment. A series of single frames from a videotape showing a LY dye transfer experiment. *A* shows the cell pair (cell diam = 23 μm) prior to dye injection. The shadow of the electrode is visible above the cell to be injected. (*B–E*) The fluorescent images of the two cells at 0, 3, 12, and 60 sec, respectively, after the end of the microinjection. The microelectrode was removed from the injected cell at $t = 0$. *Note*: The photographs do not give an adequate impression of the visible decrease in fluorescence in the injected cell during the transfer

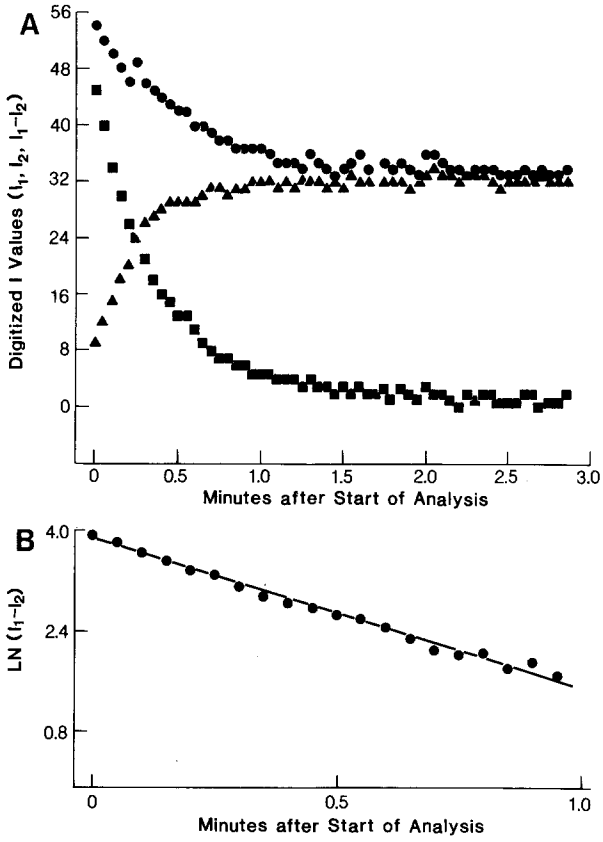

Fig. 5. Analysis of dye transfer data. (*A*) A graphical representation of the LY transfer depicted in Fig. 4. Data points show the decrease in fluorescent light intensity in the injected call (I_1, ●), the increase in intensity in the neighboring, recipient cell (I_2, ▲), and the difference ($I_1 - I_2$, ■) as a function of time after the end of the microinjection. Note that the dye transfer is nearly complete at 1 min after the end of the microinjection. (*B*) The data points from *A*, $I_1 - I_2$, are shown in a ln plot for the first 60 sec of transfer. Note the high correlation of the points to the computer-fit best straight line. The slope of the line is -0.038 sec^{-1}: the corresponding *PA* value is 12.1×10^{-11} cm^3/sec

Videotapes of the dye transfer experiments were replayed and data points (I_1 and I_2) collected at regular intervals (3 or 5 sec typically; occasionally, 10 or 30 sec intervals were used for slower transfers). As indicated in Materials and Methods, pairs of I_1 and I_2 values for each sampling time, t, are utilized by the computer to calculate the slope of the line relating $\ln(I_1 - I_2)$ to time elapsed after the first digitized sample was taken (Fig. 5). A slope value was accepted if the following two criteria were met: (i) The sum of I_1 plus I_2 (provided in the computer analysis) did not change more than 10% during the dye transfer. (A constant sum would indicate that the dye did not leak significantly from either cell and was not appreciably bleached during the continuous illumination. Furthermore, a constant sum would indicate that the cells had essentially equal volumes, and that the dye intensity values were indeed measured at points of equal thickness.) (ii) The correlation coefficient of the least squares calculation was greater than 0.92. (This was an arbitrary cut off. In practice, the correlation coefficient was usually greater than 0.97.) Dye permeance (*PA*) for each acceptable experiment was calculated from the slope and the cell volumes, as in Eq. (1) above.

Variability of Dye Permeance in Isolated Cell Pairs

The distribution of dye permeance values within the sample population consisting of isolated pairs of Novikoff cells is quite asymmetrical or, more precisely, is skewed positively (Fig. 6). Thus, *PA* values range from 0.08×10^{-11} to 27×10^{-11} cm^3/sec with the median value of 2.8×10^{-11} and the mean value of 4.5×10^{-11}, being much closer the lower end of the range. The skewed shape of the PA histogram is like that of estimated junctional conduc-

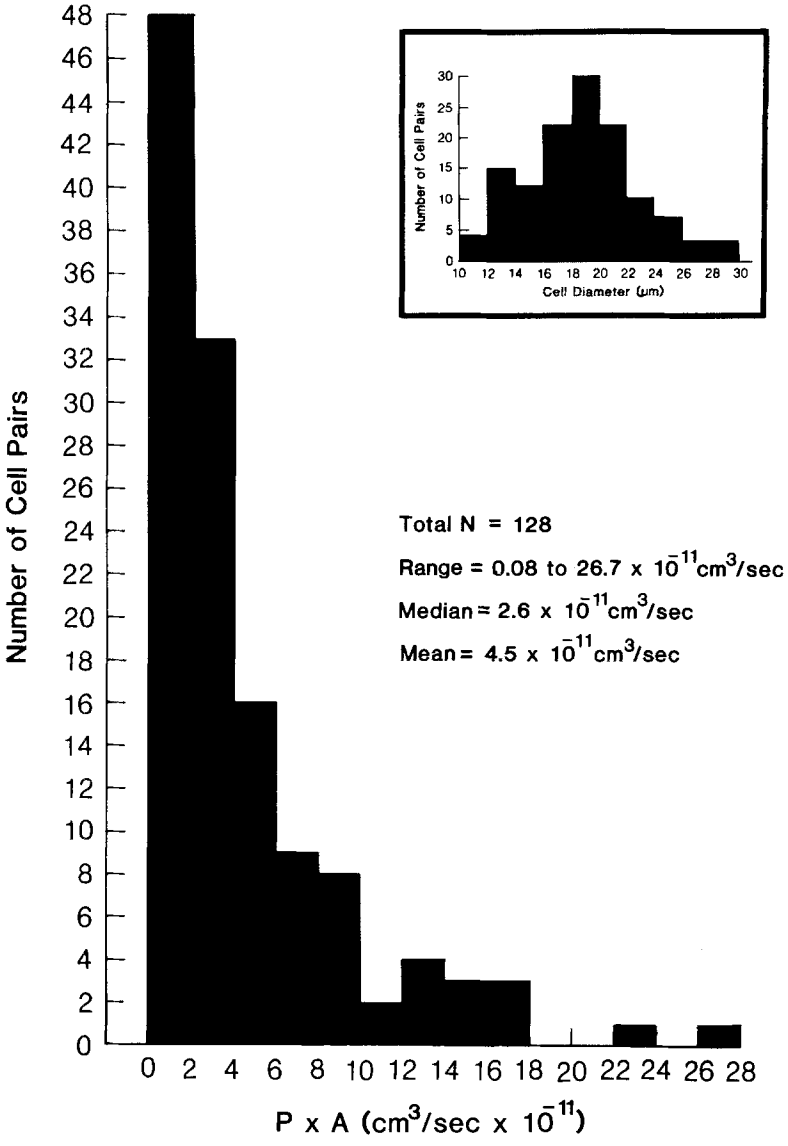

Fig. 6. Histogram of the LY *PA* values. Each determination was made on a separate cell pair as described in Materials and Methods. Note that the distribution of *PA* values is highly skewed toward lower values (only *non-zero* values are included). The insert shows the corresponding histogram of cell diameter values for the cell pairs used in the experiments summarized in the main histogram. The distribution of cell diameters is more normal: the mean diam. is 19.0 μm, identical to the median value

tances and gap junctional areas reported in an earlier study of Novikoff cells (Sheridan et al., 1978). The distribution of cell diameters is normal (Fig. 6, insert).

The wide range of permeance values is probably not a reflection of inherent variability in the measurement procedure itself. In four separate experiments, two PA determinations were made in quick succession in the same cell pair in the following way: first, a relatively small amount of dye was injected and, after dye redistribution was essentially complete (within 5 min), a second injection was made in the same cell. The data from the video sequences of each injection were analyzed, and in each double injection experiment the two *PA* values were quite similar, i.e., within 20% (Table). These results also indicate that repeated injections can be done without causing massive cell damage. More-

Table. Repeated determinations of *PA* on individual Novikoff cell pairs

Set #	Cell diam. (μm)	*PA* ($cm^3/sec \times 10^{-11}$)		Corr. Coeff. of Slope
1	20	1st	4.4	0.97
		2nd	4.0	0.95
2	28	1st	15.0	0.98
		2nd	18.0	0.95
3	19	1st	1.2	0.96
		2nd	1.0	0.93
4	29	1st	2.8	0.95
		2nd	3.3	0.95

This table shows sets of *PA* determinations in which the LY injection was repeated in the same cell pair. For each set, the consecutively determined *PA* values are within ±20% of one another. In two of the sets, *PA* went down in the 2nd trial and in two sets *PA* went up. This data indicates the high reliability of the determinations of *PA* made in this study.

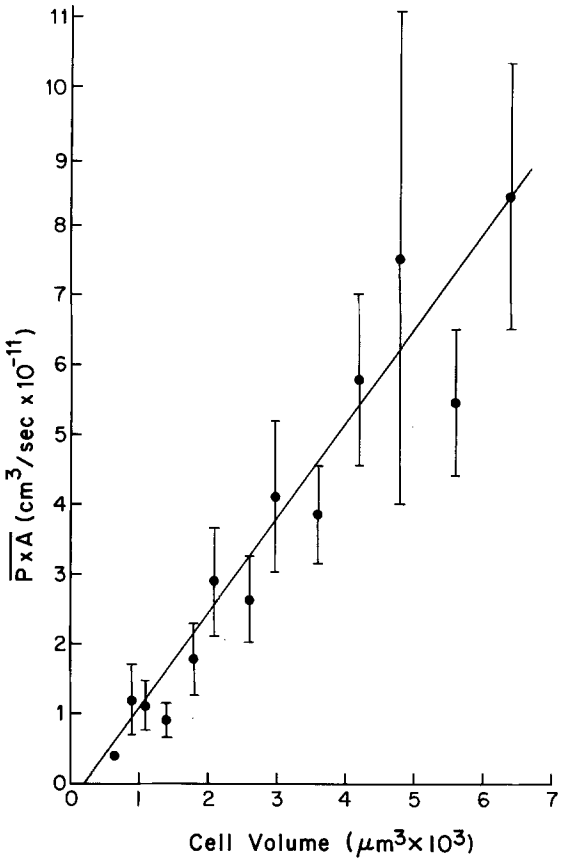

Fig. 7. Linear regression of *PA vs.* cell volume. The mean and standard error of the *PA* determinations are shown for each volume category, for the data on cells below 23 μm diam. Because the diameter measurements were made to the nearest 1 μm (= 1 mm on the screen), and the volumes were calculated from the diameters, the volume categories were not of constant size, but varied from 190 to 831 μm^3. (Total $N = 113$; note that the dot closest to the origin represents *one* data point.) The equation of the regression line is $y = 1.3x - 0.2$, where y is in units of 10^{-11} cm^3/sec and x is in units of 1000 μm^3; the correlation coefficient, $R = 0.42$, is highly significant, $p < 0.001$. The slope value, given the dimensions of y and x, has the following meaning: every 1000 μm^3 volume increase (within the range shown) results in an increase in mean *PA* of 1.3×10^{-11} cm^3/sec

over, if LY binds to the permeable channel, it does not interfere noticeably with subsequent dye transfer, at least within the time frame of these experiments.

Relationship of Dye Permeance and Cell Size

In an earlier review, one of us suggested that the "capability" of cells to carry out "junctional communication" might be determined by the ratio of junctional permeance to cell volume (Sheridan, 1973). Because our cell population showed considerable variation in cell diameter, from 11 to 30 μm, it seemed of interest and feasible to test the relation between permeance and cell volume. (Since the two cells in each pair were the same volume, a single volume term is associated with each *PA* measurement.) Indeed, although the relation shows considerable scatter, there is a positive correlation for cells up to 23 μm in diameter (Fig. 7). With linear regression analysis, a slope of 1.3×10^{-11} cm^3/sec per 1000 μm^3 is obtained with a correlation coefficient = 0.42, which is statistically significant ($p < 0.001$). The y intercept is -0.2×10^{-11} cm^3/sec, which is not significantly different from zero, i.e., the line passes near the origin, suggesting direct proportionality. As an alternate means of testing proportionality, we determined the best curvilinear fit for a large set of log, square, exponential, square root, and cos/sin functions. The best fit was for the equation:

$$\log (PA) = 1.16 \log(V) - 0.48 \qquad R = 0.51$$

or

$$PA = 0.61 V^{1.16}$$

which suggests nearly a direct proportionality. There were 15 measurements of *PA* made on cell pairs in which the cell diameters exceeded 23 μm (24 to 30 μm). The average *PA* values in this group did not continue to increase, but rather reached a plateau at the maximum average value for cells of 23 μm diameter (*not shown*).

Discussion

Numerous studies have used fluorescent dyes to evaluate the distribution and sieving characteristics of permeable junctions in a wide variety of cell systems (Loewenstein, 1981). In most of these studies, however, the measures of junctional patency have been qualitative, simply indicating the number of instances of dye transfer, or the presence of "dye coupling." Some quantitative measures have been reported, e.g., "transit time" (Simpson, Rose & Loewenstein, 1977) or "transfer time interval" (Atkinson et al., 1981), which are related indirectly to junctional permeance, but in only a few cases have there been attempts to estimate junctional transfer kinetics either for a population of cells within an intact tissue (Pollack, 1976; Safranyos & Caveney, 1985) or for single cell pairs (Atkinson & Sheridan, 1985; Zimmerman & Rose, 1985). In this

paper, we have used a method developed in our laboratory (Liu et al., 1982; Atkinson & Sheridan, 1985; Biegon et al., 1986) to study the junctional transfer of LY between cultured Novikoff hepatoma cells. Our method utilizes a computer-assisted video system that provides a digital output linearly related to intensity of cell fluorescence (Figs. 2, 3 and 5) and that exploits Fick's first law applied to loss-free transfer of tracer in an isolated pair of cells. The utility of the substitution of fluorescent dye intensity, which may be detected quite readily and reliably for dye concentration in Fick's equation, has been remarked previously (Socolar & Loewenstein, 1979, section 4.3.1, pp. 154–156).

In the present study, permeances have been calculated from the transfer kinetics; this calculation is possible because volume is easily estimated in spherical cells. Moreover, by selecting cell pairs for which $(I_1 + I_2)$ remains constant and $I_1 = I_2$ at steady state (Figs. 4 and 5), we have avoided any complications from binding of the dye to cytoplasmic elements or digitizing over regions of unequal thickness [nevertheless, such deviations from ideality can be handled mathematically, as will be discussed in a subsequent publication (M.M. Atkinson, *in preparation*).

Permeance, PA, contains two terms, permeability coefficient, P, and junctional area, A. Consequently, variations of permeance in the cell population can, in principle, be related to variations in either or both terms. Variations in P can only come from variations in mean channel diffusion coefficient, D, which would occur if there are variations in interactions between the tracer and the channel walls. Variations in A can arise from variations in the numbers of open junctional channels or in the mean diameter of the channels. We cannot directly distinguish these possibilities. We can, however, estimate the average diffusion coefficient needed to relate the average permeance value to measured junctional area, expressed as particles/interface, assuming that all channels are open and have a fixed diameter. We have previously reported a mean of 2700 ± 540 junctional particles per interface in Novikoff cell cultures (Preus et al., 1981*b*). Although this value represents junctions between cells in chains and clumps as well as pairs, there is no reason to believe that the value for pairs alone is substantially different. Using this value and an estimated channel diameter of 1.6 nm (Schwarzmann et al., 1981), an open channel area of 5.4×10^{-11} cm^2 per interface is obtained. Because the distribution of particles/interface was skewed and resembled that for our permeance values, to a first approximation, the permeability coefficient, P, is given by the ratio of mean PA to mean junctional area: i.e.,

$$4.5 \times 10^{-11}/5.4 \times 10^{-11}\ cm^2 = 0.83\ cm/sec = P.$$

Since $P = D/\text{thickness}$:

$$D = (0.83\ cm/sec) \times (170 \times 10^{-8}\ cm)$$
$$= 1.4 \times 10^{-6}\ cm^2/sec.$$

This value is rather close to that estimated for LY in the cytoplasm of giant axons from the earthworm (2×10^{-6} cm^2/sec; Brink & Ramanan, 1985).

The substantial spread of PA values in the sampled population, more than two orders of magnitude, may result from many factors. Besides intrinsic variability, it is likely that some of the smaller permeances reflect junctions that are newly formed or in the process of formation (Johnson et al., 1974; Pederson et al., 1980; Preus et al., 1981*a,b*). With a 10-hr doubling time and a formation process that may be completed within an hour or less (Johnson et al., 1974; Preus et al., 1981*a,b*), however, most of the junctions are presumably mature and therefore other reasons for the range must be sought. It has been suggested that cells utilizing permeable junctions for "nonelectrical communication" might achieve a more stable junctional capability by maintaining a relatively constant ratio between junctional permeance (described in terms of overall junctional area, although varying the proportion of open channels at constant area would suffice) and cell volume (Sheridan, 1973). As indicated by Eq. (1) above (Materials and Methods), with a constant permeance/volume ratio, any transferable substance would undergo a constant rate of concentration change for any given concentration difference between the two cells [substituting concentration for intensity in Eq. (1)]. Whereas it was originally argued that the junctional capacities of different cell types might depend on their respective permeance/volume ratios, it is interesting to ask if a single cell line shows any tendency to increase its permeance in proportion to increased cell volume. Previous attempts to test this idea with Novikoff cells were equivocal because the junctional conductances were measured on cells with a narrow range of volumes (Sheridan et al., 1978).

In the present study, with a wider range of cell volumes, we have found a significant correlation between PA and cell volume as determined by two separate approaches. In the first approach, the data for cells less than 23 μm in diameter were fitted to the best straight line, yielding a correlation coefficient of $r = 0.42$ which was highly significant ($p < 0.001$). Because the y intercept (i.e., PA value at zero volume) was very near zero, direct proportion-

ality was indicated. In the second approach, a more direct test of proportionality was made by fitting a double log function to the data. The best fitting curve had the following parameters:

$$PA = 0.61V^{1.16}$$

which again indicated nearly a proportional relation. (Strict proportionality would give an exponent of 1. The deviation from the ideal might simply reflect the scatter of the data.)

The proportionality between mean *PA* and volume essentially excludes a simpler explanation of an increasing junctional permeance with increasing cell size. Because the effective contact area should increase in direct proportion to surface area (*SA*), if the density of junctional channel precursors were independent of cell size, the number of available precursors for any interface would be proportional to *SA*. Thus, one would predict a proportionality between *SA* and *PA*. However, as expected from the *PA vs. V* relation, *SA* and *PA* are far from proportional:

$$PA = 2.18(SA)^{1.73}.$$

Thus, it appears that regulation of junctional permeance in relation to cell volume is more complex. It might be, for example, that the number of open junctional channels is determined by a cytoplasmic molecule that is synthesized in proportion to cell volume. The effect of this regulatory molecule could be to change either the number of junctional channels or the proportion of them which are open.

Although the correlation between cell size (volume or surface area) and *PA* explains, in part, the range of the *PA* distribution, other factors must contribute to the variability. One possible factor is the stage of the cell cycle. In the Novikoff cultures the cells appear to have a range of sizes just after cell division. Thus, two cell pairs, each with similar cell sizes, are not necessarily at the same stage of the cell cycle. This conclusion is obvious if we simply note that the cells have diameters ranging from 12 to over 24 μm, producing more than an eightfold range of volumes. At cell division, there is only a halving of volume, reflecting a decrease of only about 79% in radius. Therefore, cells 12 μm in diameter just after division arose from a cell only about 15 μm in diameter, while cells 24 μm in diameter just before division will give rise to cells about 19 μm in diameter. Thus, even if there were a simple relationship between junctional permeance increase and cell volume increase for a cell pair (presumably daughter cells) progressing through the cell cycle, the relationship might vary with cells of different initial sizes, leading to the observed spread in permeance *vs.* volumes.

Another factor may simply be the probabilistic nature of junction formation when the average junctional size is relatively small. In all of our various analyses of junction formation between Novikoff cells, determined with either ultrastructural or physiological methods, a similar, skewed distribution of total junctional areas per interface or conductances has been seen (Sheridan et al., 1978; Preus et al., 1981*a*,*b*). Such distributions would be expected if individual junctions had a relatively normal distribution of areas and the probability of forming a junction were low. In such a case, the distribution of total junctional areas would be mainly influenced by the number of individual junctions per interface and therefore would always be skewed. (In fact, with very low formation probability, it would be nearly Poisson in form.)

If the relationship between cell volume and junctional permeance is a property of the cell and its position in the cell cycle, we might consider what relationship ought to occur during junction formation between reaggregated Novikoff cells. Undissociated cell pairs in our cultures are probably comprised of daughter cells, and thus both cells in each pair are likely to be at similar points of the cycle. During reaggregation, however, cells in different stages of the cell cycle would be randomly associated, and each cell in a newly-formed pair could have a different effect on the junctions formed between them. Thus, even with pairs of cells of equal volumes we might find an even greater spread in the relationship between permeance and volume. Moreover, if we were to examine pairs of cells of different size, a dependence on contact area (a fraction of total surface area of cells) would be further indicated if *PA* were more closely related to (limited by) the size of the smaller cell, whereas a closer relationship to the volume of the larger of the cells might imply the presence of a soluble (promoting) factor, a factor which in addition might be transferred from cell to cell through the forming junction. Novikoff hepatoma cells rapidly form new junctions during reaggregation and so would be an excellent system in which to study the effects of cell size, as well as other factors, on junction formation.

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