and D. Thus, in the absence of a physical contour, the stereoscopic sense imposes one.

Further, the self-luminous displaced plane appears slightly lighter (sometimes darker) than the identical self-luminous ground (*6*). In the heteronymous view, on the other hand, where the displaced plane comes forward, as in Fig. 2*D*, it now appears slightly darker (sometimes lighter) than the ground. These brightness effects may appear even before the stereoscopic edge. They are delicate, but they are reliably present to a trained eye.

Although there is no target in the center of s, it is nevertheless possible to fixate the center of the inner field reasonably well. Now a further increase of s will cause the stereoscopic edges to disappear and the floor of the window to break up into two separate parts composed of the no-longer-unified upper and lower disparate rows. The stereoscopic displacement of these rows is still perceived, however, out of the corner of the eye (best in the inferior field, in my experience), until the angles given in columns 3 and 5 ("stereopsis") of Table 1 are reached. Whatever else this may mean, it can at least be asserted that stereoscopic experience can occur in the total absence of physical contours at the central fovea.

By particularly steady fixation, one can induce a temporary local-adaptation in both eyes, a perceptual fading known as the Troxler effect. Or, using Kaufman's approach (*3*), one can induce a foveal suppression or rivalry by placing a letter (say an *e* or an *L*) at the center of s for the right eye and another rival letter (say an *a* or an *F*) at the distance δ to the left of the center of s for the left eye. In the present experiment, these two methods bring us as close as we can come to whatever is meant in clinical ophthalmology by "suppression" or "rivalry." Under the first condition, the stereopsis will fade, unless one is permitted at least some very slight sideways oscillation of fixation; but under the second condition the peripheral stereopsis remains, provided that neither is s too large nor δ too small. Thus there appears to be a difference between the effect of Troxler adaptation and the effect of "suppression." At the present time we can only conjecture what this means physiologically.

Current theories of stereoscopy hypostatize three neurophysiological events, reified under the names of "rivalry" and/or "inhibitory suppression" and/or "facilitative fusion," to account for three associated and variously distinct perceptual events. However, these neurotheoretical concepts are based upon experience with targets having completed physical contours. What can be said in the present instance where we have stereopsis in the absence of contours? There is rivalry at δ since it is monocular, and the ⊞'s of Fig. 2 float about in a disconcerting fashion. But within the empty visual field itself, there is no consistent iridescence (but see *6*), fragmentation, or fading. On the other hand, it is hard to decide what, if anything, could have been "fusing," especially in the *black* empty visual field. The fact that the displaced self-illuminous plane appeared slightly brighter (facilitation?) than the ground in homonymous view is offset by the fact that it appeared slightly darker (inhibition?) than the ground in heteronymous view. Are these effects related to the Fechner paradox or to the Hermann illusion? Perhaps the stereoscopic edge is a true Gestalt organization (specifically not derived from monocular form recognition) in which formal or probability "fusion" and/or "rivalry" and/or "suppression" and/or "differencing" occur as contingencies. A theory of this nature seems to be the only kind that could be consistent with the present finding of visual contours in homogeneous space. And, unlike these others, it could also be consistent with the concept of correspondence in the single eye (*7*).

Finally, and perhaps most important, only a Gestalt theory could account for the beautiful sharpness of this stereoscopic edge, as noted by all experimenters, despite the use of physically blurred and out-of-focus targets. This sharpness is especially apparent to the user of free-viewing stereoscopy because some accommodative blur almost invariably appears for him as an unwelcome rider on his method of observation.

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3. L. Kaufman, *Amer. J. Physiol.* **77**, 193 (1964); *ibid.* **77**, 393 (1964).
4. This experiment is visually quite striking and I recommend that the reader try it for himself, although it will take some practice.
5. W. Gogel, *J. Psychol.* **50**, 119 (1960); *ibid.*, p. 179; *ibid.*, p. 257.
6. The brightness of the dark displaced plane is particularly difficult to specify; sometimes it is iridescent, suggesting a rivalry. Similar iridescence occurs with Julesz's fig. 36 (*1*, p. 1156), which is inconsistent with his statement (*1*, p. 1159) that ". . . as long as depth is seen, no rivalry can be present." Iridescence is one form of rivalry. See especially Kaufman's fig. 5 (*3*, p. 399), which is conclusive on this point.
7. G. L. Walls, *Vision Res.* **2**, 69 (1962).
8. I am grateful to Susan Smith for her assistance in this work.

28 June 1965

Constitution, Viability, and Lactate Dehydrogenase in Stationary-Phase L-Cell Suspension Cultures

Abstract. *Starved suspension cultures of L cells exhibit a plateau phase of short duration followed by loss of key cellular constituents and rapidly decreasing viability. In contrast, regularly fed, undiluted cultures remain stationary at a high cell density for prolonged periods without structural alterations or loss of viability. The L cells contain a single lactate dehydrogenase isozyme with an electrophoretic mobility similar to that of lactate-dehydrogenase-5. High-density stationary cultures show a tenfold increase of the specific activity of this enzyme and a recurrent biphasic pattern of carbohydrate utilization with, first, production and, later, consumption of lactate.*

It has been suggested that the study of the transition of a bacterial or mammalian cell culture from logarithmic growth to the plateau phase might yield information relevant to the regulation of growth in complex organisms (*1*). Plateau-phase cells, however, have been known to undergo structural alterations and loss of viability (*2*), and therefore comparisons with nongrowing tissues *in situ* are open to criticism (*3*). We are now reporting that, when the plateau phase is induced by starvation in suspension cultures of L cells, there is a rapid loss of key cellular constituents and a progressive decrease of viability. These changes are absent when the plateau is the result of increased cell density in regularly fed undiluted cultures. In addition, such undiluted

cultures exhibit an apparent adaptive response from one of the enzyme systems known to be under epigenetic control. These findings suggest that even in the case of a long-established heteroploid cell line, growth control may be present under certain conditions.

All experiments were performed with L-929 cells, clone WRL10A (*4*), grown as stock suspension cultures in Eagle's spinner minimum essential medium (sMEM), supplemented with 10 percent of horse serum. These cultures were maintained in logarithmic growth by periodic medium renewal and dilution so that the population was maintained between 3 and 10 $\times$ 10^5 cells per milliliter. Doubling time was 22 to 28 hours and plating efficiency, determined on trypsinized single-cell suspensions to avoid any errors due to clumping, 50 to 60 percent.

Experimental cultures were set up in the sMEM medium. Initial total suspension volume and population density were 200 ml and 3.5 to 4.5 $\times$ 10^5 cells per milliliter, respectively; temperature of incubation was 35°C and the *p*H was controlled with 5 percent CO_2, either in a commercial incubator or in the incubation chamber described (*5*). In the first series of experiments, the cultures were grown for the indicated periods without medium renewal; in the second, the medium was renewed daily, without dilution of the cell population, by centrifugation of the entire culture at 1000 rev/min for 25 minutes and resuspending the cells in fresh medium. In order to obtain proper dispersion of the cells, sampling began 2 hours after the cultures were set up or the medium was renewed and continued at desired intervals. Cells were counted with a hemocytometer. In case more than two clumps were seen in any field or more than five cells were present in any clump, the counts were repeated on a suspension of single cells that had been obtained by treatment with trypsin. Relative viability was evaluated from the ratio of the plating efficiency of the trypsin-treated suspensions from experimental cultures to the plating efficiency of suspensions from stock cultures in the logarithmic phase of growth. Cell size was determined electronically with the Coulter counter equipped with an aperture tube (100 μ in diameter) and calibrated with pollen grains of known size. DNA, total cellular RNA, and total protein were determined by the methods of Scott, Fraccastoro, and Taft and of Oyama and Eagle (*6*). The *p*H of the medium was measured anaerobically and the concentrations of glucose and lactate in the medium were determined by the glucose oxidase and the Barker methods, respectively (*7*). Oxygen dissolved in the medium was measured with a blood-gas chromatograph apparatus after replacing its original cuvette with a modified Van Slyke chamber (*8*). Lactate dehydrogenase (LDH) activity and soluble protein were determined (*9*) both in the medium and in the 10,000*g* supernatant of washed cells that had been broken up by high-frequency sound, and the results were expressed as LDH specific activity in Wrobleski-La Due units per microgram of protein (*10*). The LDH isozymes were separated by the agar-gel electrophoretic method of Wieme (*11*); for the development of the isozyme zones, the incubation mixture described by Van der Helm *et al.* was used (*12*) with the exception that *p*-iodonitrotetrazolium was used instead of nitro blue tetrazolium and the medium was adjusted to a final *p*H 7.8.

Cultures with initial densities of 3.5 to 4.5 $\times$ 10^5 cells per milliliter, grown

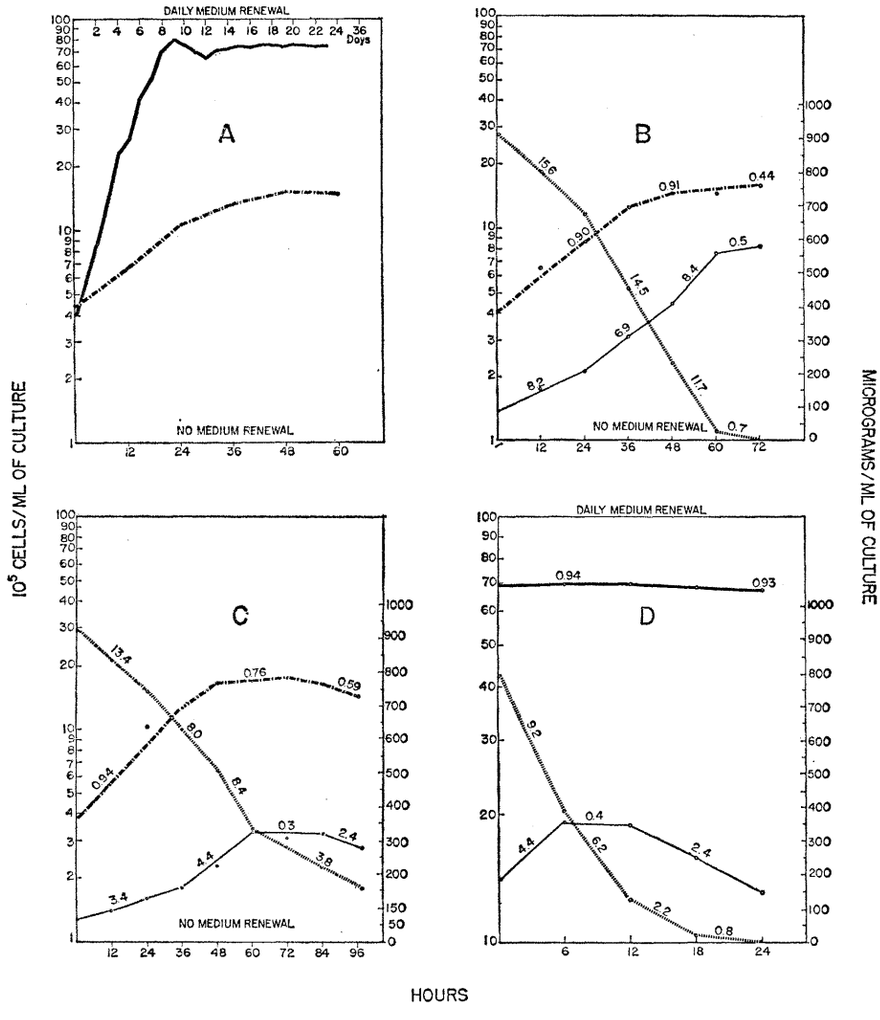

Fig. 1. Cell population kinetics: (*A*) in cultures with (boldface solid line) and without (dot and dash line) renewal of medium; (*B*) concentration of glucose (dotted line) and lactate (thin solid line) in cultures with a high glucose utilization rate; (*C*) same as *B*, except in cultures with a low glucose utilization rate; and (*D*) same as *B*, except during a typical interval between successive renewals of medium in stationary cultures of high cell density. The curves in *A* represent typical single experiments; in *B*, *C*, and *D* averages of three or more replicate experiments. In order to obtain proper dispersion of the cells the 0 time sample in *A*, *B*, and *C* was obtained 2 hours after setting up of the cultures, and in *D* 2 hours after renewal of the medium. The numbers accompanying the cell population curves indicate relative viabilities; those accompanying the glucose and lactate curves, average rates of utilization or production over the corresponding curve segments in 10^{-6} μg per cell per hour.

Table 1. Constitution and LDH activity of cells in the logarithmic phase of growth, in the starvation plateau, and in the stationary phases. Data on logarithmic growth and starvation plateau refer to the population curve shown in Fig. 1*B*; stationary phase data refer to the population curve shown in Fig. 1*D*.

Hours	Cell volume (μ^3/cell)	DNA ($10^{-6}\mu$g/cell)	RNA ($10^{-6}\mu$g/cell)	Protein ($10^{-6}\mu$g/cell)	LDH (unit/μg protein)
		Log growth and starvation plateau			
0	1414	24	41	336	1.04
24	1356	24	34	274	1.14
48	1228	23	38	324	0.92
72	1152	16	31	250	0.79
		Stationary phase			
6	1380	25	37	307	8.36
12	1496	22	36	365	9.23
18	1514	21	30	287	10.5
24	1459	28	33	363	9.93

for 48 hours without medium renewal, reached the plateau phase at densities of 10 to 20 $\times$ 10^5 cells per milliliter, with relative viability and cell density declining rapidly thereafter (Fig. 1*A*, lower curve, 1*B*, 1*C*, cell population curves). The final phase of these cultures, therefore, appears to be a starvation plateau of short duration.

In contrast, when carefully manipulated, cultures with daily medium renewal reached densities of 65 to 75 $\times$ 10^5 cells per milliliter within 2 to 3 weeks (Fig. 1*A*, upper curve). These densities were quite stable; daily fluctuations due to medium renewal and sampling manipulations were less than 5 percent, and there was no decrease in viability (Fig. 1*D* upper curve). These high-density cultures, therefore, represent a stationary phase of long duration.

Frequent measurements of *p*H and O_2 content of the medium showed no significant changes during the progression of the cultures from logarithmic growth to starvation plateau or the stationary phase. Average values throughout the series were *p*H 7.23 and 79 $\times$ 10^{-3} micromoles of oxygen per milliliter of medium.

Rates of glucose utilization and lactate production and utilization in cultures progressing from logarithmic growth to starvation plateau were variable (Fig. 1, *B* and *C*). There was no correlation between glucose-utilization rates and the rates of logarithmic growth or the final cell densities achieved. Also, there was no correlation between the presence or the absence of glucose in the medium and the time of the termination of the plateau phase through loss of viability.

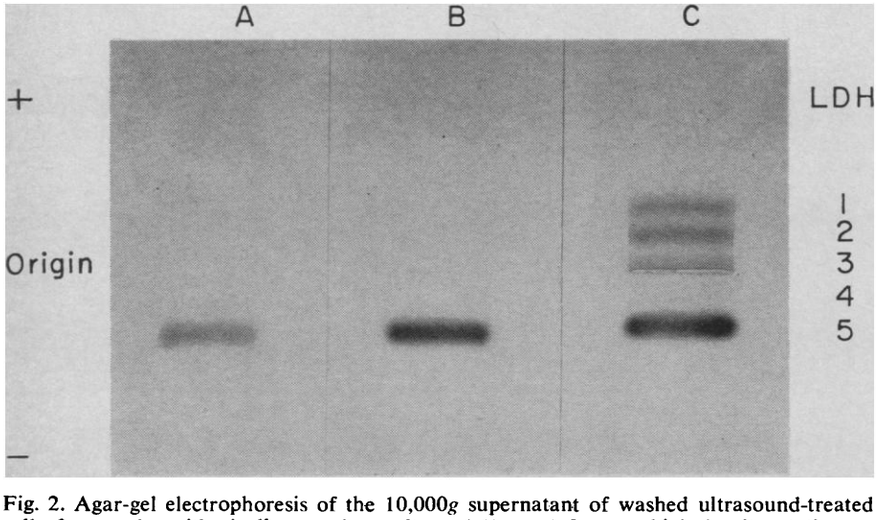

Fig. 2. Agar-gel electrophoresis of the 10,000*g* supernatant of washed ultrasound-treated cells from a logarithmically growing culture (*A*), and from a high-density stationary culture with daily medium renewal (*B*). In each case 10^6 cells suspended in 1 milliliter of Earle's balanced saline were treated with high-frequency sound and centrifuged; then 5 μl of the resulting supernatant was placed into the slot of origin. Normal mouse serum (*C*) is shown for comparison.

Rates of glucose utilization in stationary cultures were also variable and in general lower than during logarithmic growth (Fig. 1*D*). Very low levels and finally depletion of glucose occurred always between 12 and 24 hours after renewal of the medium. During this period of glucose depletion, approximately one half of the lactate, which had accumulated in the medium earlier, was also utilized.

Both the decrease of cell size and that of cellular protein, RNA, and DNA reported by earlier investigators (*2*) were also noted here as cultures progressed from logarithmic growth to starvation plateau (Table 1). Values for cell size, DNA, RNA, and protein in high-density stationary cultures remained within the limits characteristic for logarithmic growth.

Cultures progressing from logarithmic growth to starvation plateau exhibited minor fluctuations of the LDH specific activity (Table 1); but due to variation from experiment to experiment no distinct pattern similar to the one reported by De Luca and Nitowsky (*13*) could be established. In high-density stationary cultures, specific activity of LDH remained constant between changes of media, at a level approximately ten times higher than during the logarithmic phase of growth. This increase of LDH activity was not due to decreased cell leakage, because unused culture medium and media from logarithmically growing, starvation plateau, and stationary cultures had virtually identical activities averaging 40 units per milliliter of medium. Evidence that the observed differences in LDH activity were not due to the accumulation of dissociable activators or inhibitors was provided by the finding that mixing and dilution experiments on supernatants from ultrasound-treated cells of high and low specific activity gave the predicted results.

Agar-gel electrophoresis of the 10,000*g* supernatant of sonicated washed cells from the logarithmic growth, starvation plateau, and stationary phases showed only a single isozyme band migrating negatively with a mobility corresponding to the LDH-5 component of normal mouse serum (Fig. 2). The difference in the staining intensity of the isozymes from logarithmically growing and stationary cells is consistent with the findings concerning differences in total LDH activity (Table 1).

Chromosome counts and karyotype

analysis showed that, on the basis of the rigorous criteria recently developed (*3*), the chromosomal complement of cells undergoing their first division after dilution of a high-density stationary culture was identical with the complement of cells kept in continuous logarithmic growth. This finding, and the fact that a culture with typical logarithmic-phase characteristics is invariably obtained by dilution of a stationary culture, indicate that the possibility of selection is remote and that most likely, logarithmic growth, starvation plateau, and stationary phase represent different functional states of the same cell population.

The plateau phase of cultures without medium renewal appears as a transient state reflecting the decrease of cellular metabolism preceding cell death. Even with glucose present, cell proliferation ceased and relative viability invariably declined below 0.90 between 48 and 72 hours (Fig. 1*C*); thus this decline indicates exhaustion of limiting nutrients other than glucose.

The average cell density of stationary cultures was 7×10^6 cells per milliliter, approximately seven times greater than the geometric-mean of the density of cells grown in culture without medium renewal from 0 to 72 hours. Exhaustion of glucose and other limiting nutrients, resulting in structural changes of the cells and loss of viability, would therefore be expected to occur within 10 hours after medium renewal. The fact that these high-density cultures were maintained for prolonged periods on a daily medium renewal schedule (Fig. 1*A*, upper curve) without changes in cell size, cellular DNA, RNA, protein (Table 1), and viability (Fig. 1*D*, upper curve), indicates that nutrient utilization rates were lower and that adaptive metabolic changes had taken place. Thus, because its availability per cell was lower, glucose was utilized at lower rates in these high-density stationary cultures. This, however, did not prevent the virtual exhaustion of glucose from the medium at 12 to 18 hours, which in turn led to extensive utilization of lactate (Fig. 1*D*). During the maintenance of these cultures, lactate utilization was a recurrent event characteristic of the second 12-hour interval between medium renewals. Figure 1, *B* and *C*, on the other hand, shows that in cultures progressing from the logarithmic to the starvation plateau phase, lactate utilization occurred only as a terminal event concomitant with loss of cell viability. This indicates that in cultures kept in the log phase of growth by periodic medium renewal and dilution, utilization of the accumulated lactate does not occur. The striking increase of the LDH activity in the high-density stationary cultures could therefore represent an adaptive response related to the recurrence of glucose exhaustion and lactate utilization in these cultures.

The finding in L cells of only one isozyme with an electrophoretic mobility similar to LDH-5 and presumably containing a majority of M (muscle)-type subunits is consistent with the shift from H (heart) to M subunit production observed in other tissues explanted in vitro (*14*). On the other hand an adaptive tenfold increase of the synthesis of this isozyme related to increased lactate utilization would seemingly be at variance with the hypothesis that the M enzyme is primarily concerned with production of lactate (*15*). The high-density stationary-cell culture system described seems well suited for further investigation of these questions and of the factors involved in the apparent shift of the emphasis of cellular metabolism from growth to maintenance. This last point is of interest in that it involves suspension cultures of a long-established heteroploid cell line where contact inhibition (*15*) would be absent.

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17 September 1965

Cellular Origin of Hyaluronateprotein in the Human Synovial Membrane

Abstract. *Bright fluorescent staining, which indicates the presence of hyaluronateprotein, was observed in the lining cells of the synovial membrane following application of rabbit antiserum to hyaluronateprotein and a fluorescein-labeled antiserum to rabbit γ-globulin. Staining was shown to be specific and due to antigenic determinants on or closely associated with the protein moiety of hyaluronateprotein.*

The anionic polysaccharides of connective tissue appear to exist as compounds with protein called proteinpolysaccharides (*1*). Most histologic methods to localize proteinpolysaccharides in tissues depend on interaction of a dye with the anionic groups of the polysaccharide (*2*). When the cationic groups of some thiazine dyes, such as toluidine blue, combine with anionic groups of the polysaccharide, the dye undergoes a metachromatic color change from blue to reddish violet. In the Hale method (*3*) a trivalent ferric cation complex becomes fixed to anionic groups of the polysaccharide, and the iron is stained by the Prussian blue technique. More recently, Alcian blue (*2*), a dye with a tetravalent cation, has been used to demonstrate the presence of polyanionic polysaccharides. To identify the polysaccharide, additional steps are needed, such as incubation of the tissue with a stain having a low *p*H (< 2), or the use of alcohol in the dye or in the wash solution to abolish metachromatic staining of hyaluronate (*2*), or removal of hyaluronate from tissue sections before staining by digestion with streptococcal hyaluroni-