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CELL MOVEMENTS IN A CONFLUENT MONOLAYER ARE NOT CAUSED BY GAPS: EVIDENCE FOR DIRECT CONTACT INHIBITION OF OVERLAPPING

L. TIMPE*, E. MARTZ† AND M. S. STEINBERG‡
Department of Biology, Princeton University, Princeton, New Jersey 08540, *U.S.A.*

SUMMARY

According to the hypothesis of contact inhibition of movement, cells in a confluent monolayer are restrained from major overlapping by a directional inhibition of locomotion. This explanation of monolayering proposes that contact between 2 cells locally paralyses the locomotory function, preventing movement in the direction that would lead to overlapping. Consequently, a cell in contact on all sides with neighbouring cells should be immobilized. Yet in strictly monolayered cultures of confluent chick liver or mouse 3T3 cells, we have previously observed both translational cell movements and re-shufflings of relative cell positions. The 'confluence' was not perfect, however, and it seemed possible that the movements observed were due to release from contact inhibition by occasional transitory gaps seen to open up between cells.

In the present study, detailed gap experiences and cell movements were recorded for 31 cells over a total of 1637 cell-hours. There was no significant correlation between frequency of gaps experienced and the extent of cell movement measured as neighbour-exchanges. We conclude that gaps are not a major cause of the movements observed. The hypothesis based on contact inhibition of motion, which attempts to explain monolayering indirectly by imposing a restraint on cell locomotion, cannot explain the substantial cell movements seen in the confluent cell monolayer studied here. To explain contact inhibition of overlapping, the evidence favours a more direct hypothesis which places no restriction on cell movement other than that overlapping be avoided. Such direct avoidance of overlapping could result from differences in the strengths with which cells adhere to one another and to the substratum.

INTRODUCTION

A cell 'monolayer' was defined by Abercrombie & Heaysman (1954) as a 2-dimensional distribution of cells attached to a culture substratum, in which nuclei overlap less than would be expected if their distribution were random. They proposed that monolayers form because of 'contact inhibition', a 'directional prohibition of movement' (not to be confused with a variety of other phenomena later given the same name; reviewed by Martz & Steinberg, 1973). According to this hypothesis, when one cell collides with another it stops; later it may start up again and move off

* Present address: Department of Neurobiology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, U.S.A.
† Present address: Department of Pathology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, U.S.A.
‡ Author to whom correspondence and off-print requests should be sent.

in some other direction. This phenomenon has been observed with cell pairs. The contact inhibition ('of movement') hypothesis as it applies to monolayering extrapolates this behaviour of cell pairs to a whole population of cells in tissue culture, drawing the inference that cells do not form multilayers because they do not move over (or under) their neighbours.

We have previously referred to the tendency of certain cell populations to monolayer as 'contact inhibition of overlapping' in order to distinguish this behaviour from other contact inhibitions (reviewed in Martz & Steinberg, 1973), and to provide an operationally specific, but mechanistically non-committal term.

Possible mechanisms for contact inhibition of overlapping fall into at least 2 classes: (1) there exists an inhibition of the cell's *motility-generating apparatus*, at least toward a contacted cell, so that one cell lacks the locomotory activity required to move bodily over (or under) another; or (2), *overlapping* itself is energetically unfavourable (Martz, Phillips & Steinberg, 1974). In the latter case, if the locomotory apparatus is uncompromised, it would have insufficient power to produce overlapping.

We regard the first hypothesis as *indirect* contact inhibition of overlapping, since it is not overlapping itself which is inhibited, but rather the locomotory apparatus required to produce overlapping. In the second hypothesis, by contrast, the inhibition of overlapping is a direct result of the energetics of overlapping *per se*.

In a series of studies, we have been concerned with determining which of these 2 classes of mechanism is operating. We reasoned that if cell movement in the direction of a contact is prohibited, the relative positions of cells in a confluent monolayer (cells contacted by neighbours on all sides) should be fixed. On the other hand, if it is only overlapping (rather than movement *per se*) that is inhibited, lateral movements of cells within the monolayer would be permitted.

We found substantial evidence that cell movement is not prevented by all-around cell contact. In their original research on the role played by number of neighbour contacts in inhibiting cell movement, Abercrombie & Heaysman (1953) found that the maximum number of neighbours, 6 or more, reduced chick heart fibroblast velocity, but only to between 40 and 50% of its uncontacted value. Martz (1973) found similar values of around 50% for restriction of 3T3 (fibroblast-like) cell speed within a monolayer. In another analysis of the same film, Martz & Steinberg (1974) measured neighbour-exchanges as an indication of large-scale cell movement. In a confluent, stationary density culture, the average cell exchanged 36% of its neighbours during 2·2 days. In the same time an average cell moved $1{\cdot}1 \pm 0{\cdot}65$ mean cell diameters in a random direction.

In another experiment, Steinberg & Garrod (1975) plated chick embryonic limb bud mesenchyme and liver parenchyma cells together at subconfluent densities. The 2 populations sorted out, even after confluence, forming islands of liver cells in a sea of limb-bud mesenchyme. The average island of liver cells increased its population by a factor of 1·59 during a 24-h period after confluence had been reached, while the number of islands decreased by a similar factor during the same time. In other experiments (Garrod & Steinberg, 1975), neighbour-exchanges were observed within

confluent monolayers of liver parenchyma (epithelioid) cells. An average cell exchanged 46% of its neighbours (the sum of neighbour losses and gains) in 6 h. During the same period it moved 7·8 μm relative to the other cells in its island. These observations all indicate that there can be appreciable cell movement within confluent cell monolayers of several kinds.

Observations of cell locomotion in a confluent monolayer at stationary density would eliminate inhibition of cell movement as an explanation for the observed monolayering, except for one possibility (Abercrombie, personal communication; Goldman, 1973). Sometimes gaps appear between cells in a monolayer (Martz & Steinberg, 1974). Such gaps could release inhibition of movement locally. Though the gaps might appear to be small and brief, they might nevertheless be sufficient to cause the amount of cell movement observed. If a cell's inhibition were released by a gap, it might move toward that gap and fill it – perhaps similarly mobilizing other cells in its wake, in a chain reaction. In this manner, one primary gap could affect several cells in sequence.

Our purpose here is to decide whether or not gaps are a sufficient cause for the observed cell movements. We have collected data on the numbers of neighbours exchanged by certain cells in a 'confluent' monolayer at stationary density, and on the numbers of gaps experienced during that time by these same cells. The linear correlation between the 2 sets of data has been examined. A positive correlation would not prove that gaps cause neighbour-exchanges, although that would be one possibility. A lack of correlation, however, would constitute strong evidence that the gaps are not adequate releasers of contact inhibition. In that case, an explanation other than direct contact inhibition of movement would have to be found for the avoidance of overlapping by the cells.

MATERIALS AND METHODS

We have used neighbour-exchanges rather than velocities to quantitate cell movements. Cell velocity, as measured by displacement across the substratum, may include a large component due to the motion of the cell group (Martz & Steinberg, 1974). Individual cells within such a group could be contact inhibited by their neighbours, yet have substantial velocities. Neighbour-exchanges, on the other hand, reflect the movements of each cell with respect to those other cells immediately surrounding it.

Data were obtained from the same film (no. 18) used in the neighbour-exchange analysis of Martz & Steinberg (1974). This film, photographed using Nomarski optics and a 10 × objective, shows the growth to confluence of a constantly perfused culture of 3T3 mouse fibroblast-like cells (Todaro & Green, 1963). Exposures were made at 150-s intervals from the beginning of culture through exponential growth and confluency until stationary density was reached. At that point, the interval between exposures was extended to 240 s. The culture medium was Dulbecco-Vogt's modification of Eagle's basal medium plus 3·3% foetal calf serum. The film spanned 7·6 days, during the last 2·5 of which the monolayer was at stationary density (Fig. 1). More details of the experiment and filming are in Martz & Steinberg (1972).

For the neighbour-exchange and gap analyses, 2 intervals of the film were chosen (Fig. 1). Each interval lasted 2·2 days. The pre-stationary density interval ('Interval I') extends from the beginning of confluence – the first time at which no substratum in the field of view is uncovered by cells – to the achievement of the stationary cell density. The stationary density interval ('Interval II') begins at the end of Interval I and continues to the end of the film.

Since cytoplasmic boundaries were often indistinct, the positions of cell nuclei were studied. Tracings of nuclei were made from frames at the beginning and end of each interval. An objective protocol was designed to decide which cell nuclei were *neighbours*, which were *non-neighbours*, and which had a relationship *intermediate* between neighbour and non-neighbour (Martz & Steinberg, 1974). Cells were chosen for neighbour analysis if they satisfied 2 conditions: (1) they were far enough from the edge of the frame that all neighbour relationships

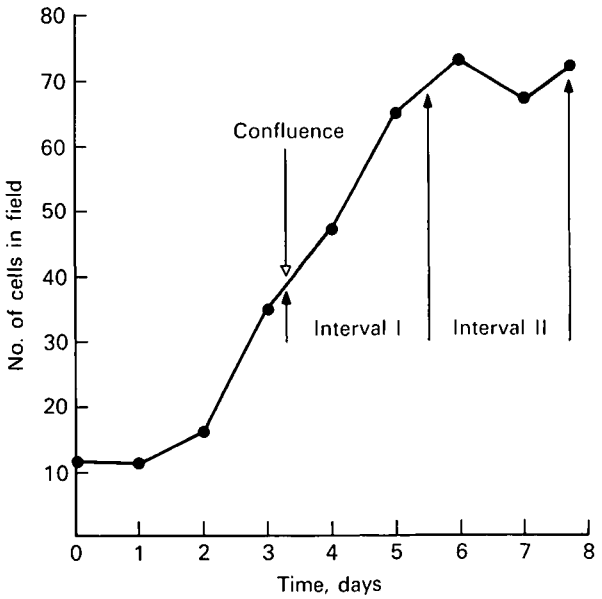

Fig. 1. No. of 3T3 cells in a fixed photographic field during 7·6 days of continuous filming, starting with a sparse culture. (The initial lag in cell number is due to departures of cells from the field; there was no lag in the mitotic rate (Martz & Steinberg, 1972).) Two intervals were chosen for gap/cell movement analysis. Interval I extends from the time of initial confluence to the time of achievement of the stationary density. Interval II extends from the end of Interval I to the end of the film. No nuclear overlapping was observed at any time throughout the film. [Film 18; perfused culture; Nomarsky optics; 10 × objective lens (see Martz & Steinberg, 1972 for technical details).]

could be assigned, and (2) they were in the field of view for the entire interval. This left a total of 10 nuclei in Interval I and 21 in Interval II. The total number of nuclei which changed from the *neighbour* to *non-neighbour* category during an interval was added to the number which changed from *non-neighbour* to *neighbour*. This sum, divided by 2 because each exchange involves 2 neighbours, was the value of 'neighbours-exchanged'. Entrances into and departures from the *intermediate* category were not recorded as neighbour-exchanges. More details about this process are in Martz & Steinberg (1974).

Gaps come from various sources. Some are the result of mitosis; when a cell gathers up its cytoplasm before dividing it leaves a gap. Others are caused by the detachment of dead cells from the substratum. The remainder have unknown causes.

It was not always apparent to us which shufflings of the cytoplasm should be considered as gaps. Large, clearcut gaps were observed, in which an area of substratum was apparently free of cytoplasm for intervals from 5 min to an hour or more. There were also small thinnings of cytoplasm which never appeared to reveal bare substratum and which lasted only 5 to 10 min. Between these extremes were 'gaps' of every size, morphology and duration. Since we were examining the hypothesis that gaps influence the amount of cell motion in a confluent monolayer, we wished to include in our data any event that might be construed as a gap so that

no possible correlation between gaps and motion would be overlooked. Yet the possibility existed that too much 'noise' in the form of miscellaneous small events might obscure a real correlation between the clearcut gaps and cell movement. To examine both possibilities, we put each gap into one of 4 classes – each class distinguished by the size, duration and structure of its gaps. The data could then be analysed class by class or all together.

Each cell from the population that was neighbour-analysed was also gap-analysed. Every gap that occurred near a cell in a particular interval was noted, and the following descriptive information was recorded: (1) when the gap opened and closed; (2) whether or not it was caused directly by a mitosis; (3) an estimate of the percent of the perimeter of the cell observed that was exposed to the gap; and (4) a classification of the gap. Each gap was classified as belonging to one of 4 categories explained in Table 1.

These data were obtained from the film by the use of an analytical projector (L-W Photo, Inc., Van Nuys, California, Model 224-A).

Much of the numerical work was done with the help of the Princeton University Computer Center. A statistical package named SNAPIEDA was used to make scatter-plots and to calculate coefficients of correlation.

Table 1. *Gap class definitions*

Gap class	Minimum duration	Size relative to a small* cell	Description
A	20	≥	Substratum apparently clear of cytoplasmic extensions for some part of the gap's life
B	20	≥	Substratum never entirely clear of thin, cytoplasmic extensions
C	10	<	May or may not have thin cytoplasmic extensions over the substratum
D	Not specified	Not specified	Events which are questionably gaps: odd shapes and sizes; no substratum visible

* A small cell was about 50 μm in diameter.

RESULTS

Sixty-eight gaps were recorded in Interval I. The mean number of gaps per cell was 6·8; the maximum was 14 and the minimum was 1. For interval II, 29 gaps were observed, with a mean value of 1·38 gaps per cell. The maximum number of gaps per cell was 4, and the minimum was zero.

In Interval I, a total of 14 neighbour-exchanges were observed for the 10 cells. The mean number per cell was 2·8, the maximum was 6 and the minimum was 1. In Interval II, a total of 20·5 neighbour-exchanges were observed adjacent to 21 cells. The mean number per cell was 1·95, the maximum was 4 and the minimum was zero. As noted previously (Martz & Steinberg, 1974), these data indicate that there is less cell movement in Interval II than in Interval I, perhaps due to contact inhibition of movement.

Because there was wide variation in size and duration among the gaps, we analysed various combinations of the different classes. The method of analysis was to choose a class or group of classes, such as 'A+B', and make a scatter-plot of the neighbour-

exchanges adjacent to a given cell against the number of gaps experienced by that cell. Each point on such a plot represents the experiences of one cell during the interval investigated. Gap data were analysed for class groups A, A+B, A+B+C and A+B+C+D. Coefficients of correlation were calculated. We chose to show the scatter-plots of A+B and A+B+C gaps in Fig. 2 because they showed the highest correlations found, and because they seemed most useful for our purposes. Class A alone has substantially fewer data, while class group A+ B+C+D contains some very minor gaps and perhaps some events that are not gaps at all.

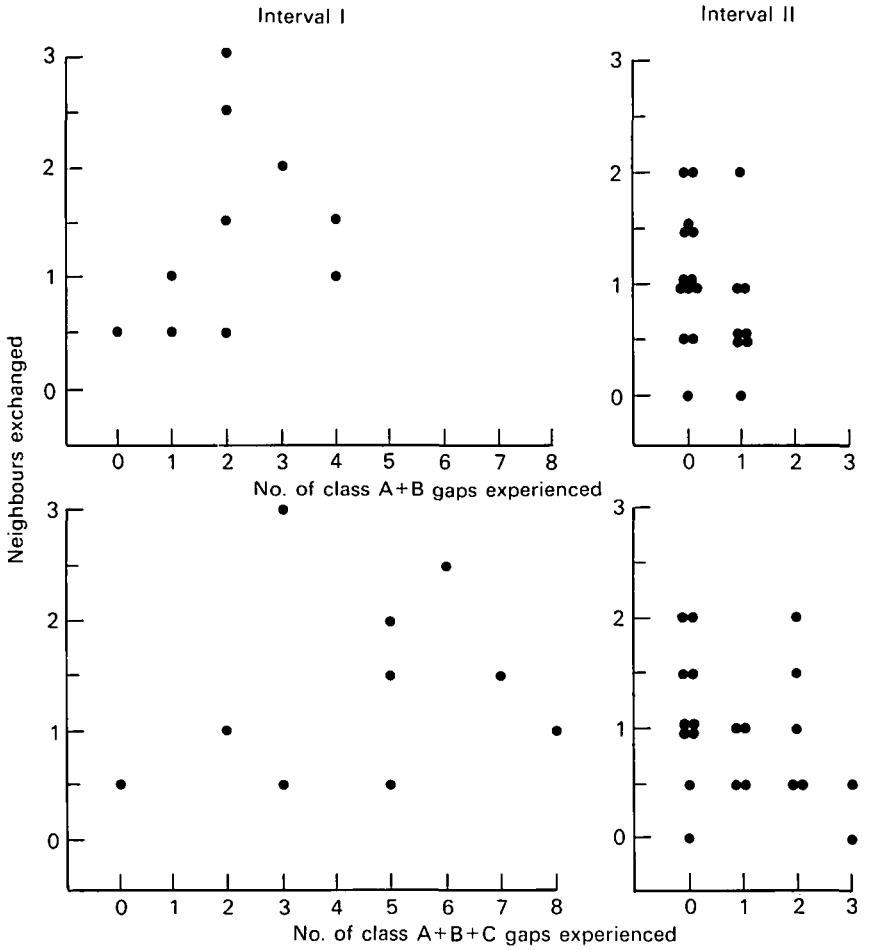

Fig. 2. Scatter-plots of neighbour-exchanges vs. number of gaps experienced by individual 3T3 cells in Film 18 between confluence and stationary density (Interval I) and during stationary phase (Interval II). See text for gap classification. Correlation coefficients are given in Table 2.

A surprising property of the correlation coefficients is that they are all negative in the stationary density interval. We see no obvious biological or statistical explanation for this. We note, however, that 29 gaps are distributed among 21 cells in this interval. This gives different cells little variation in their gap experiences. Scatter-plots and

co-efficients of correlation are probably inappropriate for these data, but we include them for completeness' sake.

The very paucity of gaps suggests that gaps were unimportant in causing the observed cell movements. During Interval II, 29 gaps would have to induce the cell movements which bring about 20·5 neighbour-exchanges. Of the 21 cells considered, no. 6 was the only one which experienced the maximum of 4 gaps (in class group A+B+C+D). Cell no. 6, however, exchanged no neighbours during this interval. Cell no. 9 was the only cell to experience 3 gaps; it exchanged one half of a neighbour. Three cells exchanged the maximum of 2 neighbours. The same 3 cells experienced 0, 1 and 3 gaps respectively. (The mean gap experience overall was 1·38 gaps-all classes-per cell.)

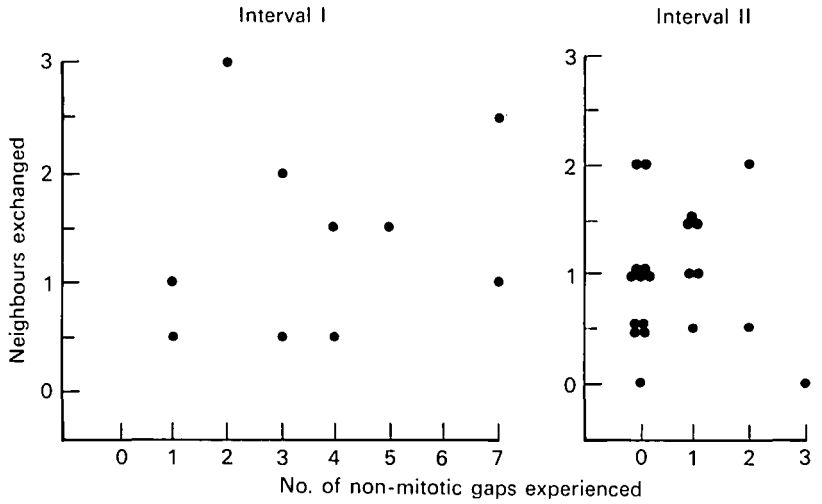

Fig. 3. Scatter-plots of neighbour-exchanges vs. number of non-mitotically caused gaps of all classes experienced by individual 3T3 cells after confluence. Correlations are smaller than before exclusion of mitotically caused gaps. Correlation coefficients are given in Table 2.

During Interval I, a similar situation prevailed. Cell no. 31 experienced the greatest number of gaps (14); but its value of 1 neighbour-exchange was less than the mean of 1·4. With 3 neighbour-exchanges, cell no. 22 had the largest number in Interval I, yet it experienced only 4 gaps compared with the mean number of 6·8 gaps. Since contact inhibition of movement is defined at the level of individual cells, and since the individuals with the highest values of one variable almost always had low values for the other variable, we feel that these examples provide particularly convincing evidence that temporary gaps within the confluent monolayers did not release cells from this contact inhibition.

Many gaps are initiated by mitosis. Most, if not all, are closed when the daughter cells re-spread upon the substratum. Since these gaps are not closed by movement of neighbour cells we thought that their occurrence might add noise to our data. Therefore, the A+B+C+D data were re-analysed after exclusion of mitosis-caused gaps. The results show even smaller correlations than do the complete data. Fig. 3

contains the scatter-plots for both intervals, and Table 2 (p. 301) lists the correlation coefficients.

We considered the possibility that it is not the number of gaps experienced by a cell that is important in inducing it to move, but the total amount of time during which a cell experiences these gaps. To examine this possibility, we added together the total

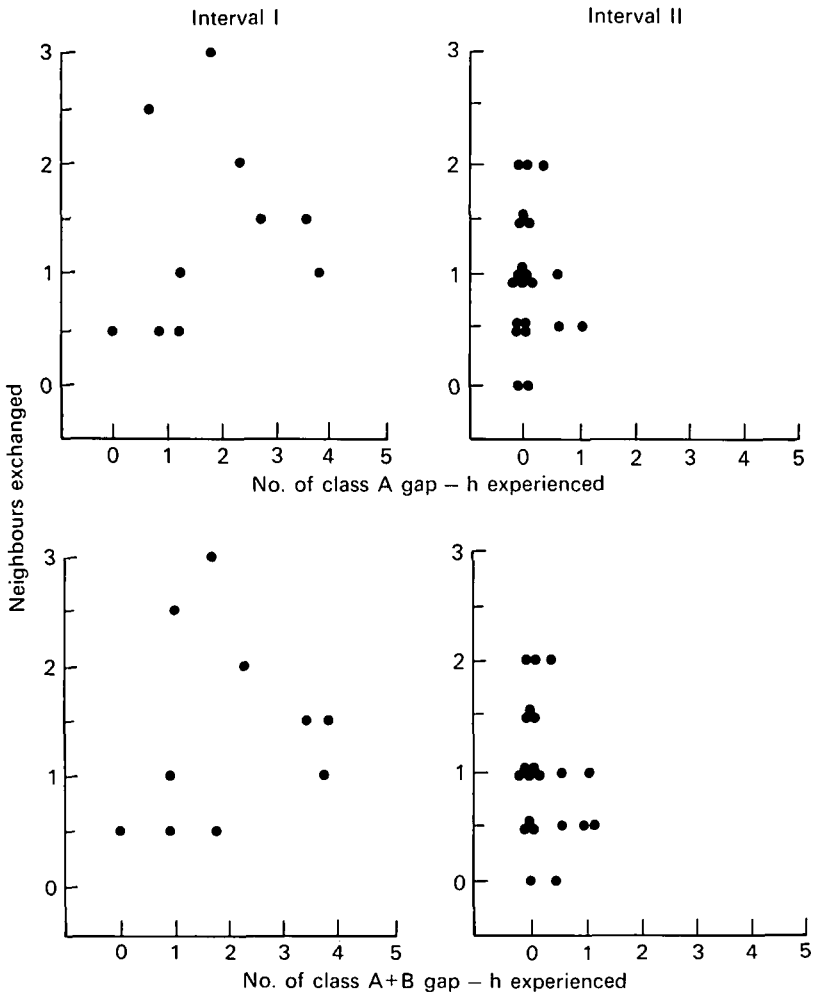

Fig. 4. Scatter-plots of neighbour-exchanges vs. total number of class A and Class A + B gap-minutes experienced by individual 3T3 cells after confluence. Correlation is not significant (see Table 2).

minutes of gap experience per cell and then compared this to the number of neighbours exchanged. It would be unreasonable to consider equal exposures to gaps of different classes as potentially having similar effects on release of inhibition. Class A gaps, for example, are larger and more distinct than Class C gaps. Rather than make an *ad hoc* weighting scheme and thereby expose the data to an element of arbitrariness, we chose

to restrict this analysis to class groups A and A+B. These gap-minutes thus apply to similar sorts of gaps. Fig. 4 shows scatter-plots of neighbours exchanged by cells experiencing both classes of gaps for both intervals. The correlations, shown in Table 2, are not significant.

In the course of gathering gap data, we noticed that there seemed to be almost no individual occurrences of the phenomenon we were looking for. Non-mitotic gaps closed by *cytoplasmic* movements, but the nuclei of adjacent cells did not participate in these movements. There was not a single clear example of a previously contact-inhibited cell nucleus moving into, or substantially in the direction of, a gap.

Table 2. *Correlations of neighbour-exchanges with gaps*

	Interval I		Interval II	
Gap class(es)*	ρ†	P	ρ	P
A	0·30	> 0·3	−0·07	> 0·8
A+B	0·31	> 0·3	−0·3	> 0·1
A+B+C	0·23	> 0·5	−0·31	> 0·1
A+B+C+D	0·24	> 0·5	−0·09	> 0·7
Non-mitotic A+B+C+D	0·19	> 0·5	−0·07	> 0·8
Time-weighted A	0·18	> 0·6	−0·12	> 0·6
Time-weighted A+B	0·18	> 0·6	0·29	> 0·2

* For definitions of gap classes see Table 1.

† Correlation coefficients (ρ) for the number of neighbour exchanges vs. the gaps experienced by each cell. These correlations represent the experiences of 10 cells in Interval I and 21 cells in Interval II, and are based upon the data illustrated in Figs. 2–4. Probabilities (P) indicate that none of the correlations are statistically significant.

DISCUSSION

Examination of individual cells with the extreme values of neighbour-exchange or gap experience shows a surprising lack of correlation between the two. In each interval, those cells with the most gap experience have fewer than the average number of neighbour-exchanges. Similarly, 3 of the 4 cells with the highest neighbour-exchange values experience fewer than the average number of gaps. These observations conflict with the idea that the gaps we observed were sufficient to release contact inhibition of movement in the monolayer.

None of the scatter-plots shows much correlation between these 2 variables. Even for the data showing the highest positive correlation (the class group A+B gaps), the probability that the distribution occurred by chance, $P > 0{\cdot}30$, is quite large. It is possible that the correlation we have been seeking might not be linear; but the scatter-plots give no hint of any sort of positive correlation at all. The low correlation coefficients obtained correspond to high probabilities that the hypotheses tested are false. Nevertheless, in view of the small number of cells analysed, it remains possible that there is a low correlation between neighbour-exchanges and gaps. Only a much larger-scale analysis of neighbour-exchanges and gaps could establish such a low correlation. However, the present evidence does establish that gaps are

not sufficiently correlated with neighbour-exchanges to be a major cause of the movements observed in the postconfluent monolayer studied here.

Although cell contacts have been demonstrated to reduce the speed of cell locomotion in subconfluent cultures (Abercrombie & Heaysman, 1953; Martz, 1973; Gail & Boone, 1971), this 'contact inhibition of cell speed' (Martz, 1973; Martz & Steinberg, 1973) was not observed to immobilize even those cells surrounded by other cells. Moreover, the observation of substantial cell movement in confluent monolayers (Garrod & Steinberg, 1975; Martz & Steinberg, 1974; Steinberg, 1973) confirms that cells are not necessarily immobilized when surrounded. Thus, any cell

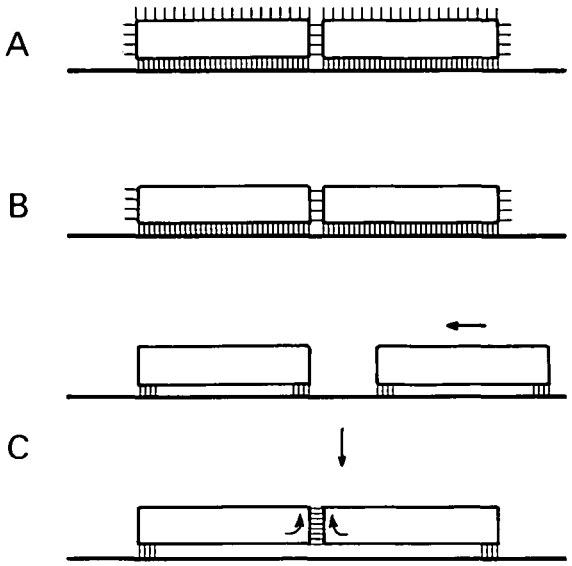

Fig. 5. Representations of direct inhibition of cell overlapping by 3 varieties of differential adhesiveness. A, B, cells adhere more strongly to substratum than to each other's upper surfaces. In B, upper cell surface is totally non-adhesive to other cells. C, adhesive regions localized at cell periphery or poles detach from substratum for preferred mutual apposition (stronger adhesion).

contact-induced paralysis of the motility-generating apparatus would appear to be partial or transient. Such a partial or transient inhibition of the locomotory apparatus does not readily account for the observed nearly perfect lack of nuclear overlapping. The present finding that the cell movements in confluent monolayers are not due to temporary breakdowns in confluence increases this difficulty.

It is for this reason that we find the direct hypothesis for contact inhibition of overlapping most satisfactory. It explains lack of overlapping by a mechanism which places no restriction on movements which do not lead to overlapping, and hence is readily consistent with all findings. Such an inhibition of overlapping could arise from cellular adhesive differentials of several kinds (Fig. 5). Cells might adhere more strongly to the substratum than to one another's 'upper' surfaces (Fig. 5A), causing them to 'prefer' the former over the latter (Abercrombie, 1961; Carter, 1967; Weston & Roth, 1969; Steinberg, 1973; Martz *et al.* 1974; Garrod & Steinberg, 1975).

In an extreme form of the above situation (Fig. 5B), cells' upper surfaces might be altogether non-adhesive toward other cells (DiPasquale & Bell, 1974; Middleton, 1973), like the external surface of an amphibian embryo (Holtfreter, 1943). There is also evidence (reviewed in Harris, 1973) that cells may adhere to other cells or surfaces only at scattered attachment regions, which tend to be concentrated at polar or peripheral sites on attached cells. In Harris' study, when such regions on 2 cells came together upon cell contact, cell-substratum adhesions appeared to be lost as cell-cell adhesion was established (Harris, 1973), suggesting a transfer of cell attachments from the substratum to the cell-cell interface (Fig. 5C), possibly due to differential adhesion.

Although contact inhibition by the direct restraint of locomotion toward a contacted cell remains a theoretical possibility for still-uninvestigated cell populations, every investigation of this matter familiar to us has revealed evidence that *cells in full lateral contact on a substratum continue to locomote, even while simultaneously avoiding major mutual overlapping*. Thus we have suggested (Martz & Steinberg, 1973, 1974; Steinberg, 1973; Martz *et al.* 1974; Garrod & Steinberg, 1975) that the expression 'contact inhibition of overlapping' is more descriptive of the observed behaviour than is 'contact inhibition of movement'.

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