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Cell Junction and Cyclic AMP: II. Modulations of Junctional Membrane Permeability, Dependent on Serum and Cell Density

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Summary. Junctional molecular transfer (as indexed by the number of cell interfaces transferring fluorescent-labelled molecules) and concentration of endogenous cAMP were determined in mammalian cells in culture at varying serum concentration and cell density. In several cell types, on stepping the serum concentration from 10% (the concentration to which the cells had been adapted) to zero, the junctional transfer rose (reversibly) within 48 hr, as the endogenous cAMP concentration rose. The junctional transfer was inversely related to serum concentration over a range, most steeply so the transfer of large and charged molecules. One cell type showed no junctional change in response to serum; it showed also no endogenous cAMP change. Junctional transfer varied inversely with cell density over the range of 0.7–7 (10^4 cells/cm^2) in 3T3 cells. In cultures seeded to various densities, or growing to various densities on their own, junctional transfer fell with rising density, and so did the endogenous cAMP concentration. Upon downstep from high density, junctional transfer rose over 24–48 hr. In B cells, junctional transfer was independent of cell density over the aforementioned range, and so was the endogenous cAMP concentration. These results, in conjunction with the effects of exogenous cAMP described in the preceding paper of this series, point to a cAMP-mediated junctional effect; a possible teleonomy for control of membrane junction is discussed.

Key words: Cell junctions, gap junction, junctional permeability, membrane permeability, cell-to-cell channels, cyclic AMP, cell density, serum

In this paper, we deal with the junctional effects of two factors of cell culture that influence the cell-endogenous cyclic AMP. The concentration of cAMP is known to rise in a variety of cells when they are deprived of the serum to which they have adapted (Grimm & Frank, 1972; Otten, Johnson & Pastan, 1972; Seifert & Paul, 1972; Kram, Mamont & Tomkins, 1973; Sheppard & Prasad, 1973; Ooey, Vogel & Pollack, 1974), and to rise with decreasing cell density in some cell types (when serum is not a significant variable) (Ooey et al., 1974; Burstin, Renger & Basilico, 1974; Haslam & Goldstein, 1974; *cf.* Chlapowski, Kelly & Butcher, 1975). Here we examine the effects of serum and cell density on junctional molecular transfer together with those on endogenous cAMP in several fibroblastic and epithelioid cell types. We find that the junctional transfer changes in a manner predictable from the changes in endogenous cAMP.

Materials and Methods

Mouse 3T3-BalbC and 3T3-42, and rat B and RL (liver) cell cultures and rat primary liver cells were used. The culture media and culture conditions, the medium for cell dissociation, the procedures for junctional probing and scoring, and for determinations of fluorescence tracer loss, endogenous cAMP, and incidence of cytoplasmic intercellular bridges are described in the first paper of this series (Flagg-Newton, Dahl & Loewenstein, 1981). The medium (and serum complement) for 3T3-42 was the same as that for 3T3-BalbC. The glutamic series of junctional probes is listed in Table 1 of the first paper; other probes used are in Table 1 here.

In the experiments in which cell density was the variable, the medium was changed daily to prevent serum depletion, which was particularly important at high densities; the last change was 10–12 hr before the junctional testing. Cell density was determined with the aid of a hematocytometer.

All sets of experiments for a given experimental variable, as subsumed by a given table or figure, were performed on parallel subcultures from the same stock, unless stated differently.

Results

Serum Deprivation

Junctional Transfer

The various cell types, except for RL, stopped growing after a day or so in serum-free medium. However, they generally stayed well attached to the dishes and

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Table 1. Junctional probes (additional probes are in Table 1 of the first paper of this series)

Probe	Charge[a]	Mol. wt.
Carboxyfluorescein	1–2	376
LRB	0	559
LRB Gly-Gly-Gly-Gly-Gly-Gly OH	1	901
LRB Arg-Pro-Arg		990

[a] Net negative charge.

LRB = Lissamine rhodamine B (red fluorescent); Gly, glycine; Arg, arginine; Pro, proline.
For preparation, characteristics and purity of the probes, *see* Simpson et al., 1977, and Socolar and Loewenstein, 1979. For molecular dimensions, *see* Schwartzmann et al., 1981.

to each other for at least 3–4 days. We tested junctional tracer transfer over this time on subconfluent cultures.

Serum deprivation led to increase of junctional transfer in B, 3T3-BalbC, and 3T3-42 cultures. For example, on omitting the usual 10% serum from the medium of the 3T3 cultures, the incidence of junctions transferring LRB(Glu)OH about doubled within 72 hr; and a similar rise ensued in the incidence of junctions transferring LRB$(Glu)_2$OH in B cultures where the junctions (somewhat less restrictive to start with) passed this molecule in controls with about the same incidence as the 3T3 cultures passed the LRB(Glu)OH (Table 2). As one would expect, the increase was most pronounced with probe molecules near the limit for cell-to-cell channel permeation (Flagg-Newton, Simpson & Loewenstein, 1979). Thus, after serum deprivation, LRB$(Glu)_2$OH was transferred in 3T3-42 cultures and LRB$(Glu)_3$OH in B cultures, whereas before they were not; even the long LRB$(Leu)_3(Glu)_2$OH was then transferred in B cultures. Figure 1 illustrates a typical experiment on 3T3-BalbC cells probed with LRB(Glu)OH, and Table 2 gives the junctional incidence data and their frequency distribution for the three cell types.

This junctional effect took on the order of a day to develop. In 3T3-BalbC cultures, probed with LRB$(Gly)_6$OH, the permeable-junction incidence rose between 24 and 48 hr after serum deprivation; by 24 hr it had not yet risen. Reversal, as tested in B cultures, took place within a day of serum restoration (Fig. 2).

Dose-response curves, as determined with probe molecules of different length and charge, are shown in Fig. 3. In these experiments, parallel B-subcultures were for 72 hr exposed to medium with concentrations of serum varying between 0 and 10%; the cell densities were about the same at the time of the probings. The steepest changes with serum concentration were registered in the transfers of the larger and more charged molecules; for instance, an increase in serum concentration from 0 to 2% sufficed to bring the LRB$(Glu)_3$OH permeable-junction incidence from 60% down to 0% (Fig. 3).

The increase in the incidence of transferring junctions upon serum deprivation went generally hand in hand with an increase in the fluorescence intensity in the sink cells, the cells contiguous to the injected one (*see* Fig. 1). Thus, as in the experiments of the

Table 2. Permeable-junction incidence and serum (table continues on facing page)

Cell type	Serum concentration (%)	LRB Glu OH					LRB$(Glu)_2$OH				
		Permeable first-order junctions					Permeable first-order junctions				
		Incidence (%)[a]	Frequency[b]				Incidence (%)	Frequency			
			0	10–49	50–89	90–100		0	10–49	50–89	90–100
3T3 Balb-C	10*	**31** (30: 90; 14, 3)	5	4	4	1	**3** (2: 62; 12, 2)	11	1	0	0
	0	**79** (106:135; 18, 7)	1	0	14	3	**36** (27: 75; 9, 2)	4	1	4	0
3T3-42	10*	**24** (16: 66; 12, 2)					**0** (0: 30; 6, 1)	6	0	0	0
	0	**54** (72:125; 19, 6)					**13** (19:134; 20, 5)	15	3	2	0
B	10**						**30** (26: 87; 14, 3)	6	3	5	0
	2**						**66** (35: 53; 8, 2)	0	0	8	0
	1**						**71** (29: 41; 6, 1)	0	1	1	3
	0						**79** (134:170; 18, 4)	0	2	9	7

All permeability tests were on subconfluent cultures 72 hr after serum deprivation. Not included are LRB, LRB$(Glu)_2$OH- and LRB Arg-Pro-Arg-permeability data of RL cells which showed no significant change upon serum deprivation.

[a] Incidence (%) of first-order permeable junctions, cumulative scores. In parentheses, in the following order: the number of permeable first-order junctions; the number of first-order junctions; the number of microinjections (trials); the number of culture dishes examined.

preceding paper, the rise in incidence seemed to reflect a rise in the rate of junctional transfer. Indeed, there was no sign of a reduction in nonjunctional permeability to the probe molecules associated with the effect; the rates of fluorescence loss (exponential phase) for $LRB(Glu)_2OH$ and $LRB(Glu)_3OH$, as determined in two-celled preparations of B cultures were not significantly changed. Nor were there signs of increased frequency of coarse cytoplasmic-bridge transfer between cells; the control incidence of 0.3% of B cell interfaces transferring the cell-to-cell channel-impermeant FITC-fibrinopeptide (Simpson, Rose & Loewenstein, 1977) did not change on serum deprivation.

Variability. We used the same calf serum (two batches) throughout the aforegoing experiments, and in these conditions the effect of serum deprivation was consistently found in B and 3T3-42 cultures. The effect was also consistently present in 3T3-BalbC cultures which had undergone less than 30 passages in our laboratory. Among 3T3-BalbC cultures with more passages, there were occasionally unresponsive ones.

We also would like to mention a variation found after the present work had been completed. In a still ongoing search for the factor in the serum responsible for the junctional response, some (calf) sera were come across in which the junctions behaved differently. When cells were adapted to these sera and then deprived of them, there was no junctional effect; but then, at a serum concentration of 10%, the permeable-junction incidence (B and 3T3-BalbC) was about as high as that at 0% with the cells adapted to the present serum (D. Ben-Haim and W.R. Loewenstein, *unpublished*).

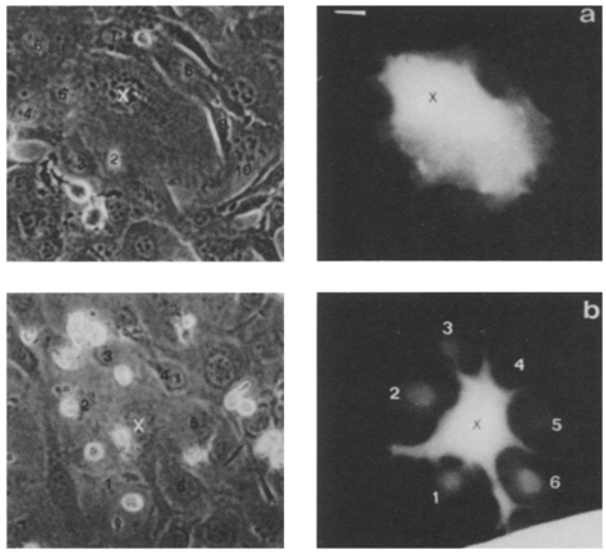

Fig. 1. Serum deprivation and cell-cell transfer. LRB Glu OH is injected into a cell (*x*) of a 3T3-BalbC culture. (*a*): In serum (10%)-containing medium, the medium to which these cells have been adapted. The tracer has been transferred to two (cells *1* and *8*) out of the ten first-order cell neighbors; their fluorescence is just above detection threshold. (*b*): In a culture deprived of serum for three days, the tracer is seen to have been transferred to all six first-order neighbors of the injected cell (*x*), five of which show strong fluorescence. *a* and *b* illustrate experiments on different cell clusters; the injected cell is the largest one in each cluster. Calibration, ~50 μm

$LRB(Glu)_3OH$					$LRB\ Leu_3Glu_2OH$				
Permeable first-order junctions					Permeable first-order junctions				
Incidence (%)	Frequency				Incidence (%)	Frequency			
	0	10–49	50–89	90–100		0	10–49	50–89	90–100
0 (0: 28; 5, 1)	6	0	0	0	**0** (0: 71; 6, 2)	6	0	0	0
0 (0: 33; 6, 1)	6	0	0	0					
8 (6: 77; 10, 2)	8	0	2	0					
56 (122:218; 31, 8)	5	3	23	0	**10** (14:138;13, 4)	10	1	1	1

[b] The frequency distribution of the incidence of permeable junctions, arranged in intervals of 10–49, 50–89, 90–100, and 0% .The interval 1–9 is unoccupied for all cell types because of the minimum scoring criterion of 2 permeable first-order junctions; the maximum number of first-order 3T3 and B cell neighbors was 20 (*see* Methods in the first paper of this series).

* Calf serum.

** Fetal calf serum.

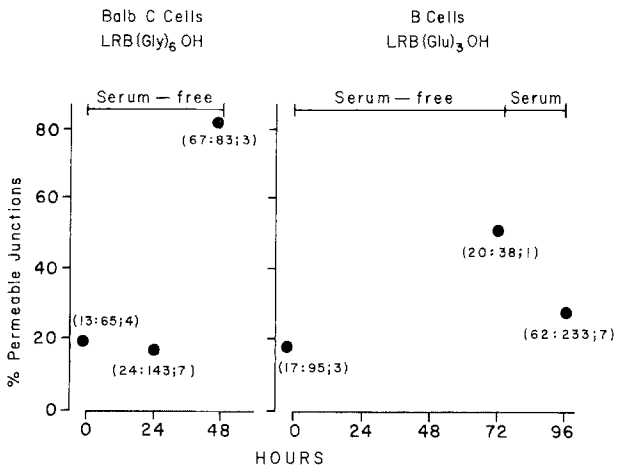

Fig. 2. Serum effects on permeable junction. *Left*: LRB(Gly)$_6$OH transfer between BalbC cells following serum deprivation. *Ordinates:* The incidence of transferring first-order junctions. *Abscissae:* Time after the exposure to serum-free medium. The first data point was obtained in serum (10%)-containing medium just before time zero. In parentheses in the following order: the number of first-order transferring junctions, the number of first-order junctions tested, and the number of culture dishes examined. (The number of injection trials for data points of 0, 24, and 48 hr were 14, 19 and 11, respectively; of these trials, 9, 12 and 0 gave 0% incidences, respectively.) *Right*: LRB(Glu)$_3$OH transfer between B cells. Here the serum was restored at time 72 hr. All data are from parallel subcultures from the same stock

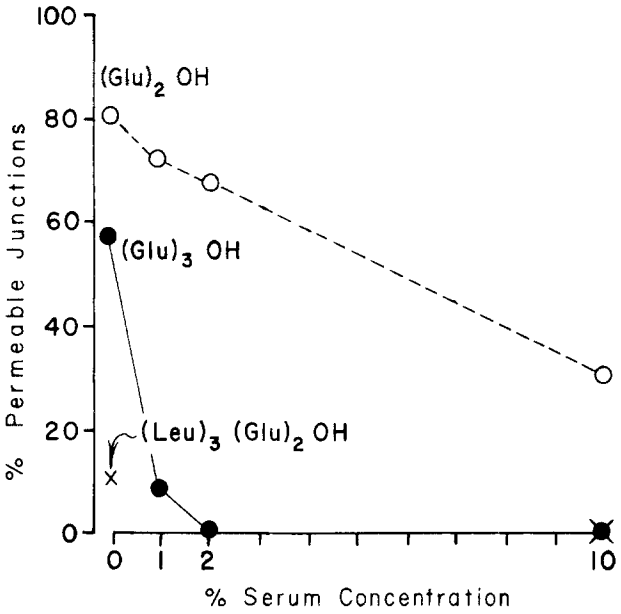

Fig. 3. Junctional transfer at various serum concentrations. The incidence of first-order junctions of B cells transferring LRB(Glu)$_2$OH (○), LRB(Glu)$_3$OH (●), and LRB(Leu)$_3$(Glu)$_2$OH (×) at serum concentrations of 0, 1 and 2% (for 72 hr) and in the control conditions of 10%. (All three data sets are from parallel cultures from the same stock.) Data detail in Table 2

Table 3. Endogenous cAMP and serum concentration. Means[a]

Serum (%)	cAMP (pmol/10^6 cells)[b]			
	3T3 BalbC	3T3-42	B	RL
0	8.6	7.9	1.2	8.0
10	3.2	3.1	0.7	7.9

[a] SE ranged 7–11%.

[b] Expressed per mg protein, the cAMP concentration ratios between the various experimental conditions were about the same as between the corresponding concentrations per 10^6 cells given.

Unresponsive Cell Types. The liver RL cell line [probed with LRB, LRB(Glu)$_2$OH and LRB-Arg-Pro-Arg] and primary liver cells [probed with LRB(Glu)OH] showed no detectable junctional response to serum deprivation.

Endogenous cAMP

The endogenous cAMP concentrations were measured in the cells grown in the medium containing the usual 10% serum (control) and after a 72-hr serum deprivation, under conditions matching those of the experiments on junctional transfer. Table 3 gives the results obtained with the various cell types. The cAMP concentrations were markedly elevated in serum-deprived 3T3-BalbC, 3T3-42 and B cells, but not in serum-deprived RL cells.

It should be noted that our cAMP data are, in a sense, relative values. The standard errors of the various mean values were below 11% (Tables 3 and 6), but this relatively low individual fluctuation was contingent on the uniformity of cell material and assay conditions; it ensued as we used parallel subcultures from the same stock for the assays on a given cell type and ran the assays for the two serum conditions (or the three density conditions) for each cell type in parallel at the same time. As demonstrated by a special series of control experiments, the fluctuation could be much larger when the cultures were from different stock or from different passages (3T3-BalbC) or even when only the assays were not in parallel – not an uncommon experience with cellular cAMP determinations (*cf.* Ryan & Heidrick, 1974; Chlapowski et al. 1975). *This means that one may validly compare here the cAMP values only from each cell type of each table, but not those from different types, nor the values from the same cell type of different tables* (including Table 5 of the preceding paper).

Cell Density

Junctional Transfer

3T3 Cells. The junctional transfer varied with the cell density in 3T3 cultures (Fig. 4). We studied this phenomenon in two kinds of experiments. In one kind, we seeded 3T3-BalbC cells to various densities; and in the other, we let them grow to various densities on their own. The results were essentially the same: the incidence of permeable junctions diminished with increasing density.

The protocol for the first kind of experiment was to dissociate semiconfluent or confluent stock cultures, to seed them to various starting densities, and to probe the junctions with LRB 48 hr thereafter. The cell densities were determined at the time of the seedings and, again, at the time of the junctional probings (the density values tabulated, Table 4). In the case of the seedings to high starting densities, the cells were at all times at high density, except for a few minutes of dissociation and re-suspension. The junctions were then probed also at 24 and 72 hr after the seedings. The medium was exchanged every day to avoid depletion of the serum (as it was in the second kind of experiment).

Figure 5*I* summarizes the results. In sparse cultures, at densities below 1 [10^4 cells/cm^2], more than 90% of the junctions tested were LRB-permeable. In confluent cultures, at densities above 4 (10^4 cells/cm^2), the incidence was reduced to 20% or less; and at intermediate densities (semiconfluent), the incidence was somewhere in between (*see* Table 4 for data detail).

In the second kind of experiment, we dissociated confluent stocks, seeded the cells to densities <0.5 (10^4 cells/cm^2) and let them grow undisturbed, some for several generations, to reach various densities. Here again (allowing enough time after the transition from high to low density, 48 hr) the permeable-junction incidence fell with rising cell density (Fig. 5*II* and Table 5). A similar trend was seen in a more limited series of experiments with carboxyfluorescein as the probe (Table 5).

The permeable-junction incidence stayed low as long as the cultures were at high densities. The incidence rose again upon transition from high to low density, and this recovery took more than a day: when confluent cultures were re-seeded to low densities (≤ 1 [10^4 cells/cm^2]), they regained maximum incidence 24–48 hr after the density transition (Fig. 7; *see also* Fig. 4*c*). This time may be compared with that required in experiments in which cells from low-density cultures were re-seeded to low density. The maximum incidence then developed within 6 hr, which presumably is the time needed for the formation of maximum cell appositions and cell-to-cell channels.

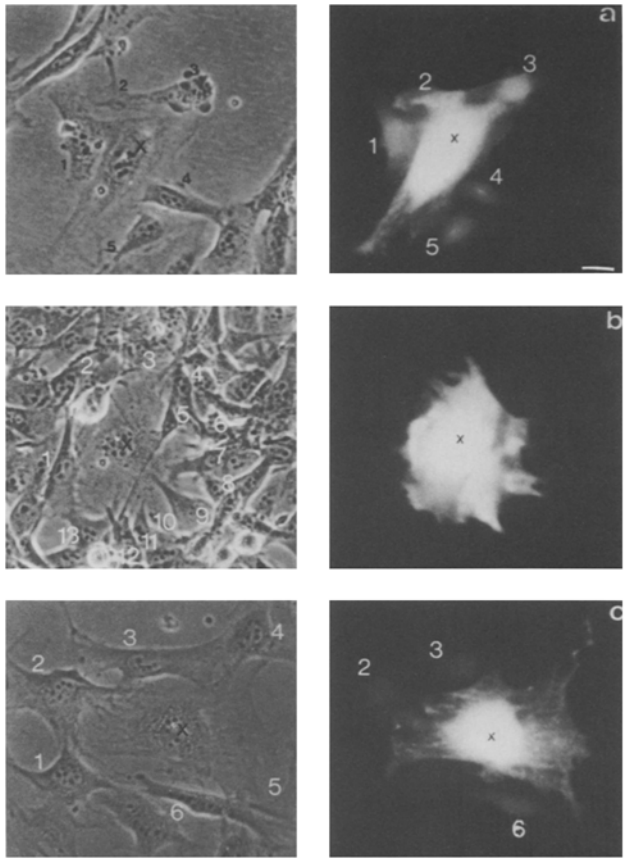

Fig. 4. Junctional transfer at different cell densities. 3T3-BalbC cell junctions are probed with LRB (*a*) at a density of ~ 1 (10^4 cell/cm^2). The tracer is seen to have been transferred to all five first-order neighbors of the injected cell (*x*); (*b*) at a density of 4 (10^4 cell/cm^2) there is no transfer detectable in any of the thirteen first-order neighbors of the injected cell. (*a* and *b* are parallel subcultures. The original stock was confluent; it was passaged 48 and 72 hr, respectively, before the permeability probing.) (*c*): 21 hr after passaging a high-density culture like the one in *b* and at a density of ~ 1 (10^4 cell/cm^2), the junctional transfer is still depressed. Three out of six first-order neighbors are fluorescent and only just above detection threshold; compare with *a*. Calibration, ~ 50 µm

Evidently, the cellular state determining the reduced junctional transfer at high cell density, outlasted the high-density condition by more than 18 hr. Therefore, in experiments of the second kind (Table 5), we consider only data at 48 hr or later after the seedings, as representing steady state. However, there are no reasons for applying this limit to the data from experiments of the first kind, such as the 24-hr datum of Fig. 4, where the cells had been at high density before and after the seedings. This datum was omitted from Figure 5*I* only for consistent presentation of 48-hr data. In the final summary of the results, in Fig. 6, we lump all data that may be

Table 4. Incidence of permeable junctions in 3T3 BalbC cultures at various cell densities. Cells seeded to various starting densities

Culture density		Time after seeding (hr)	LRB				
	(10^4 cells/cm^2)		Permeable first-order junctions				
			Incidence (%)	Frequency			
				0	10–49	50–89	90–100
Sparse[a]	$\leqq 1$	48*	**93** (39: 42; 7, 2)	0	0	4	3
Semiconfluent	$>1 \leqq 4$	48†	**69** (90:130; 20, 6)	2	0	16	2
Confluent	>4	24*	**0** (0: 47; 5, 1)	5	0	0	0
		48†	**20** (23:117; 17, 3)	10	6	1	0
		72†	**3** (3: 106; 14, 5)	13	1	0	0

The cultures tested originated from one passage of a confluent (*) or semiconfluent (†) stock. No secondary passages were made. Time zero is the time of the cell seeding in the primary passage.

[a] Same data as in experiment series #1, 48 hr, Table 5.

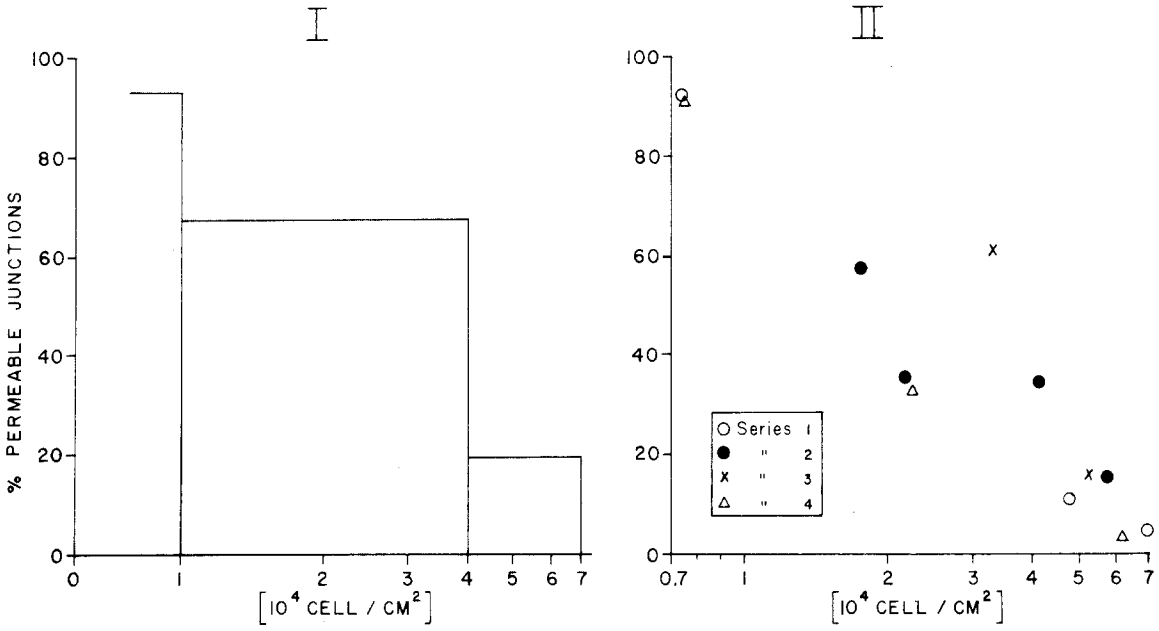

Fig. 5. The permeable-junction incidence in BalbC cultures decreases with increasing cell density. The proportion of first-order junctions permeable to LRB (ordinates) at various cell densities (abscissae). (*I*): Data from experiments in which the cells were seeded to various densities and the permeability was tested 48 hr after the seedings. The permeability data are lumped for three categories of cell density (in units of 10^4 cells/cm^2): $\leqq 1$ (sparse), $>1 \leqq 4$ (semiconfluent), and >4 (confluent). The data detail, including frequency distributions, appear in Table 4 (24 and 72 hr data are omitted in the figure; *see* text). (*II*): Data from experiments in which the cells (seeded initially to low densities) had grown to various densities (abscissae). Data points ○, ●, ×, and △ correspond with series 1, 2, 3, and 4 of Table 5, respectively

presumed to correspond to steady state, incorporating the 24 and 72 hr data of Table 4.

A set of tests with 3T3-42 cells, probed with LRB(Glu)OH, showed a similar decrease of permeable junction incidence as the cultures grew from sparse to confluent conditions.

B Cells. There was no significant change in junctional transfer in B cultures over the aforegoing density range. (The data are given in Table 6 together with those of endogenous cAMP.) Higher cell densities were not tested because identification of the cell boundaries, a necessary condition for the junctional transfer determinations, was then no longer unambiguous.

RL Cells. We used no RL cells for these density-related studies. These cells tend to grow in clusters

Table 5. Incidence of permeable 3T3-BalbC junctions at various densities. Cells growing to various densities

Expt. series #	Time after seeding (hr)	Cell density (10^4 cells/cm^2)	LRB					Carboxy-fluorescein				
			Permeable first-order junctions					Permeable first-order junctions				
			Incidence (%)	Frequency				Incidence (%)	Frequency			
				0	10–49	50–89	90–100		0	10–49	50–89	90–100
1	48	0.739	**93** (39: 42; 7, 2)	0	0	4	3					
2	48	1.55	**58** (44: 76; 14, 3)	3	0	8	3					
3	48	3.35	**62** (48: 77; 9, 2)	1	0	8	0					
4	48	0.75	**92** (37: 40; 8, 2)	0	0	4	4					
1	72	4.76	**11** (7: 63; 8, 2)	7	0	1	0	**55** (35:64; 8, 2)	3	0	4	1
2	72	2.16	**36** (43:118; 15, 4)	7	2	5	1					
3	72	5.3	**18** (18:100; 11, 3)	7	2	2	0					
4	72	2.23	**33** (34:104; 13, 3)	6	2	5	0					
1	96	6.96	**5** (3: 58; 7, 2)	6	1	0	0	**13** (8:64; 7, 2)	5	0	2	0
2	96	4.09	**35** (36:103; 11, 2)	5	1	5	0					
3												
4	96	6.10	**4** (4: 98; 12, 3)	10	2	0	0					
1												
2	120	5.66	**16** (8:115; 12, 2)	7	4	1	0					
3												
4												

The numbers 1, 2, 3 and 4 identify four series of experiments, each a sequence of permeability tests on continuously growing parallel cultures derived from one passage of the same stock culture in each series. No secondary passages were made.

on the dishes, with grossly uneven density distributions and, therefore, do not lend themselves to analysis in terms of overall cell density.

Cytoplasmic-Bridge Transfer

The frequency of transfer between cells via coarse cytoplasmic bridges seemed to increase with cell density; the incidence of cell interfaces passing the cell-to-cell channel-impermeant FITC-fibrinopeptide rose from 0.11 ± 0.02 to $0.21 \pm 0.05\%$ as the cell density increased from 0.6 to 5 [10^4 cell/cm^2]. (A similar rise occurred in B cells. Perhaps there are more cell membrane fusions at the higher densities.) The change is negligible compared to the change in the incidence of junctional transfer. In any event, the two changes are in opposite direction; neither one can account for the other.

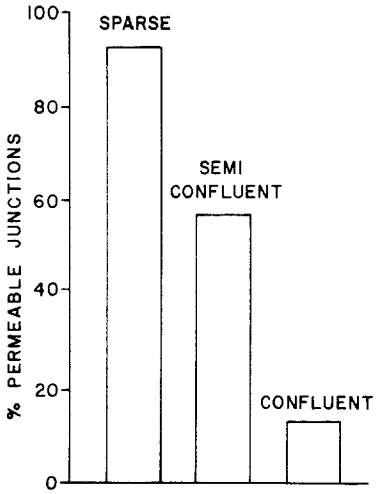

Fig. 6. LRB-permeable BalbC junctions and cell density. Summary of all permeability data presumed to reflect 'steady state' (*see* text)

Endogenous cAMP

3T3-BalbC intracellular cAMP was measured at various densities. The assays were done on subcultures parallel to those on which the junctional transfer was determined (series 4 of Table 5). The cellular cAMP concentration fell with rising cell density (Table 6), a trend already shown for 3T3 and other cell types (Ooey et al., 1974; Burstin et al., 1974; Haslam & Goldstein, 1974; *cf.* Chlapowski et al., 1975).

In B cultures, there was no significant cAMP change with cell density (Table 6). We did not run the cAMP assays and junctional tests on parallel subculture sets here (they were run in matching conditions on cultures from different stock), but both sets showed the independence from cell density.

Table 6. Endogenous cAMP and permeable junction incidence at various cell densities

Cell type	Density (10^4 cells/cm^2)	Junction incidence (%)	cAMP (pmol/10^6 cells)
3T3 BalbC	0.8–0.9	92	13.2
	2.1–2.3	33	5.2
	6 –6.2	4	3.5
B	<1	38	3.6
	1 –4	35	3.9
	4 –6	35	5.1

Junctional probe for 3T3-BalbC is LRB; for B, LRB$(Glu)_2OH$. The endogenous cAMP data for 3T3-BalbC are the means of assays on 24 culture dishes (SE $\leqq 9\%$), parallel subcultures from the same stock as those on which junctional transfer was determined (experiment series #4, Table 5). cAMP data for B cells are the means of 12 cultures dishes (SE $\leqq 11\%$), cultures in the same conditions as those on which junctional transfer was determined, but not parallel subcultures. Each series of determinations (cAMP on the one hand and junction incidence on the other), however, were on parallel subcultures.

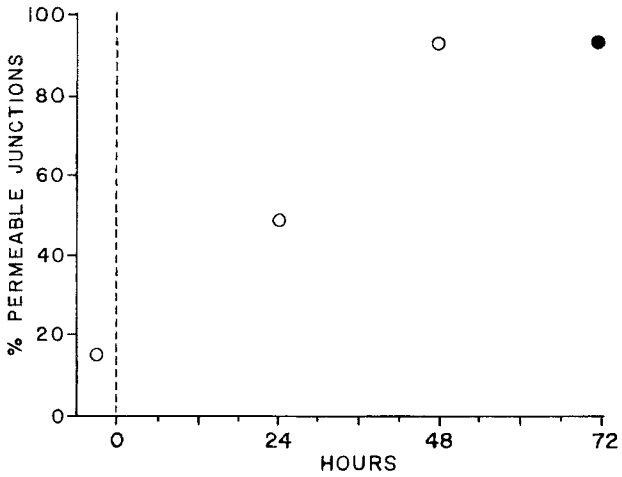

Fig. 7. Recovery of junctional transfer following the dispersion of a confluent culture. A high-density 3T3-BalbC culture is dissociated and the cells are seeded sparsely (time=0). The incidence of LRB-permeable junctions at various times after this passage. ○, ● are data from two different series (the cells in ● had undergone one secondary passage). The first data point (○, before time zero) corresponds to the confluent stock. The cell densities in order of time were: 6, 0.61, 0.74, and 0.72 [10^4 cell/cm^2]; the first 3 data belong to experiment series 1, Table 5

Discussion

The same reasoning as that followed in the preceding paper and the same kind of evidence of stable non-junctional permeability lead us to conclude that the apparent change of junctional transfer dependent on serum reflects a change of junctional permeability. In the case of the change dependent on cell density, the special question presents itself whether an enlargement of the sink volume at high density, i.e., tracer dilution via more junctions, gave rise to an apparent change in junctional transfer rate. This is unlikely: the junctional transfer was unchanged in high-density B cultures (Table 6). Thus, it would seem that also in the density-related effect, the junctional permeability is modified. This is further shown in the following paper by measurements of electrical coupling between contiguous cells of another type where ensemble topology (the counterpart of sink volume here) cannot account for the change in coupling (Azarnia, Dahl & Loewenstein, 1981).

The junctional responses to serum and cell density showed some similarities. The responses to increases of serum concentration and to increases of cell density had the same general trends (Figs. 3 and 5*II*) and the responses to downsteps from high serum concentration and to downsteps from high cell density were similar (Figs. 2 and 7). But, most revealingly, the junctional responses correlated well with changes of endogenous cAMP. With both serum and cell density as the experimental variables, junctional transfer rose when the level of cAMP rose, and only then (Tables 3 and 6). This correlation takes on an etiological tenor when it is viewed in the light of the experiments of the preceding paper (Flagg-Newton et al., 1981). These experiments showed that by supplying the cells with cAMP one can raise the junctional permeability. Thus, more then a pleiotropy, the endogenous cAMP-junctional permeability relationship seems causal.

In this light, we may envision a junctional action of serum and cell density along the following lines

$$s + r \rightarrow \downarrow \text{cAMP} \rightarrow \downarrow \text{cell-to-cell channels}$$

where endogenous cAMP plays the upregulatory role in the cell-to-cell channel formation process discussed in the preceding paper, and s, the extracellular stimulus, is a factor related to serum or cell density, down-regulating cAMP by way of a receptor process r, located, say, in cell membrane. Thus, with increasing serum or cell density, over a range, the rate of channel formation and, hence, eventually, the number of channels would diminish. The channel turnover time would not have to be faster than of the order of a day to account for the junctional response times to upsteps or downsteps of serum, such as those of Fig. 2.

A mechanism of this kind might play a physiological role in the regulation of permeable junction formation. Cell-to-cell channels can form in seemingly unspecific cell membrane regions when these are brought close enough together (Loewenstein, 1967; Ito, Sato & Loewenstein, 1974) and one naturally wonders how

the extent of permeable junction is physiologically kept within bounds. As part of a control loop, the aforegoing downregulatory mechanism may conceivably serve such a role – perhaps most simply if both *s* and *r* were on the cell surface. At this time there is not much to say about the *s* factor, but a cell surface molecule that can interact with a surface receptor molecule on a contiguous cell would not seem an implausible stimulus in a cell density-dependent phenomenon.

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