Intercellular Communication and Tissue Growth

V. A Cancer Cell Strain that Fails
to Make Permeable Membrane Junctions with Normal Cells

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Summary. A method is described for testing communication between a normal and a cancerous cell in culture without inserting microprobes into either cell; microprobes are put into other normal cells coupled to the normal cell in question. It is shown with this method that a cell strain (class-A), of epithelial morphology, isolated from Morris' liver tumor (H-5123) fails to make communicative junctions with several types of normal cells; small inorganic ions and fluorescein do not pass from the normal cells to the class-A cells (they do pass from the normal cells to normal cells, even between normal cells of different type). The class-A cells also appear incapable of junctional communication among themselves. The cells of class-A are cancerous: they are not 'contact inhibited' by each other or by the normal cells and they form malignant tumors when injected into test animals. Another cell strain (class-B), of fibroblastic morphology, derived from the same liver tumor as class-A makes communicative junctions readily. This strain is 'contact inhibited' and does not produce tumors when injected into the animals.

This paper is part of a series in which we explore the possibility of the cell junction serving as a passageway for growth controlling substances (Loewenstein & Kanno, 1966, 1967; Loewenstein & Penn, 1967; Jamakosmanovic & Loewenstein, 1968; Borek, Higashino & Loewenstein, 1969). The general approach is to search for defects in junctional communication in cells with uncontrolled (cancerous) growth. The approach is based on the consideration that if junctional communication is indeed involved in the regulation of cellular growth, genetically determined interruption of junctional communication *(uncoupling)* should lead to cancerous growth (Loewenstein, 1968 *a*). It is, of course, *a priori* extremely unlikely that the many forms of cancer should all have the same cause; but, since the junction is a vulnerable bottleneck (Loewenstein, Nakas & Socolar, 1967; Politoff, Socolar & Loewenstein, 1969; Rose & Loewenstein, 1971), we hoped that uncoupling

might be a cause frequently enough to give a reasonable chance of finding some uncoupled kinds of cancerous cells.

The first evidence of a possible uncoupling was obtained in certain liver (Loewenstein & Kanno, 1967), thyroid (Jamakosmanovic & Loewenstein, 1968), and stomach tumors (Kanno & Matsui, 1968). In contrast to their normal counterparts, the cells in these tumors showed no communication as determined by electrical measurements with intracellular probes. Similar results were later obtained with three types of cancerous cells in culture (Borek *et al.*, 1969). These latter results were the most satisfactory ones, because in culture one can measure junctional communication under conditions closely matched for normal and cancerous cells. Even so, the doubt remained, however slight, as to whether the uncoupling reflected the actual state of the cancer cells or merely a greater susceptibility to uncoupling due to intracellular probing. We now employ a method in which such probing into the cancerous cells is avoided. The method makes use of the finding that, in culture, junctional communication is readily established between normal cells of different type (Michalke & Loewenstein, 1971). The method consists essentially of determining the flow of electrical current and of fluorescent molecules between two sets of coupled normal cells bridged by a set of cancerous cells; only the normal cells are probed (Fig. 1). It will thus be shown that cancerous cells from Morris' liver tumor establish no junctional communication with normal cells.

Methods

Cells and Media

The following cell types were used. *Epithelial:* (*i*) rabbit lens cells (Shapiro, Siegel, Scharff & Robins, 1969), (*ii*) rat liver cells (Coon & Weiss, 1969; *see* Borek *et al.*, 1969), (*iii*) liver tumor cells A', (Borek *et al.*, 1969), (*iv*) liver tumor cells A; *Fibroblastic:* (*v*) BHK-21 cells (MacPherson & Stoker, 1962) and (*vi*) liver tumor cells B.

Cells *i* were cultured in Eagle's medium as modified by Dulbecco (E-D medium, Vogt & Dulbecco, 1960) supplemented with 15% (v/v) calf serum; cells *ii*, *iii*, and *vi*, in E-D medium supplemented with 10% fetal calf serum, cells *v*, in BHK-21 medium (MacPherson & Stoker, 1962) with 10% calf serum and 10% tryptose phosphate broth; cells *iv*, in Ham's (1965) F-12 medium containing a twofold concentration of aminoacids and 10% fetal calf serum. The cells were grown in plastic Petri dishes (Falcon Plastics) at 37 °C in an incubator, equilibrated with a moist CO_2-air mixture.

Cells *i*, *ii*, *iii* and *v* were from several-years old lines. The A-cells (*iv*) and the B-cells (*vi*) were freshly derived from explants (0.1 to 1 mm diameter) of solid Morris' (1965) rat hepatomas H-5123. The two cell classes were distinguishable by their morphology within 12 hr of culturing of the explants (Fig. 2). The B-cells were isolated by taking advantage of differences in adhesiveness to the dishes between the two cell classes. In E-D medium, the B-cells, but not the A-cells, adhere to the dishes; the A-cells only

adhere to B-cells. Thus, by repeated dissociation (with trypsin, 0.05%, EDTA, 0.02%, in modified Puck's saline A, solution 1 X, Grand Island Biological Co.) and passaging of the cultures (and consequent elimination of the unattached cells during the changes of medium), pure B-cell cultures were obtained. For isolation of A-cells, the cultures were kept in F-12 medium where both cell classes adhere to the dishes; pure A-cell cultures were obtained from clones.

Cell combinations were prepared by growing cells of one type (lens, liver or BHK) to the desired density and by adding then a suspension of cells of the second type (A- or A'-cells) to the dishes for co-culture. All co-cultures were in E-D medium (with fetal calf serum complement in all combinations containing liver or BHK cells; and with calf serum in the combinations containing lens cells), except for those containing A-cells which were in F-12 medium.

For testing of tumorigenicity, cell suspensions (10^3–10^6 cells), in their respective media, were injected into the thighs of rats of the same strain (Buffalo) from which the tumor and normal cells had originally been derived.

Electrical Measurements and Fluorescein Injections

Cell Combinations. Cancer Cell Bridges. For electrical measurements on mixed cultures, cell groups were used wherein two sets of coupled normal cells were bridged by one or more cancerous cells, after the pattern diagrammed in Fig. 1. Single-celled cancer bridges were fashioned as follows. Configurations consisting of normal cell chains interrupted by a gap about one cell-diameter wide were selected among sparsely populated normal cell cultures. A few cancerous cells in suspension were pipetted onto the vicinity of the chain. As soon as the cancerous cells reached the bottom of the dish, the cell closest to the gap was floated onto it by micromanipulation and allowed to settle. This entire procedure was done at 37 °C under microscopic observation. After the cell had adhered to the bottom (the cancer cells adhered within 5 min), the culture dishes were placed in the incubator for 2 hr before the start of the measurements (Fig. 3). Controls showed 2 hr to be sufficient time for formation of communicative junctions in normal-liver-cell/lens-cell and normal-liver-cell/BHK-cell bridges.

For experiments involving multicelled cancer bridges, cell groupings were selected among spontaneously occurring configurations in mixed cultures (Fig. 5). In this case, the cancerous and normal cells were in co-culture in the incubator for 4 hr to 2 days.

With the aid of microelectrodes, current was pulsed (10^{-8} amp; 100 msec duration) into one of the normal cells (*1*, Fig. 1). The resulting voltages were recorded in a normal cell *3* on the other side of the cancerous cell bridge (hatched) and, in most cases, simultaneously in a normal cell (*2*) contiguous to cell *1*. In many measurements, current was injected in a subsequent measuring step (hereafter *step 2*) also into another normal cell (*4*), contiguous to cell *3*. (For general aspects of the method of electrical measurement of coupling, *see* Loewenstein & Kanno, 1964, 1967). At least one normal cell junction intervened between the cancerous cell and any cell containing a microelectrode.

Fluorescein was injected into the cells simultaneously with many electrical measurements at step 1. To this end, the current delivering microelectrode was filled with fluorescein-Na (100 mM) and KCl (100 mM); the fluorescein anion was driven into the cell by the current (50 to 200 pulses of 100 msec duration over periods of 2 to 10 min). (For further details, *see* Oliveira-Castro & Loewenstein, 1971.) The voltage recording electrodes and usually also the second current-passing electrode were filled with KCl (3 M). In the experiments in which cell-to-cell flow of fluorescein alone was examined, only one intracellular microelectrode was used. For this purpose, cell groupings were chosen in which small clusters of normal cells were surrounded by cancerous cells in
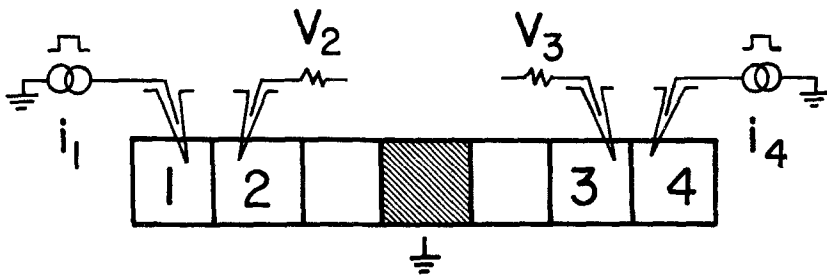
Fig. 1. Schematic representation of the cellular and electrical arrangements for measure-
ments of coupling across a cancerous cell bridge. Normal cells, white, cancerous cell,
hatched. Cells *1* and *4* contain microelectrodes for passing of current between cell interiors
and external medium (grounded). Cells *2* and *3* contain microelectrodes for voltage
recording between cell interiors and medium. The two cancer-cell/normal-cell junctions
are buffered from the disturbance of electrode insertion by one or more unimpaled
normal cell

visible contact or vice versa (Figs. 6 and 7). The co-culture times in the incubator ranged
from 4 hr to 2 days.

Homogeneous Cultures. For electrical measurements and fluorescein injections in
homogeneous cultures, the microelectrodes were placed in contiguous cells as diagrammed
in Fig. 10 (*b*). First a measurement of coupling was taken with a current probing electrode
i_1 and a voltage recording electrode V_2 in contiguous cells. (The limit of resolution of
the measurements was a voltage$_2$/current$_1$ slope of $2 \times 10^4 \, \Omega$). Then to check cell mem-
brane integrity — and obligatorily in the case of the uncoupled class-A cells — additional
microelectrodes i_2, V_1 were inserted for measurement of the resistance between interior
and exterior of these cells *(input resistance)*; the acceptance standard was an input
resistance $\geq 10^6 \, \Omega$ in each cell. This standard was twice the minimum input resistance
found in a series of measurements on A-cells not in contact (Borek *et al.*, 1969).

All measurements and injections were made at room atmosphere and temperature
(ca. $23^\circ C$), unless stated otherwise.

Cell Marking

The cancerous cells were readily distinguishable from the normal ones by their
morphology in the live cultures (e.g., Fig. 6 (*a*)). In addition, radioactive thymidine
labelling was used as an independent means of cell identification. The nuclei of one of the
cell types (generally the cancerous type) were labelled before cell mixing by a 24-hr
exposure to 1 μC/ml tritiated thymidine in the medium (specific activity 6,000 mC/mmole).
Following the electrical measurements or fluorescein injections, the cells were fixed in a
1 % glutaraldehyde solution in phosphate buffered saline in the culture dishes, and covered
with a film of Kodak NTB-2 photographic emulsion in preparation for standard radio-
autography. Upon completion of radioautography (10-day exposure, 10 °C), the dishes
were stained with Giemsa (5 %, 3 min). A double vernier built into the microscope stage
provided a convenient coordinate system for localization of the cells in the dishes. Photo-
graphs of the relevant live cell regions (phasecontrast microscopy) were routinely taken
immediately after the coupling measurements. The radioautographs could thus be easily
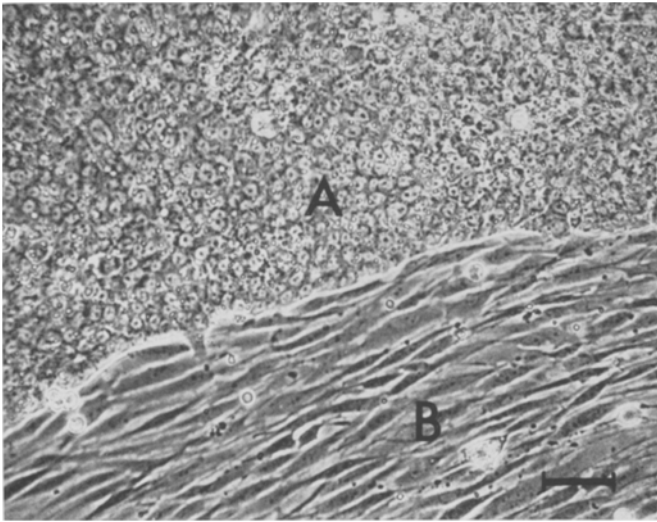matched with the photographs (Figs. 5–7).

Fig. 2. A- and B-cells. Phasecontrast photomicrograph of a 12-day old culture of an explant of Morris' liver tumor. The cells are in F-12 medium, wherein both classes of cells adhere to the culture dish. Calibration 50 µ

Results

Two classes of cells were isolated from tissue explants of Morris' tumor. One class, of epithelial morphology, was cancerous (A- and A'-cells); the other, of fibroblastic morphology, had normal growth characteristics (B-cells) (Fig. 2). The two classes differed radically in their ability to establish junctional communication, as shown by the following experiments.

A- and A'-Cells (Cancerous)

Cell Combinations

Electrical Measurements. Fig. 3 shows the result of a basic measurement of coupling taken on two sets of normal liver cells joined by a single cancerous A'-cell. The cancerous cell is the only cell bridge between the two normal sets. The distance between measuring electrodes across the bridge is very much smaller than the distance (>20 cell diameters) over which the imposed voltage signal is detectable in normal coupled liver cells. The result is simple: the normal cells are electrically coupled to each other on either side of the cancer bridge, but not across this bridge.

We examined 15 situations of this kind with one or two normal cells intervening between the A'-cell and the normal cells containing the measuring
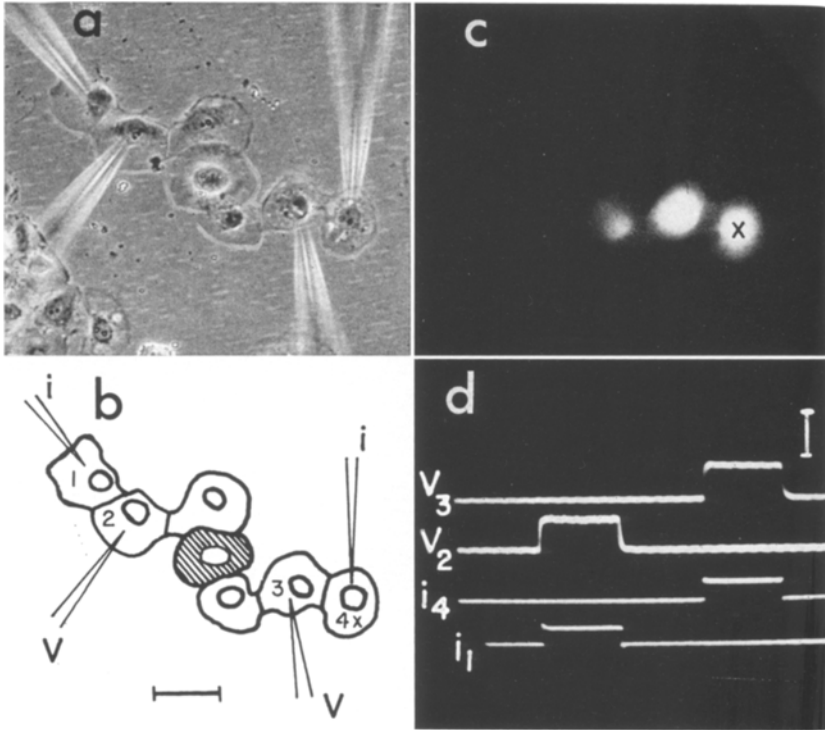
Fig. 3a–d. Lack of coupling between normal liver cells and a cancerous A′-cell from a liver tumor. Measurement across a single-cell cancer bridge. The bridge was made by manipulating a cancerous A′-cell into a gap in a chain of normal liver cells (spontaneously occurring configuration) and by incubating the cell assembly for 2 hr before the measurements. (a) Phasecontrast photomicrograph of the liver cells taken at the end of the electrical measurements (step 2, see Methods), showing four microelectrodes in intracellular position. (b) Tracing of the micrograph, cancer cell hatched; calibration 50 μ. (c) Dark-field photomicrograph of the fluorescence after injection of fluorescein into cell 4 (x). The injection was simultaneous with the electric measurement in step 1. (d) Oscilloscope records of inward currents i_1, i_4 (3×10^{-8} amp; 100 msec pulse duration) injected into normal cell 1 and, with a delay of 100 msec, into cell 4; and of the resulting membrane voltages (V) in cells 2 and 3. The record is from a 4-beam oscilloscope with common time base; the rectangular current pulses are of 100 msec duration. The record is from measuring step 2; step 1 not shown. Calibration 500 mV

electrodes. All situations gave the same results. Evidently the cancerous A′-cell fails to establish junctional communication with the normal cells. This contrasts sharply with the behavior of the normal cells, which make junctional connections readily in culture with cells of their own kind, as well as with normal cells from different organs and animal species (Michalke & Loewenstein, 1971). Fig. 4 illustrates this for a control experiment in which a normal lens cell bridges two sets of normal liver cells.
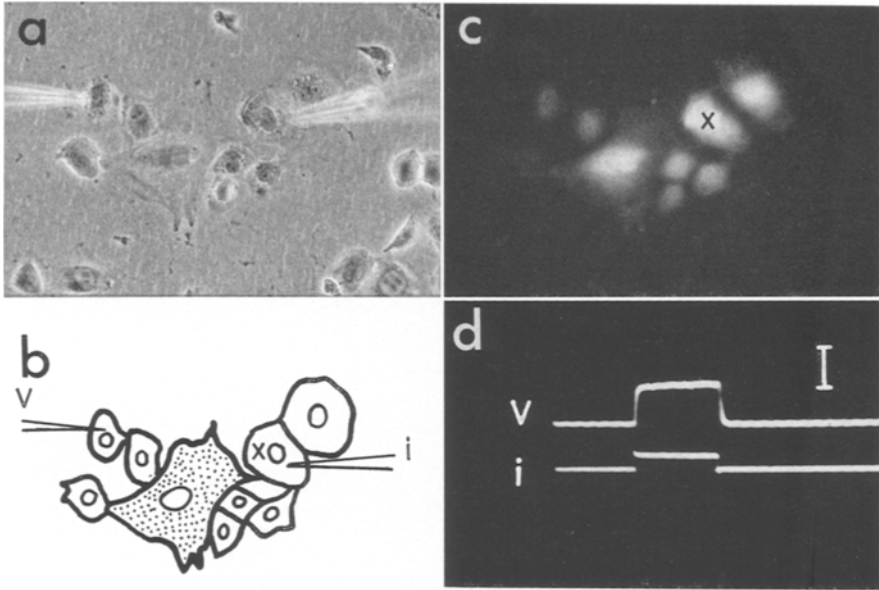
Fig. 4a–d. Control measurement of coupling across a normal heterotypic cell bridge. The lens cell bridge between normal liver cells was fashioned like the cancer bridge of the experiment of Fig. 3. Co-culture time, 2 hr. (*a*) Phasecontrast photomicrograph of the cell ensemble. (*b*) The corresponding tracing. Lens cell, dotted; liver cells, white. Calibration 1 cm = 55 μ. (*c*) Darkfield photomicrograph after fluorescein injection into cell *x*. (*d*) Oscilloscope records of a current pulse *i* driving the fluorescein anion (2×10^{-8} amp; 100 msec duration), and the resulting V in a liver cell on the other side of the bridge. Calibration 500 mV

Aside from the aforegoing experiments, situations with multicellular cancerous bridges were examined (Fig. 5). The situations included combinations of A'-cells with normal liver cells (29 cases), with normal lens cells (17 cases) and with normal BHK cells (12 cases); and combinations of A-cells with normal liver cells (11 cases). Again, at least one normal cell, and usually 2 to 4, lay between the bridge and the normal cells containing the microelectrodes, and the distance between measuring points was well within the range of detectability of voltage signals in normal cell cultures. The results were the same as in the case of the single cell bridge.

Fluorescein Injections. Cellular communication was probed also with fluorescein. Aside from broadening the information to another order of magnitude of molecular size, the fluorescein method offers the advantage over the electrical measurement of permitting one to scan for communication at several identifiable cell junctions with a single microinjection. Fluorescein, a fluorescent anion of 330 mol wt has already been used successfully in studies of cellular communication in several organized tissues (Loewen-
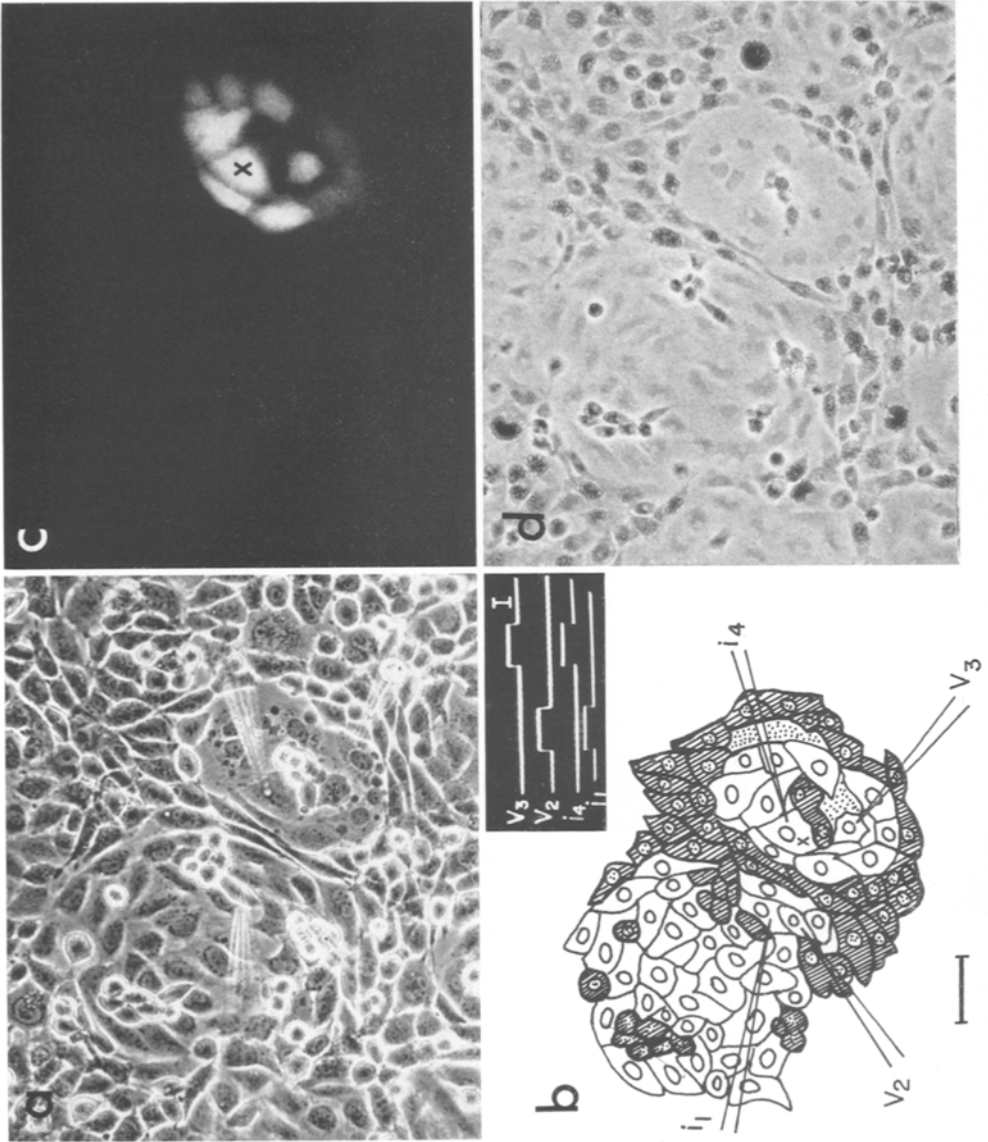
Fig. 5a–d. Lack of coupling between normal and cancerous cells. Measurements across a multicell cancer bridge. A spontaneously occurring cell configuration in a co-culture of liver and cancerous A'-cells. The cells had been in co-culture for 6 hr before the measurements. (a) Phasecontrast photomicrograph of the cells at measuring step 2. (b) Tracing of the photomicrograph. Cancer cells hatched; normal cells white; unoccupied spaces, dotted. Calibration 50 μ. *Inset:* oscilloscope records of i_1, i_4 (2×10^{-8} amp; 100 msec duration) and V_2, V_3 in measuring step 2. Calibration 500 mV. (c) Darkfield photomicrograph after fluorescein injection into cell 4 (x). (d) Radioautograph; A'-cells are ^{3}H-labelled

stein & Kanno, 1964; Pappas & Bennett, 1966; Rose, 1971; J. Tupper, *personal communication*) and in tissue cultures (Potter, Furshpan & Lennox, 1966; Furshpan & Potter, 1968). There are good reasons for assuming that fluorescein takes the same junctional route as the small inorganic ions that carry the current in the electrical measurements: (1) the present cells take up detectable amounts of fluorescein from the outside only if the fluorescein concentration in the medium is above 10^{-2} M, far above the visible level (10^{-11} M), and even then only after exposures of the order of 1 day; intracellularly injected fluorescein was seen to pass within 20 sec from one cell to another, and it never reached visible levels in the medium even many hours after cell injection. Moreover (2), in salivary gland it has been shown that several procedures that block the passage of the small ions through the cell junction also block the passage of fluorescein (Kanno & Loewenstein, 1966; Oliveira-Castro & Loewenstein, 1971; Rose & Loewenstein, 1971).

Figs. 3 (c) and 5 (c) illustrate the results of fluorescein injections made simultaneously with the electrical measurement and into the same cells on
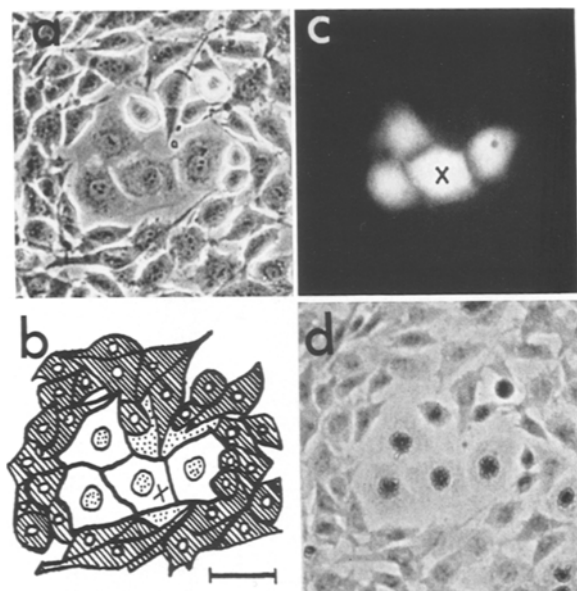

Fig. 6a–d. Non-passage of fluorescein between normal and cancerous cells. A spontaneously occurring configuration of 4 normal liver cells surrounded by cancerous A'-cells. One of the normal cells (x), the only cell impaled with a micropipette, was injected with fluorescein. The normal and cancerous cells were in co-culture for 26 hr before the injection. (a) Phasecontrast micrograph. (b) Tracing of the micrograph; normal cells white, cancerous cells hatched, unoccupied spaces dotted; calibration 50 μ. (c) Darkfield photomicrograph after fluorescein injection. (d) Radioautograph; the nuclei of the normal cells are ³H-labelled
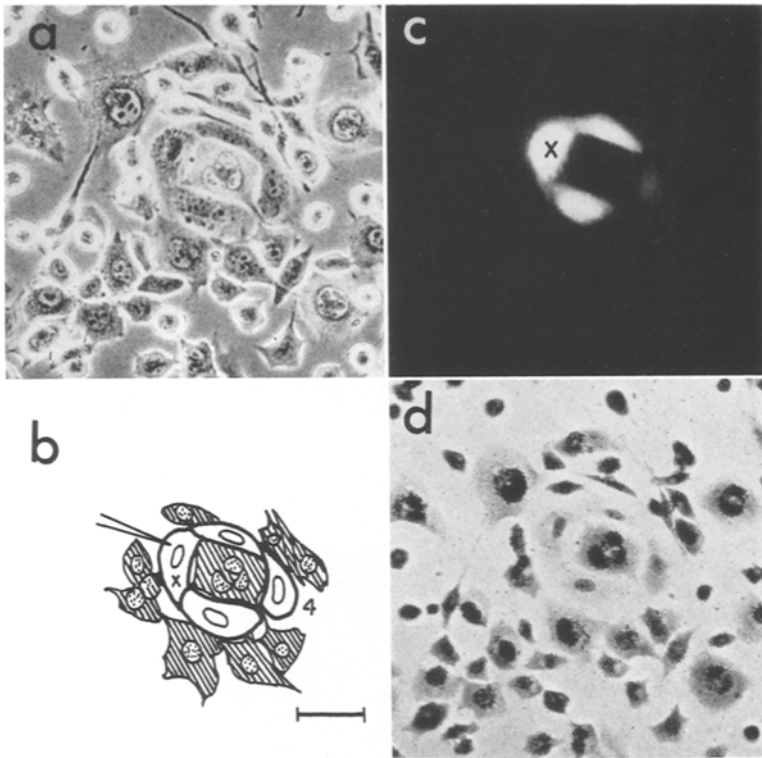
Fig. 7a–d. Non-passage of fluorescein between a cancerous A′-cell and 4 surrounding normal cells. Spontaneously occurring configuration. *x*, normal cell injected with fluorescein (this was the only cell containing a micropipette). Co-culture time before injection, 14 hr. (*a*) Phasecontrast photomicrograph. (*b*) Tracing of the photomicrograph; normal cells white, cancerous cells hatched; calibration 50 μ. (*c*) Darkfield photomicrograph. (*d*) Radioautograph; the nuclei of the cancerous A′-cells are ^{3}H-labelled. Note that fluorescein passed to all normal cells in contact, including cell *4*, the most distant and most faintly fluorescent of the three cells

which the aforedescribed electrical measurements were taken. Fluorescein is seen to spread throughout all normal cells in visible contact with the injected normal cell (without detectable leakage to the exterior), but not across the cancerous A′-cell bridges. The lack of communication is particularly clear in situations of the kind shown in Figs. 6 and 7 where the injections are made into normal cells belonging to groups with multiple A′-cell contacts. In the case of Fig. 6, the normal cells are surrounded by many cancerous cells in visible contact; in Fig. 7, the reverse applies. In either case, fluorescein stays within the confines of the normal cells. These results are typical of 84 experiments. The results of a control experiment on a normal-liver-cell/normal-lens-cell combination are shown in Fig. 8.
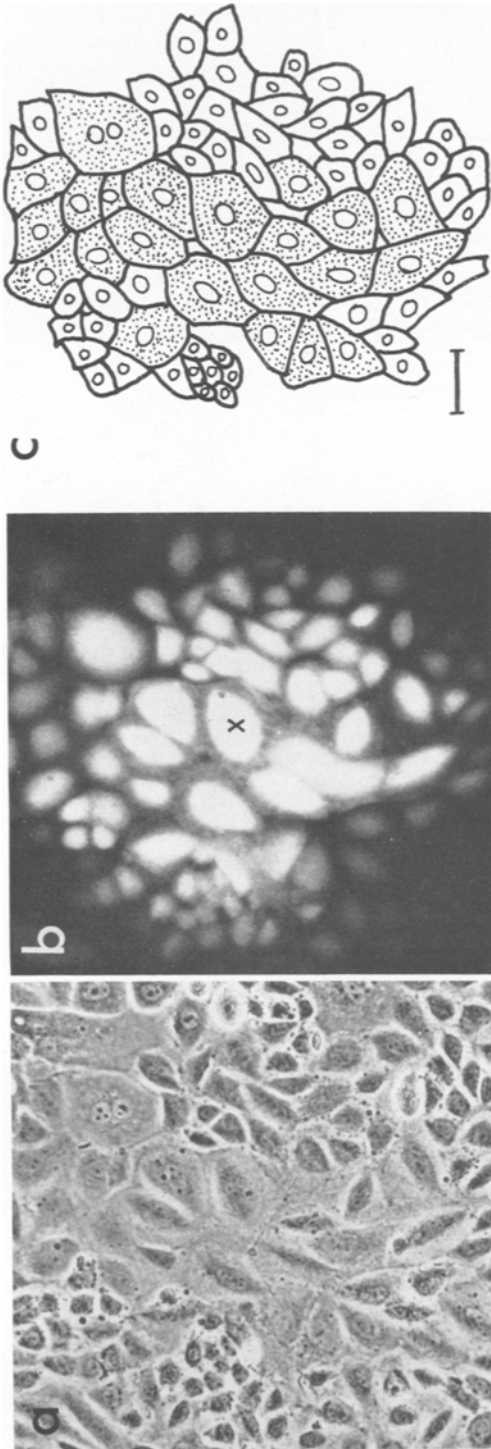
Fig. 8a–c. Fluorescein passage between normal cells of different type. Control experiment on normal lens cells in contact with normal liver cells; spontaneously occurring configuration. Co-culture time 22 hr before fluorescein injection into one of the lens cells (x). (a) Phasecontrast photomicrograph. (b) Darkfield photomicrograph. (c) Tracing of the micrograph; lens cells, dotted; liver cells, white. Calibration 50 μ

The A'-cells failed to couple in medium containing fetal calf serum, the serum used in the combinations with liver and BHK cells (*see* Methods), as well as in calf serum, as used in the combination with lens cells. The situation is thus different from that in certain hamster fibroblast cultures where coupling depends on factors in fetal calf serum and in calf serum (Borek *et al.*, 1969). The situation differs also in this respect from that in Crocker mouse sarcoma cells where coupling depends on the schedule of medium renewal (Furshpan & Potter, 1968). The failure of coupling in A- and A'-cells ensued regardless whether the medium in the dishes was changed just before the measurements or was left unchanged for hours or days. The cells failed to couple at 23° C, the usual temperature at which measurements were made, as well as at 37° C (2 cases; fluorescein).

All electrical measurements across cancerous cell bridges were done in conjunction with fluorescein injections. This was necessary, because, like a number of other cancerous cells (e.g., Abercrombie, Heaysman & Karhauser, 1957; Abercrombie & Ambrose, 1958; Temin & Rubin, 1958), the A- and A'-cells can overgrow and conceal fine processes extending from normal cell, or even entire normal cells. The consequent pitfalls were first recognized in studies of electrical coupling on A'-cell/normal-lens-cell and A'-cell/normal-fibroblast combinations (Michalke & Loewenstein, *unpublished*). The puzzling situation was then sometimes encountered where two normal cell groups presumed to be bridged by cancerous cells only, after the pattern of Fig. 1, were electrically coupled. When the cultures were subsequently fixed and the cancerous cells peeled off (they were less adhesive than the normal cells), the connecting normal cell processes became visible. In the present experiments, such cellular overlap was spotted by the fluorescein technique (Fig. 9).

These pitfalls of the electrical method also decided us – unfortunately not before a painfully long and fruitless trial by our colleague, F. Rodesch (1969, *unpublished*) – against using a combination with beating heart cells as employed by Goshima (1969, 1970). The heart cells (chick embryo in Rodesch's work) frequently made fine long processes that were readily overgrown by the cancerous A- and A'-cells, as, indeed, were entire heart cells. We found it therefore not feasible to rely on electrical recording alone in heart-cell/cancer-cell combinations. Consequently, any experimental advantage the heart cell may have held over other cells due to its built-in electrical generator, was more than offset by the larger size and easier impalement of the quiescent liver and lens cells.

Homogeneous Cell Cultures

No electrical evidence of coupling was found in homogeneous A-cell and A'-cell cultures (Fig. 10). Of 32 measurements, none gave a result of coupling.
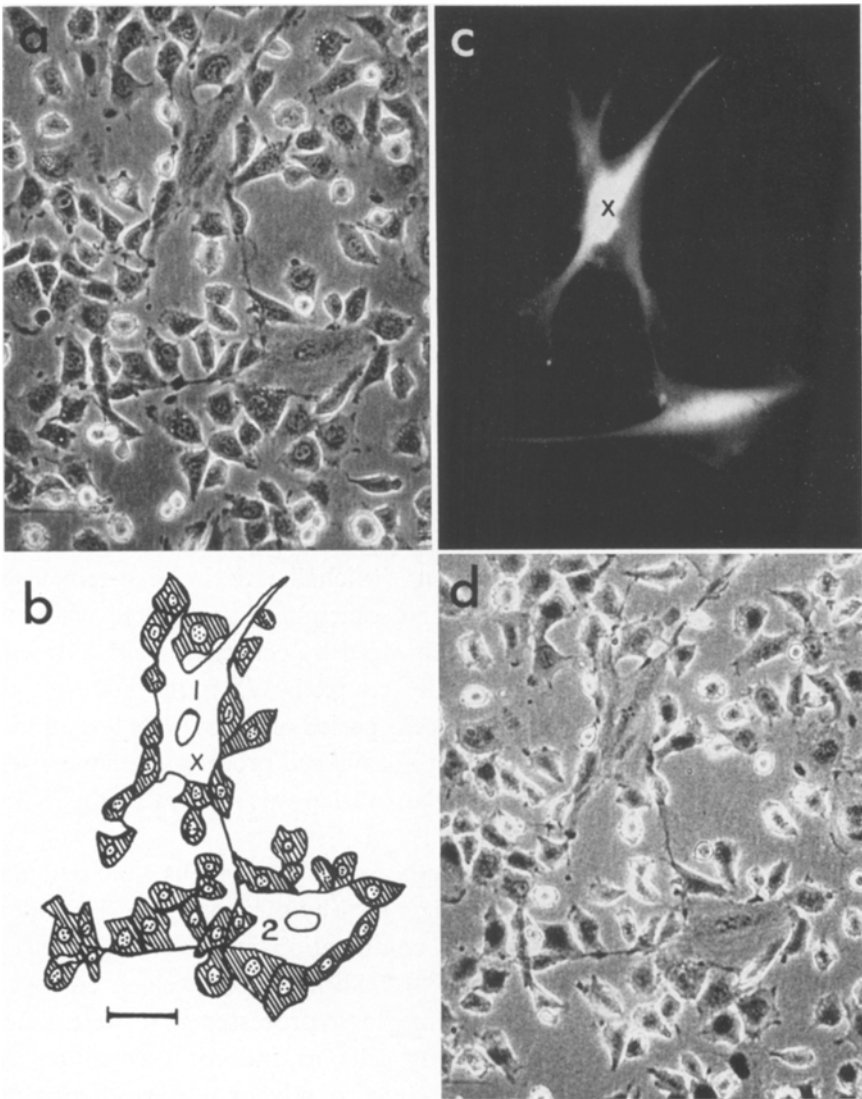
Fig. 9a–d. Fine connecting normal cellular processes underneath cancerous A′-cells. An injection of fluorescein into a normal cell (*1*) reveals fine processes connecting this cell with another normal cell (*2*) across what, before injection, looked like a gap between normal cells occupied by cancerous cells (hatched) only. (*a*) Phasecontrast micrograph. (*b*) Tracing of the micrograph; calibration 50 μ. (*c*) Darkfield micrograph. (*d*) Radioautograph; cancerous A′-cells are ³H-labelled

The A-cells and A′-cells appeared also to be uncoupled in respect to fluorescein. We made 63 fluorescein injections, some simultaneously with
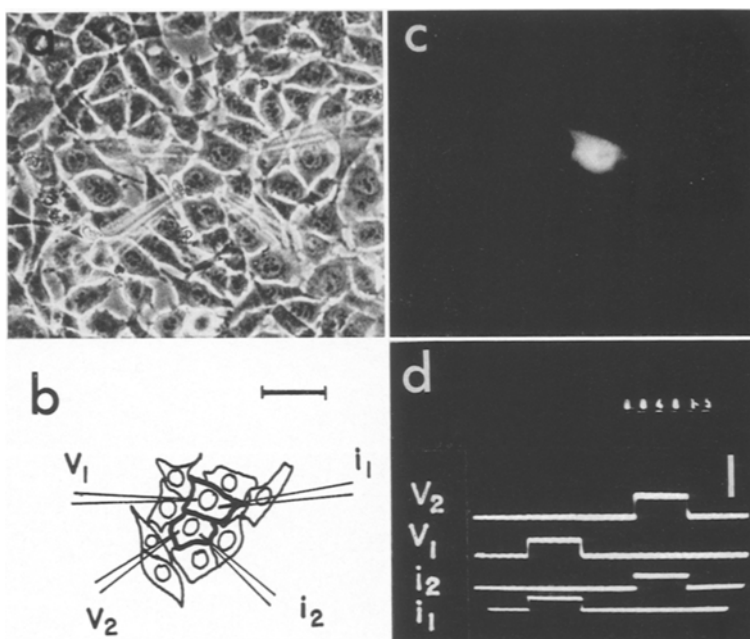
Fig. 10a–d. Lack of coupling between cancerous A′-cells. Electrical measurement and fluorescein injection in a homogeneous A′-cell culture. (*a*) Phasecontrast photomicrograph taken at final measuring step. (*b*) Tracing of the micrograph; calibration 50 μ. (*c*) Dark-field micrograph after injection of fluorescein into cell *1*. (*d*) Oscilloscope records of i_1, i_2 (2×10^{-8} amp; 100 msec duration) and V_1, V_2 from the final measuring test of coupling and of cell membrane integrity with four intracellular microelectrodes (*see* Methods). The fluorescein injection in (*c*) was simultaneous with the preceding measuring step of coupling in which only the fluorescein-containing electrode and the voltage-recording electrode were inside the cells. Voltage calibration 500 mV

electrical measurements. In no instance was fluorescein seen to pass beyond the boundaries of the injected cells (Fig. 10).

B-Cells

The B-cells are coupled. Electric current as well as fluorescein pass through their junctions (Fig. 11). They do not differ appreciably in this respect from cells of normally growing tissues.

Growth Characteristics of A- and B-Cells

The A- and A′-cells are cancerous: (1) They do not show contact inhibition; they pile up on each other and on normal cells, reaching densities
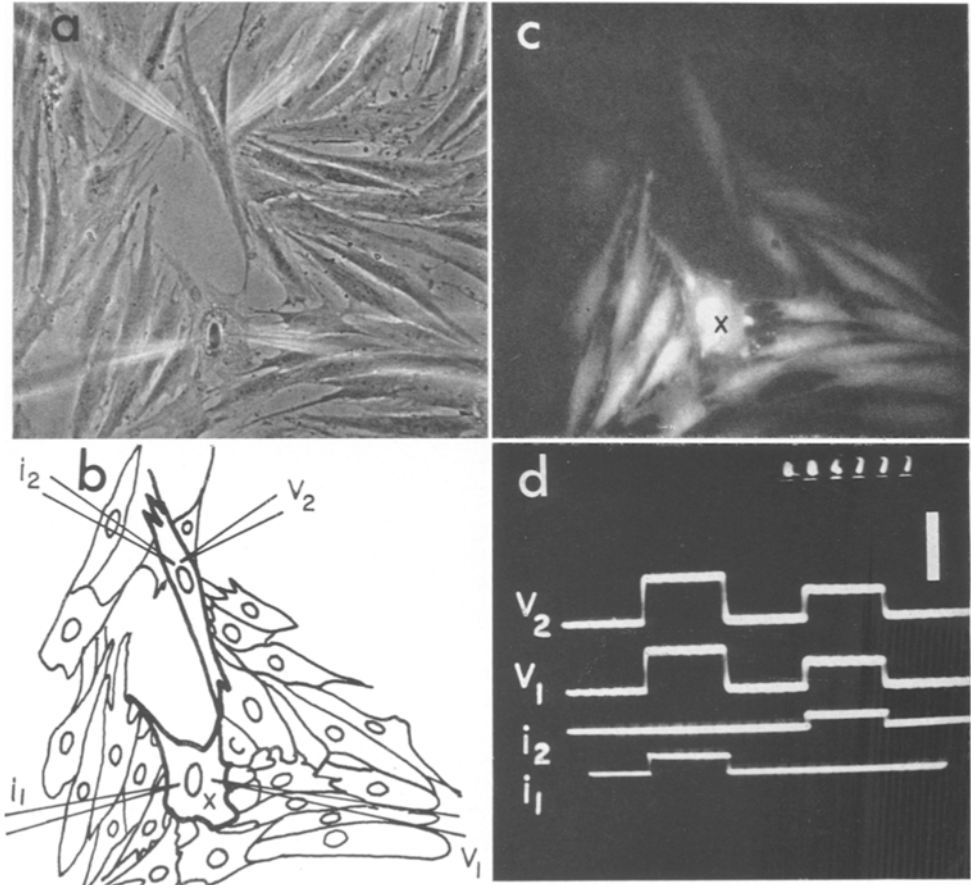
Fig.11a–d. Coupling between B-cells (homogeneous culture). (*a*)Phasecontrast photomicrograph. (*b*) Tracing of the micrograph; calibration 1 cm = 55 μ. (*c*) Darkfield; cell *x* injected with fluorescein. (*d*) Oscilloscope records of i_1 (1.5×10^{-8} amp, 100 msec), i_2 (1×10^{-8} amp) and V_1, V_2. Voltage calibration for V_2, 500 mV; for V_1, 550 mV

above 10^6 cells/cm² in the culture dishes (the normal liver cells are contact inhibited). (2) They form malignant tumors when injected into animals; 10^3 cells readily produced fatal tumors.

The coupled B-cells have normal growth characteristics: (1) They are contact inhibited, reaching densities of the order of 10^4 cells/cm² in the culture dishes. (2) They did not form tumors when injected into the animals (10^6 cells). The incidence of tumor formation with these cells was zero (7 trials) as against an incidence of 100% with the A-cells (31 trials).[1]

Fig. 12 illustrates typical growth curves of the two classes of cells.

1 Great care had to be taken to use pure B-cell suspensions for the tumorigenicity tests. Small A-cell contaminations of the inocula caused tumor formation.
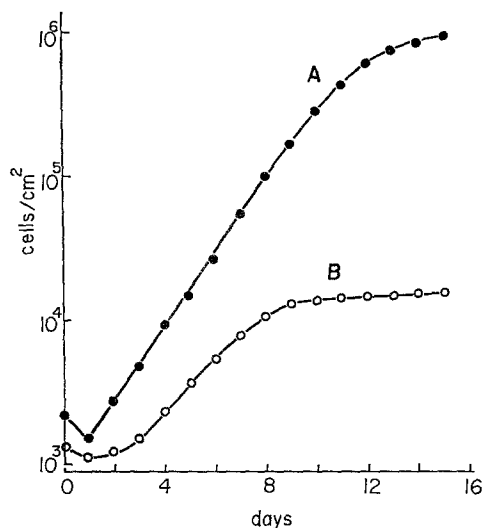
Fig. 12. Growth curves of A-(•) and B-cell cultures (○). Time zero is the time of cell seeding

Discussion

Of the two classes of cells (A and B) isolated from the tumor, class-A is the one of immediate interest in the context of the idea that the cell junction may be instrumental in conveying growth controlling signals. This class appears to be incapable of junctional communication. It fails to make communicative junctions with three types of normal cells (which make junctions readily), as shown unambiguously by the electrical measurements and fluorescent tracer results in cell combinations in which the A- and A'-cells were not directly probed. We may also be quite confident that these cells also fail to make communicative junctions among themselves, since in no instance of homogeneous cell culture was communication detectable electrically or with fluorescent tracer. Although direct probing of the uncoupled cells could not be avoided in the homogeneous cultures, there are no obvious reasons why communication in A-cells should have been more disturbed by the probing than in the normal cells; the A-cells were actually more easily impaled by the microprobes than the normal cells and they generally retained their resting potential better.

Thus, it is of great interest that these cells are also the cancerous ones, as shown by their high densities in culture and their ability to produce malignant tumors when injected into the animals; the hypothesis of a junctional involvement in growth control implies that *all uncoupled cells (by genetic defect) are potentially cancerous* (Loewenstein, 1968a). This is

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one of the few aspects of the hypothesis amenable to experimental test at this time. The converse, of course, is not necessarily true. Indeed, several kinds of cancerous cells are known to be coupled (Potter *et al.*, 1966; Borek *et al.*, 1969; P. O'Lague & H. Rubin, *personal communication*; Sheridan, 1970).

The present results may also throw some light on the question of why in earlier work cultured cells isolated from Novikoff's tumor were found to be coupled (Borek *et al.*, 1969), whereas measurements taken on the tumors indicated the cells to be uncoupled (Loewenstein & Kanno, 1967). Novikoff's tumor contains various cell types; the culture medium may have favored (and been adequate only for) the coupled cells, just as the E-D medium in the present experiments favored the B-cells from Morris' tumor. (Other possibilities are discussed by Borek *et al.*, 1969, and by Loewenstein, 1968 *b*.) For the same reason, and other difficulties aside (Jamakosmanovic & Loewenstein, 1968), the interpretation of electrical coupling measurements on solid tumor calls for caution: the predominance of one cell type over others in a tumor may conceivably vary owing to variations in the tumor environment in the animals. Perhaps the discrepancies in the measurements in solid Novikoff's tumor of Loewenstein & Kanno (1966) and of Sheridan (1970) may be attributable to such variations (*see* also Sheridan, 1970, and Loewenstein, 1968 *b* for further discussion of this point).

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