ACTIN CONTENT AND ORGANIZATION IN NORMAL AND TRANSFORMED CELLS IN CULTURE

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ABSTRACT

The amount of actin and total protein per cell in normal rat kidney (NRK) cells in culture is initially high in very low density cultures, but rapidly decreases as the cells come into contact in higher density cultures. In a viral transformant of NRK (442), the level of actin and total protein does not change significantly from low to high density cultures. NRK cells, which are flattened against the substrate, have prominent bundles of actinlike microfilaments in the basal cytoplasm adjacent to the substrate. 442 cells, which adhere poorly and are more spherical in shape, lack well-organized basal microfilament bundles, but may display microfilament bundles in cytoplasmic processes extending from the cell body. The percentage of insoluble actin is <20% in both cell lines, and 442 cells consistently contain smaller amounts than NRK cells.

KEY WORDS actin quantitation · microfilaments · NRK cells · contact inhibition · transformation

During growth and differentiation, cells may undergo dramatic changes in their shape, rate of motility, and extent of intercellular contact. Actin is thought to play a major role in these and other basic cellular activities, and it is not surprising that actin is now known to be one of the most abundant proteins in many cell lines that are currently used in biological studies (3, 24, 28). Accordingly, there has been much recent interest in the ways in which actin may vary in form, quantity, and distribution with changing patterns of cellular activity. Recent observations have suggested, for example, the possibility that alterations in the rate of cellular motility may correlate with changes in intracellular actin concentration (9, 28). A number of reports have focused on the differences that may be observed between certain types of normal and transformed cell lines in the organization of actin filaments into bundles and in the disposition of these bundles within the cell (5, 10, 13, 21, 25, 34, 35). Other reports suggest that the extent of cellular contact with the substrate or with other cells in vitro may stimulate the formation of actin filaments in cells (14, 20). We report here a study in which normal rat kidney cells (NRK) and their Kirsten viral transformants (442 cells) are compared as to the amount and organization of actin during normal log growth and in high density culture conditions.

MATERIALS AND METHODS

Cell Culture Conditions

NRK and 442 cells were obtained from the laboratory of Dr. Keith Porter (University of Colorado, Boulder, Colo.). Cultures were periodically renewed from pleuropneumonia-like organism-free frozen stocks. Both cell types were grown in Dulbecco's medium fortified with 10% fetal calf serum and 1% penicillin-streptomycin antibiotic. Cells were grown on 75-cm² tissue culture

flasks and removed by the addition of 0.5% trypsin and 0.2% EDTA in Puck's saline G. To minimize protein degradation, the cells were removed after 4 min of incubation at 37°C and the flasks were washed once in Puck's saline G to remove residual cells. An aliquot was removed from this pooled cell suspension for immediate cell counting with a hemacytometer. The cells were then spun down in a clinical centrifuge and the pellet was immediately solubilized by the addition of sodium dodecyl sulfate (SDS) sample buffer. The time for complete solubilization to occur was reduced by placing the sample in boiling water for 1 min and agitating in 3–4 vol of SDS sample buffer. The entire procedure from the time of addition of trypsin EDTA solution to complete solubilization took no more than 7 min.

Trypsinization and Cell Solubilization

Cells were plated out at differing densities from trypsinized stock cultures, grown through four cell cycles, and removed from the tissue culture flasks by another application of trypsin and EDTA. The time that the cultures were maintained after the initial trypsinization and plating was always constant. The harvested cells were solubilized immediately with SDS-mercaptoethanol sample buffer for subsequent quantitation of actin and total protein. It was necessary, however, to demonstrate that the trypsinization at harvesting did not affect these values, and experiments were undertaken to test this. Cells were removed after progressive time intervals in the standard trypsin-EDTA solution. It was found that the values of total protein and actin per cell were not altered more than 5% by exposure to trypsin for up to 15 min. After 15 min, significant alterations in the values of actin per cell were observed. These included a maximum of 30% reduction in the values of actin per cell at 30 min in trypsin-EDTA solution. The values of total protein per cell after 15 min were uninterpretable due to initiation of cellular lysis. Therefore, all experiments were done in such a manner as to lyse the cell pellet after no more than 6 min in trypsin-EDTA solution, during which time the cells were also removed from the substrate and washed.

Quantitative Polyacrylamide Gel Electrophoresis and Total Protein Quantitation

5- or 10-ml samples were applied to 0.75 mm thick 12% SDS polyacrylamide slab gels. This system and the methods of stain quantitation have been described previously (15). Total protein determination of whole cell preparations solubilized in the SDS sample buffer was carried out by a modification of the Bradford dye-binding protein assay (1). Small aliquots were removed directly from the samples solubilized in sample buffer and diluted 1 to 100 with water and assayed according to the Bradford Coomassie Brilliant Blue G dye-binding method. The bovine serum albumin protein standards were originally solubilized in SDS sample buffer and were also diluted 1 to 100 before the standard Bradford assay. Plots were initially made of the optical density versus microliters of applied sample to determine the relative amount of solubilized whole cell material which produced a linear relationship between the amount applied and the degree of dye-binding. All further assays were carried in the linear ranges of these curves. Rabbit skeletal muscle actin isolated by a modification of the method of Mommaerts (22) was quantitated by the method of Lowry et al. (18), and five to seven aliquots of actin were applied to each slab gel to serve as a standard actin protein curve when quantitating actin from banding patterns of whole cell homogenates.

Identification of Actin Bands and Demonstration of Purity

Whole cell preparations from NRK and 442 cells solubilized in sample buffer were run on preparative slab gels and were stained with Coomassie Brilliant Blue R in 25% TCA and destained in 7% acetic acid. The actin bands were then cut out with a razor blade, and the actin was eluted, iodinated, and trypsinized according to the procedure of Bray and Brownlee (2). Chromatographic analysis was carried out on silica gel-G plates eluted with ammonium hydroxide:*N*-propanol (8). Autoradiographs were made with standard X-ray film and the patterns were recorded by scanning slices of the film on a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with linear transport.

A further demonstration of the purity of the actin band was obtained by examining two-dimensional gels of whole solubilized NRK and 442 cells using the method of O'Farrell (23) as modified by Wilson et al. (37).

Percent Polymerized Actin

To obtain values for the percentage of polymerized actin in the NRK and 442 lines, cells grown on plastic flasks were washed twice with Hanks' balanced salt solution and removed with a rubber policeman after the addition of 2.5 ml (per 100-cm^2 flask) of the NP40 actin stabilization buffer (4). The cells were then sonicated for 5 s at 0°C and centrifuged for 3 h at 100,000 g. The resulting pellet and supernates were then run on one-dimensional SDS slab gels and the prominent actin bands were quantitated as described above.

To determine the extent to which the Bray and Thomas (4) method of actin stabilization allows polymerization or depolymerization of the actin in cellular homogenates during the procedure, the following series of experiments were undertaken. NRK cells were grown on 100-cm^2 plastic tissue culture flasks, washed twice in leucine-free and serum-free media, and then labeled in 4 ml of leucine-free and serum-free media to which was added 75 μCi of [^{3}H]leucine (100 Ci/mmol). The cells

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were labeled for 12 h and then separated into high-speed supernate and pellet as described above using the NP40 stabilization buffer of Bray and Thomas (4). The pellet and supernatant fractions were then added separately to live cells which had been washed in Hanks' solution. These cells with their added labeled pellet or supernatant fractions were in turn solubilized in the NP40 stabilization buffer and sonicated as described above. These preparations were finally separated into high-speed pellet and supernatant fractions such that four total fractions had now been obtained. Any shift in actin from the polymerized to depolymerized or the depolymerized to polymerized form in the course of the homogenization or subsequent centrifugation procedures could thus be detected as a loss or addition of radioactivity to the actin bands of the above-described four subfractions.

Preparation of Cells for Electron Microscopy

For transmission electron microscopy, cells grown on carbon-coated cover slips were fixed in a glutaraldehyde/paraformaldehyde mixture (17), postfixed in OsO_4, and embedded in Epon. For scanning microscopy, cells fixed upon cover slips were dehydrated in acetone and dried by the critical-point method.

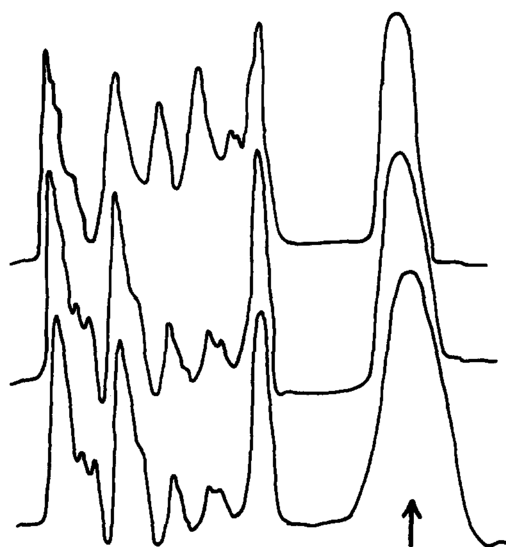

FIGURE 1 Densitometer tracings of autoradiographs taken from thin-layer chromatographic separations of ^{125}I-labeled tryptic peptides. The upper trace is from a band on one-dimensional slab gels which were run using purified rabbit muscle actin. The middle trace is from the actin band of the NRK whole cell homogenate, and the bottom trace is the presumptive actin band removed from the 442 viral transformant of the NRK cell line. Large arrow denotes origin.

RESULTS

Quantitation of Actin and Total Protein

To test the possibility that actin and total protein content vary during culture growth, we have examined values of the amount of actin and total protein per cell during log growth and at confluence of NRK cells and their viral transformant designated 442. Initially, it was necessary to demonstrate that the Coomassie Brilliant Blue R-staining band on SDS gels which coelectrophoresed with rabbit muscle actin was indeed actin and was not measurably contaminated with other polypeptides. This was accomplished as described in Materials and Methods by an iodination and tryptic peptide fragment analysis. The results shown in Fig. 1 clearly indicate that the presumed actin protein bands seen on gels of NRK and 442 samples are highly similar to rabbit muscle actin. To further examine the homogeneity of the actin band, two-dimensional electrophoresis was run on whole cell homogenates. The results indicate that the actin band is essentially free from other contaminating polypeptides of differing charge characteristics (Fig. 2).

The amount of actin per cell decreased during log growth of the NRK line. Fig. 3*A* indicates the changes in these values as the cells became confluent Cell-to-cell contact was observed in both NRK cells and the 442 transformants at ~4,000,000 cells per flask. The NRK cells showed a dramatic reduction in actin content per cell when cell contact was established (Fig. 3*A*). The scatter of the data at the lower cell densities may arise from inaccuracies in cell counts made with the hemacytometer. The amount of actin per cell in the 442 cell line did not change during the growth cycle (Fig. 3*B*).

To determine whether these changes in actin content were related to corresponding changes in the amounts of total protein, the latter was measured using the same samples on which the actin determinations were made. For the NRK line, these measurements (plotted in Fig. 4*A*) indicate that total protein per cell decreased during log growth at the same time that the amount of actin per cell decreased (Fig. 3*A*). When actin is plotted against the amount of protein per cell, no change is observed in the NRK line with growth (Fig. 4*A*). In the 442 cell line, neither total protein (Fig. 4*B*) nor actin per cell decreased as the cells approached confluence. Analysis of variance sup-

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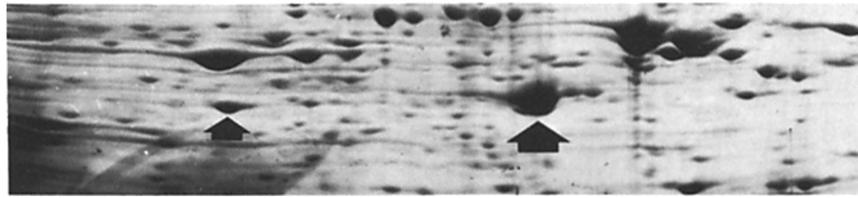

FIGURE 2 A section of a two-dimensional gel pattern of an NRK whole cell homogenate. Small arrow denotes the only major potential contaminant of the actin band on one-dimensional gels. This component runs slightly ahead of the actin band on one-dimensional gels and can be separated from it. Larger arrow denotes actin. The two small spots to the right of the actin spot are secondary charge modifications of actin. Isoelectric focusing cathode to the left. pH range 5.4–8.7 (left to right). Mol wt range 63,000–30,000 (top to bottom).

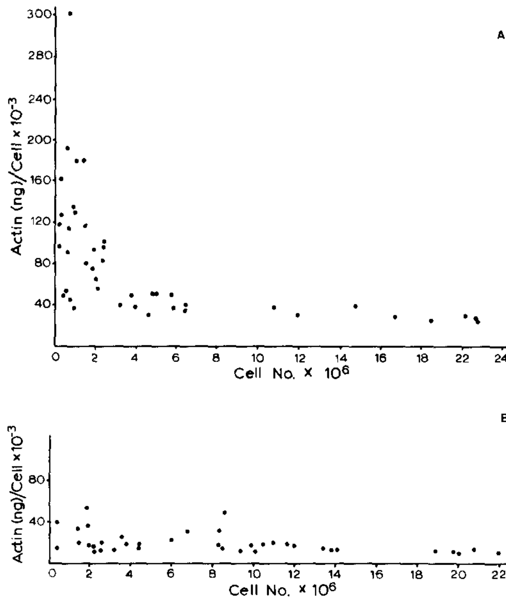

FIGURE 3 Plots of the amount of actin per cell graphed against the total cell number per flask for the NRK cell line (*A*) and the 442 viral transformant (*B*). Each point is a mean from two gels, each of which was internally controlled with rabbit muscle actin standards. More than 30% of the cells that were in contact had densities of 5,000,000 per dish or greater as revealed by phase optics light microscopy. Analysis of variance revealed a statistically significant difference ($P < 0.01$) between points taken at subconfluence (4,000,000 cells per dish or less) and those postconfluence (6,000,000 cells per dish or more) for the NRK line only. There was no statistical significance in a similar comparison made for the 442 transformant.

ports these conclusions at the 1% level. To confirm these results, total protein values were determined by a second method, the standard Lowry procedure (18). The results of this method were similar to those of the dye-binding analysis of total protein per cell.

These changes in actin and total protein per cell were not produced by conditioning of the media, because low density cells grown for two generations in conditioned media (taken from postcon-

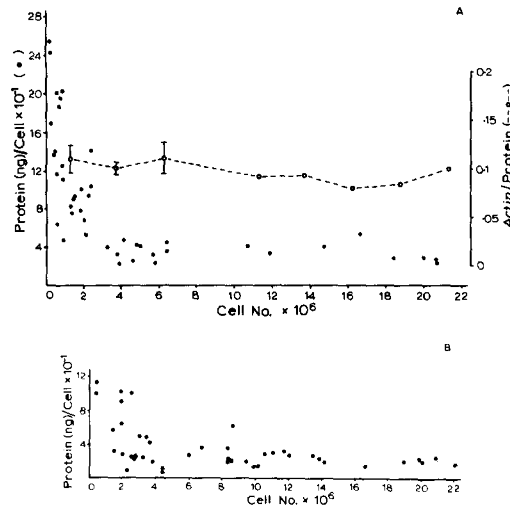

FIGURE 4 Shown are plots of total protein per cell graphed against cell number per flask for NRK (*A*, solid circles) and 442 (*B*) cell lines. Values were obtained using the modified Bradford dye-binding assay on the same samples used to generate the values of actin per cell described in Fig. 3. At the same significance level and cell number values as described in Fig. 3, the NRK line showed a significant increase in values of protein per cell at the lower cell densities. The 442 transformant failed to show this statistical difference. The ratio of actin/protein in NRK cells with growth is shown in Fig. 4*A* (open circles). The ratio does not change. The ratios (ordinate) were determined by averaging the values for actin/cell (Fig. 3*A*) and protein/cell (Fig. 4*A*) for every unit of 2.5×10^6 that the cells increased in number (abscissa).

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TABLE I

Ratio of Pelleted/Supernatant Actin in Confluent NRK and 442 Cells

Exp no.	NRK	442
1	0.1148	0.05166
2	0.125	0.115
3	0.157	0.0683
4	0.208	0.182
5	0.166	0.057
6	0.154	0.115
7	0.112	0.108
Mean	0.1495	0.0996

Analysis of variance reveals significance at $P < 0.05$. Note also that in each pair of experiments the ratio is always higher for the NRK line.

fluent cultures) had the same actin and total protein content as control low density cells.

Percentage of Pelletable Actin

Using the standard Bray and Thomas NP40 actin stabilization buffer, we consistently obtained higher values for the relative percentage of pelletable actin in the NRK cells as compared to the 442 viral transformant (Table I). It is important to note that absolute values for pelletable actin in NRK and 442 lines are not available. This is evident from the series of control experiments given in Table II and Fig. 5. When either the pellet or the high-speed supernate is re-homogenized with new cells, an appreciable portion of the actin is converted to the alternative form. Therefore, at some point during the NP40 lysis, the sonication, or the subsequent centrifugation, some of the actin shifts from the pellet to the supernate, and vice versa. The degree of interconversion appears to be dependent upon the magnesium concentration (Table II), with higher magnesium concentration favoring the pelletable form.

Structural Observations

The NRK cells show a marked tendency to spread upon the growth substrate. At low culture density, the peripheral regions of the cells are

TABLE II

Exp no.	$[Mg^{++}]$	$Pellet_1$ / $Pellet_1$ & $supernate_1$	$Pellet_2$ / $Pellet_2$ & $supernate_2$	$Pellet_3$ / $Pellet_3$ & $supernate_3$
	mM			
1	10	24.2	98.7	27.2
2a	6	33.9	82.4	18.8
2b	6	32.2	87.7	11.7
2c	6	34.4	74.0	13.4
3	3	10.2	67.7	4.8

The percentages given in the columns express the ratio of actin in each pellet at stages 1, 2, and 3 of Fig. 5 to the total actin at each stage (the pellet combined with its respective supernate). The amounts of actin in each pellet or supernate were determined from radioactive actin bands on electrophoretic runs of each pellet and supernate.

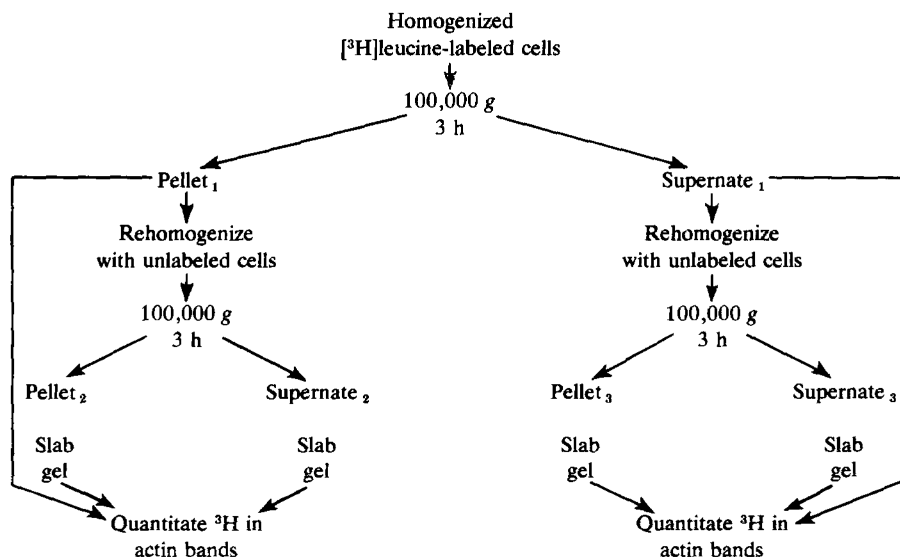

FIGURE 5 Flow sheet for double homogenization experiment.

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extremely thin. The basal cytoplasm contains a dense matte of microfilaments organized into prominent bundles similar to those described in other nontransformed cells in culture (13, 21). In cells allowed to grow past confluence (15×10^6 cels/100-cm^2 culture dish), the cells become somewhat thicker and their lamellae overlap. Under these conditions of extensive intercellular contact, microfilament bundles are found throughout the cell cortices and significant numbers of short microvilli form on the upper surfaces of the cells. Cells plated at low density (5,000 cells/2.5-cm dish) but placed in media taken from confluent cultures (10^7 cells/flask) possessed the same morphologies as low density cells.

442 cells are spherical or spindle-shaped and display minimal contact with the substrate. In contrast to the NRK cells, the morphology of the 442 cells does not change with increasing cell contact even though the cells may form thick clumps rising from the culture dish. In addition, the microfilaments in 442 cells are poorly organized and form cortical bundles only in occasional cytoplasmic processes that extend from the spindle-shaped cells.

DISCUSSION

The behavior of the NRK and 442 cell lines in our laboratory is consistent with the general pattern observed in similar cell lines by others (6, 7, 19, 27). Although the NRK cells may overlap after confluence, their growth is inhibited, and the alterations in cell and microfilament organization we observe are similar to those reported by Fonte and Porter (12). The 442 cells are less inhibited by contact, and do not respond morphologically to contact in any way that we could detect.

A unique finding of our study is the decrease in actin content during growth of the NRK cells. The period of most rapid reduction corresponds to the onset of intercellular contact, with little further change occurring subsequently over a number of cell generations. This reduction in actin content parallels a similar reduction in total protein, which in turn could be a reflection of metabolic degradation and/or a reduction in total cell volume at confluence. Our results are different from those of Bray and Thomas (3), who reported an increase in actin content during culture growth of fibroblasts. In terms of total protein content, on the other hand, results similar to ours have been reported recently for L cells in monolayer culture (33) and for HeLa cells (29). Another recent study (31) indicates that an increase in the rate of protein degradation during culture growth may affect the amount of protein in L cells.

Although it is now assumed that actin can exist in different forms in the cell, it has proven very difficult to determine the ratios of these forms to one another within the intact, living cell. In some recent studies (4, 11), efforts have been made to determine the ratio of particulate to soluble actin by centrifugation of cellular homogenates. In our own experiments, we have undertaken a further test of the method used by Bray and Thomas (4) to stabilize filamentous and nonfilamentous actin in cellular homogenates. The results of these experiments show that the extent of interchange between particulate and soluble pools of actin can vary considerably with different ionic conditions. Because of this, we must emphasize that, even though the buffer systems and homogenization procedures used in this work were designed to stabilize actin, our data for the ratios of particulate to soluble actin are subject to considerable error in terms of absolute values. However, since the NRK and 442 cells were treated in exactly the same manner, we believe that useful comparisons of the relative extent of actin pelletability in the cell lines can be made. For this reason, we conclude that our observation of a greater amount of particulate actin in NRK cells is meaningful.

Fine and Taylor (11) have reported that the ratio of particulate to soluble actin in 3T3 cells is 2:1, whereas the ratio is reversed in the transformant. Since these workers used buffers and procedures that were different from ours, and did not report on the extent to which their procedures stabilized actin in its different forms, it is appropriate to note here only that both studies show the normal cells to contain more particulate actin than the transformed cells. The results of both studies, moreover, are compatible with the conclusion that cells which appear to contain more microscopically detectable organized actin filament bundles also yield greater amounts of actin in pellets from high-speed centrifugation of cell homogenates. Since quantitation of the extent of actin organization into filaments is difficult from microscope data alone, refinement of the biochemical techniques employed in this and other recent studies should greatly facilitate future comparative studies of the state of actin in cells.

It is now evident that actin is a very abundant protein in the cell. It also seems likely that a significant fraction of the actin in NRK, 442, and

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other cell types that have been studied exists in the soluble form (presumably G-form) at all stages of cell growth. In terms of the organization of actin into filamentous form in the cell, we have observed that the appearance of actin filament bundles in NRK cells is apparently not a function of the total actin in the cells. 442 cells, while having amounts of actin comparable to those of NRK cells, appear to have greater surplus of the soluble form of actin. It follows that the amount of actin that is polymerized in the cell is not regulated at the level of actin synthesis but rather is controlled by local cytoplasmic conditions. The factors that control this polymerization are not presently apparent, but it seems likely that accessory proteins (16, 26, 30, 32) and the extent to which the cell adheres to and spreads upon the substrate (36) will be found to play major roles.

We wish to thank Mrs. Yolanda Palacio for her technical assistance.

This project was supported by a research grant from the National Cancer Institute 1 RO1 CA19855-01.

Received for publication 14 October 1977, and in revised form 10 February 1978.

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