**References and Notes**

1. S. Udenfriend, *Science* **152**, 1335 (1966); D. J. Prockop and K. I. Kivirikko, *Ann. Intern. Med.* **66**, 1243 (1967).
2. L. N. Lukeus, *Proc. Nat. Acad. Sci. U.S.* **55**, 1235 (1966); K. I. Kivirikko and D. J. Prockop, *Biochem. J.* **102**, 432 (1967).
3. E. Hausmann, *Biochem. Biophys. Acta* **133**, 591 (1967); K. I. Kivirikko and D. J. Prockop, *Proc. Nat. Acad. Sci. U.S.* **57**, 782 (1967).
4. K. I. Kivirikko and D. J. Prockop, *J. Biol. Chem.* **242**, 4007 (1967); R. S. Bhatnagar, J. Rosenbloom, K. I. Kivirikko, D. J. Prockop, *Biochim. Biophys. Acta* **149**, 273 (1967).
5. J. Greenberg, L. Fishman, M. Levy, *Biochemistry* **3**, 826 (1964).
6. K. A. Piez and R. C. Likins, *J. Biol. Chem.* **229**, 101 (1967); in *Calcification in Biological Systems*, R. F. Sognnaes, Ed. (AAAS, Washington, D.C., 1960), p. 411.
7. E. J. Miller, G. R. Martin, K. A. Piez, M. J. Powers, *J. Biol. Chem.* **242**, 5481 (1967).
8. P. Bornstein, *Biochemistry* **6**, 3082 (1967).
9. W. T. Butler, K. A. Piez, P. Bornstein, *ibid.*, p. 3771.
10. W. T. Butler and L. W. Cunningham, *J. Biol. Chem.* **241**, 3882 (1966); L. W. Cunningham, J. D. Ford, J. P. Segrest, *ibid.* **242**, 2570 (1967).
11. N. A. Kefalides and R. J. Winzler, *Biochemistry* **5**, 702 (1967); R. J. Spiro, *J. Biol. Chem.* **242**, 1915 (1967).
12. W. T. Butler, in preparation.
13. A. Nordwig and F. K. Pfab, *Biochim. Biophys. Acta* **154**, 603 (1968).


14. Part of this work was done at NIDR, Bethesda, Maryland. I thank Dr. K. A. Piez for advice and discussion.

23 May 1968


## Cell Proliferation: Enhancement by Extracts from Cell Surfaces of Polyoma-Virus-Transformed Cells


Abstract. *Dispersion of confluent monolayers of BHK21 cells with ethylenediaminetetraacetate yields a material that inhibits cell proliferation, whereas identical extraction of polyoma-virus-transformed cells provides material which enhances cellular proliferation. The material was partially characterized.*


The loss of contact inhibition and subsequent uncontrolled growth demonstrated by tumor-virus-transformed cells has been correlated with the neoplastic process (*1*). Defendi and Gasic (*2*) have reported that polyoma-virus-transformed cells histochemically exhibit an abnormally heavy layer of acid mucopolysaccharide at the cell surface, and they suggested that this increase of material could be correlated with the loss of contact inhibition demonstrated by those transformed cells. Stoker (*3*) has reported that incorporation of thymidine into polyoma-virus-transformed cells is inhibited when they are in contact with stationary, nondividing BHK21 cells, and he has suggested that this effect results from the passage of molecules between the contiguous cells. Addition of conditioned medium obtained from high concentrations of chick embryo cells to small numbers of these cells enhances their growth (*4*). Furthermore, conditioned medium obtained from either very crowded cultures of these cells or from chick cells transformed with Rous sarcoma virus contains a substance which inhibits growth (*4, 5*). These active factors are suggested to be derived from the cell surface as an exudate, and to reflect conditions at the cell periphery.

I now describe the isolation, some chemical characteristics, and the growth-enhancing effect of extracts derived from the surfaces of polyoma-virus-transformed cell by treatment with ethylenediaminetetraacetate (EDTA).

Cultures of hamster kidney cells (BHK21) demonstrating contact inhibition were used as controls. Two polyoma-virus-transformed derivatives of this line, TC-1 and H-6, demonstrating loss of contact inhibition and continuous mitosis, were also used as sources of cell surface material (*6*). Ethylenediaminetetraacetate was used to disperse confluent cell cultures and to obtain the extracellular material because it removes from the coating of the BHK21 cells and its transformed derivatives that material which is detected with Hale's stain, and does so as completely as the various enzymatic treatments reported by Defendi and Gasic (*2*). Viability of these cells, as determined by the trypan-blue dye-exclusion method usually exceeded 95 percent; any preparations revealing less than 95 percent viability were discarded. These preparations were centrifuged, and the clarified supernatants were divided into high-molecular-weight (HMW) and low-molecular-weight (LMW) moieties by ultrafiltration (*7*). The HMW and LMW fractions from transformed and nontransformed cells were tested for biological activity by addition of constant volumes of each isolate to either the transformed or the nontransformed cell cultures. Stock EDTA solutions, subjected to the ultrafiltration and concentration procedures, were used as controls.

Addition of LMW fractions derived from either transformed or nontransformed cells, as well as the HMW fraction obtained from BHK21 cells, markedly inhibited cell proliferation. However, addition of HMW fractions obtained from the polyoma-transformed cell line TC-1 enhanced cell growth (Fig. 1 and Table 1). The extent of inhibition or enhancement was dependent upon the number of cells, as well as upon the volume of material added to this test system. The effects were greatest when larger volumes of test materials (0.3 ml) were added to lesser numbers of cells ($< 150{,}000$).

Inhibition of growth was transitory, and eventually all cell cultures attained confluency. The morphology and contact orientation of BHK21 cells stimulated by addition of transformed cell HMW material were normal when such cells reached confluency. It is unclear whether any loss of contact inhibition occurred during the stages of rapid proliferation. No cytomorphological effects were noted when test samples were added to confluent monolayered cultures. Addition of control solutions neither enhanced nor inhibited cell proliferation, even when volumes as large as 0.4 ml were added to the test system. The dry weights of the test samples were not significantly different; each sample contained 30 to 40 $\mu$g of material per 0.1 ml.

Table 1. Effect of EDTA extracts obtained from dispersed, viable cells, on rates of cell growth. To each Leighton tube containing 350,000 cells in 1.5 ml of MEM was added 0.1 ml of the fraction to be tested. All tests were performed in duplicate, and the average elapsed time necessary for a confluent monolayer to be attained was determined microscopically. Cell numbers were determined in a hemocytometer at 24 hours and at the time confluency was attained.

| Source of added material | Cell counts ($\times 10^5$) | | | | | | Hour confluency reached | | |
|---|---|---|---|---|---|---|---|---|---|
| | BHK21 | | H-6 | | TC-1 | | | | |
| | 24 hours | Confluency | 24 hours | Confluency | 24 hours | Confluency | BHK-21 | H-6 | TC-1 |
| None added | 6.25 | 10.1 | 7.00 | 16.8 | 7.30 | 16.2 | 75 | 48 | 52 |
| Control EDTA HMW | 7.35 | 12.3 | 8.20 | 17.5 | 7.10 | 15.1 | 75 | 55 | 55 |
| Control EDTA LMW | 6.90 | 12.5 | 8.50 | 17.3 | 8.10 | 17.1 | 70 | 50 | 60 |
| BHK21 HMW | 4.90 | 10.3 | 5.70 | 15.5 | 7.10 | 14.7 | 95 | 60 | 75 |
| TC-1 HMW | 8.95 | 13.5 | 9.70 | 17.2 | 8.80 | 16.2 | 62 | 40 | 45 |
| BHK21 LMW | 5.10 | 11.2 | 6.20 | 16.9 | 5.50 | 15.1 | 85 | 65 | 60 |
| TC-1 LMW | 5.90 | 10.7 | 6.50 | 14.9 | 6.50 | 16.2 | 82 | 55 | 65 |

Colorimetric assays on HMW fractions from both TC-1 and BHK21 cells revealed the presence of amino sugars (Elson-Morgan procedure), hexose (orcinol test), and protein (microbiuret reaction); no sialic acid was detected by Warren's thiobarbituric acid assay. Sedimentation-velocity analysis of HMW materials derived from each cell type revealed similar single broad peaks in the analytical ultracentrifuge. This heterogeneity is confirmed by gel-filtration on Sephadex G-100 columns. Five subfractions (detected by assays for protein and hexose) were detected, with 0.1*M* NaCl used as the eluant. Subfractions from the two cell types were not qualitatively different.

The ultraviolet absorption spectra of the unfractionated HMW materials revealed the presence of a 260 nm peak. The optical density at 260 nm was reduced after HMW materials were incubated with ribonuclease and then dialyzed. It was not reduced when the HMW material was incubated with deoxyribonuclease. RNA, as a structural component of the cell surface, has been reported (*8*). Whether the RNA detected in the material extracted with EDTA resides in the extracellular matrix is unknown. It is not yet clear if the materials effecting cellular proliferation are related to any of those reported by other investigators (*5*, *9*).

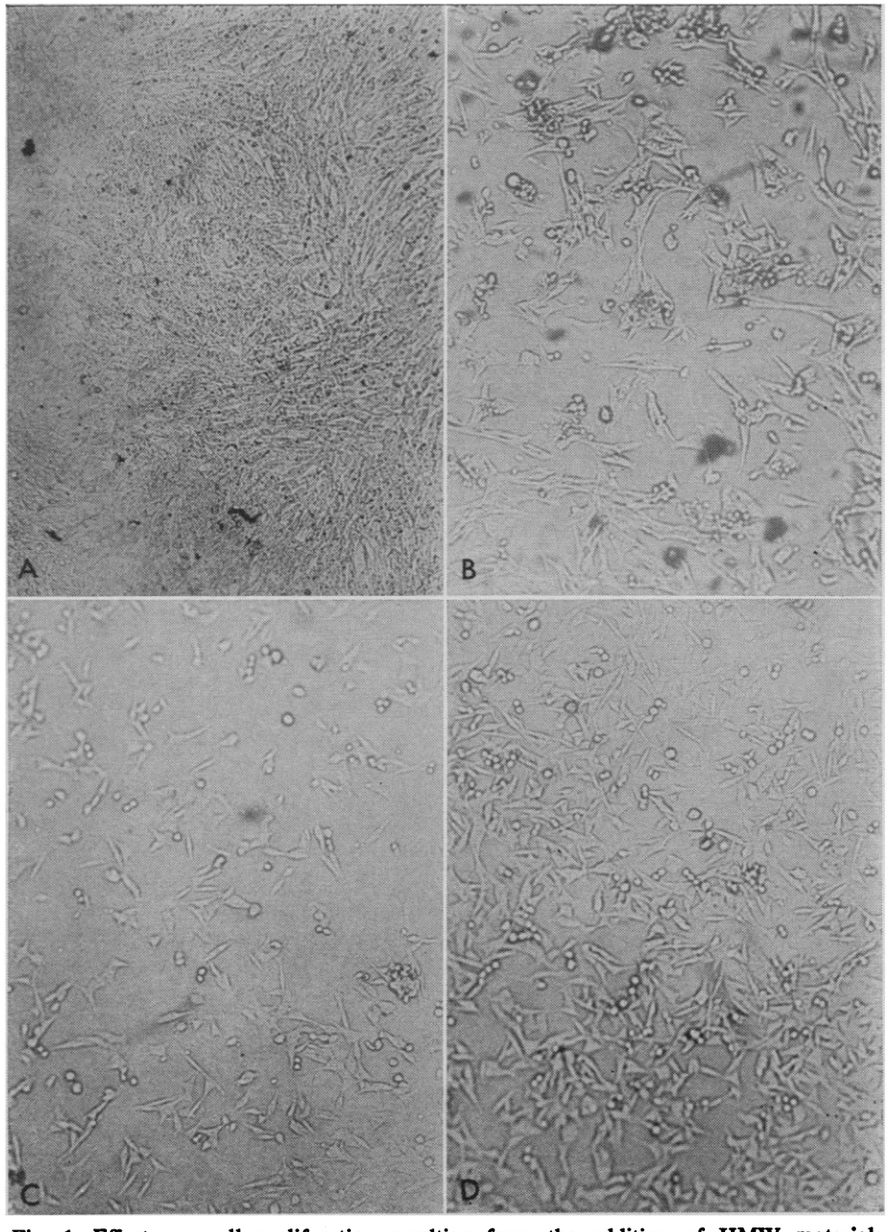


Fig. 1. Effects on cell proliferation resulting from the addition of HMW materials derived from transformed and nontransformed cells dispersed with EDTA. Test materials (0.3 ml) were added to innoculums of 600,000 cells in 1.5 ml of MEM. All cells were in test for 24 hours. (A) TC-1 cell HMW material added to BHK21 cells (~ × 320); (B) EDTA HMW control material added to BHK21 cells (~ × 480); (C) BHK21 cell HMW material added to TC-1 cells (~ × 480); (D) EDTA HMW control material added to TC-1 cells (~ × 480).

JOHN W. BEIERLE
*Allan Hancock Foundation,*
*University of Southern California,*
*Los Angeles 90007*

**References and Notes**

1. M. Abercrombie and E. J. Ambrose, *Cancer Res.* **22**, 525 (1962); A. S. G. Curtis, *J. Nat. Cancer Inst.* **26**, 253 (1961); L. Weiss, *The Cell Periphery, Metastasis and Other Contact Phenomena* (North-Holland, Amsterdam, 1967).
2. V. Defendi and G. Gasic, *J. Cell. Comp. Physiol.* **62**, 23 (1963).
3. M. Stoker, *J. Cell Sci.* **2**, 293 (1967).
4. H. Rubin, *Exp. Cell. Res.* **41**, 138 (1966).
5. ———, *ibid.*, p. 149.
6. BHK21 cells were obtained from the Cell Culture Repository, Rockville, Marvland; TC-1 cells were supplied by Dr. K. Habel, (National Institutes of Health); H-6 cells were obtained from Dr. M. Stoker, Institute of Virology, Glasgow. Both control and transformed cells were cultivated in stoppered milk dilution bottles containing 10 ml of Eagle's minimum essential medium (MEM), newborn calf serum (10 percent), and 200 units of penicillin and 200 μg of streptomycin per milliliter (Grand Island Biologicals). After the cells developed into confluent monolayers, they were washed four times with 50-ml volumes of physiological saline. Concentrates of these washings, tested immunologically by the Ouchterlony agar double-diffusion method with antiserums prepared against MEM, revealed only a trace of MEM after the third washing, and none after the fourth. The washed monolayered cells were then dispersed with 10 ml of 0.02 percent disodium ethylenediaminetetraacetate (Eastman Organic) in calcium- and magnesium-free phosphate-buffered saline (*p*H 7.2) for 20 minutes.
7. Cells from 15 bottles were pooled and treated with EDTA; the cell suspensions were then centrifuged at 5000*g* for 15 minutes. The clarified supernatants were concentrated 20-fold through a thoroughly washed collodion-membrane ultrafiltration apparatus (Carl Schleicher and Schuell, model CBA). The material retained in the collodion bag is the HMW fraction. The LMW fraction, that fraction of the supernatant passing through the membrane, was subsequently concentrated in a Diaflo pressure dialysis apparatus (Amicon Corp.) with a UM-3 membrane of a 500 molecular weight exclusion limit. The HMW and LMW fractions, or control EDTA solutions (0.05 to 0.3 ml), were added to Leighton tubes containing 1.5 ml of MEM and various numbers of cells (50,000 to 600,000). The cells were derived from stock cultures dispersed with trypsin and were still in suspension when test samples were added. Tubes were incubated at 37°C and were monitored microscopically for 5 days; selected cell cultures were treated with trypsin, and the cells were counted (hemocytometer) in order to detect cell number changes. All assays were performed in duplicate, and experiments were repeated with several pools of EDTA-extracted materials.
8. E. Mayhew, *J. Cell Physiol.* **69**, 311 (1967); L. Weiss and E. Mayhew, *ibid.* **68**, 345 (1966).
9. W. S. Bullough, in *Cellular Control Mechanisms and Cancer*, D. Emmelot and O. Mulbach, Eds. (Elsevier, New York, 1964), p. 125; J. B. Foley, B. J. Kennedy, J. D. Ross, *Cancer Res.* **23**, 368 (1963); M. Moskowitz and D. M. Schenck, *Exp. Cell Res.* **38**, 523 (1965); A. Szent-Gyorgyi, L. G. Egyud, J. A. McLaughlin, *Science* **155**, 539 (1967).
10. Supported in part by an interdepartmental cancer grant and an NIH predoctoral fellowship. I thank Dr. M. Maxfield for his many helpful suggestions.

20 June 1968