Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5989–5992, October 1980
Cell Biology

Purification of kidney epithelial cell growth inhibitors

(BSC-1 cells/growth control/high-performance liquid chromatography/initiation of DNA synthesis/reversible G_1 arrest)

ROBERT W. HOLLEY, PETER BÖHLEN, ROY FAVA, JULIA H. BALDWIN, GARY KLEEMAN, AND ROSEMARY ARMOUR

The Salk Institute for Biological Studies, Post Office Box 85800, San Diego, California 92138

Contributed by Robert W. Holley, July 17, 1980

ABSTRACT **Two high molecular weight growth inhibitors have been isolated from the culture medium of BSC-1 cells, epithelial cells of African green monkey kidney. The purified kidney epithelial cell growth inhibitors, at ng/ml concentrations, reversibly arrest the growth of BSC-1 cells in the G_1 phase of the cell cycle. Their action is selective; they are most active on BSC-1 cells, are less active as inhibitors of the growth of rat lung and human breast epithelial cells, and do not inhibit the growth of 3T3 mouse embryo fibroblasts and human skin fibroblasts in culture. Their growth inhibitory action on BSC-1 cell cultures is counteracted by epidermal growth factor or calf serum.**

One of the areas of ignorance that remains in our broad understanding of the control of growth of mammalian cells is the subject of endogenous growth inhibitors. The existence of a number of growth inhibitors has been postulated (1, 2), and growth-inhibitory fractions have been obtained from various mammalian cells and tissues (3–14). Nevertheless, there are doubts as to the significance of endogenous growth inhibitors because most of the inhibitor preparations have low specific activity, the inhibitors have been difficult to purify, and with some preparations the causes of growth inhibition have, at least in part, turned out to be trivial (15–17). There is no doubt that highly active growth inhibitors exist for mammalian cells, because interferon and corticotropin are extremely active as growth inhibitors in certain cell cultures (18, 19), but the inhibitory activities of these two substances could be incidental to their other biological activities. Because of the potential significance of growth inhibitors and because purified inhibitors could be very useful in the manipulation of growth of mammalian cells, we have undertaken further studies in this area.

Density-dependent regulation of growth of BSC-1 cells (African green monkey kidney epithelial cells) has been found to result in part from the accumulation of growth inhibitors in the culture medium (20). The present paper describes the isolation of high molecular weight growth inhibitors from the culture medium of these cells. The growth inhibitors have very high specific activities, approximately equal to the specific activities of interferon and corticotropin in their respective cell culture assays. The new growth inhibitors appear to be selective in the types of cells on which they act.

MATERIALS AND METHODS

Cell Cultures. BSC-1 cell stock cultures were maintained as previously described (21) in Dulbecco's modified Eagle's medium (DME medium) (22) with 0.45% glucose and 10% (vol/vol) calf serum. Cells were detached from the culture dishes with 0.025% trypsin in calcium- and magnesium-free Tris/saline buffer (23) containing 0.5 mM Na_2 EDTA. Cultures of secondary rat lung epithelial cells were provided by Trudy Messmer, secondary human breast epithelial cells by Susan Potter, and secondary human skin fibroblasts by Hans-Jürgen Ristow.

Preparation of Conditioned Medium. Dense BSC-1 cell cultures, used for preparation of conditioned medium, were grown in 15-cm Falcon tissue culture plates in DME medium with 1% calf serum. The medium was changed once a week until the cells reached a density of approximately 1.5×10^5 cells per cm^2. The cultures were then maintained in DME medium with 0.1% calf serum. To prepare conditioned medium, the cultures were changed to DME medium without serum (100 ml per 15-cm plate) and this medium was left on the cultures for 1 day. The serum-free conditioned medium was then removed for concentration of the inhibitors by ultrafiltration. The BSC-1 cell cultures were returned to DME medium with 0.1% serum (25 ml per 15-cm plate) for at least 1 day before the preparation of serum-free conditioned medium was repeated. Conditioned medium was prepared repeatedly, twice a week, from the same cultures for approximately 2 months.

Concentration of the Growth Inhibitor by Ultrafiltration. The serum-free conditioned medium obtained from 60 15-cm plates of dense BSC-1 cells was concentrated to approximately 1/100th of its volume in a large (2.5 liter) Amicon unit using a 15-cm YM10 ultrafiltration membrane. The ultrafiltration membrane then was washed twice with 10-ml portions of 1 M acetic acid to recover adsorbed materials, and this wash was frozen and saved to be combined with the final concentrate. The 100-fold concentrate was further concentrated in a 400-ml Amicon unit to approximately 10 ml by using a 76-mm YM10 membrane. This concentrate was diluted with 50 ml of Tris/saline buffer and reconcentrated to 10 ml; the concentrate was diluted a second time with 50 ml of water and reconcentrated to approximately 6 ml. The final concentrate was made 1 M in acetic acid by the addition of glacial acetic acid. The 76-mm YM10 membrane used for these concentrations was then washed twice with 2-ml portions of 1 M acetic acid. The three acetic acid solutions (the 6 ml of aqueous concentrate that was acidified plus the washes of the two YM10 membranes) were combined and concentrated finally to approximately 6 ml; this 1000-fold concentrate of the conditioned medium was the starting material for gel filtration.

Gel Filtration of the Growth Inhibitor. The 6 ml of 1000-fold YM10 concentrate of the conditioned medium was chromatographed on a 2 × 25-cm column of Bio-Gel P-60 (100–200 mesh; Bio-Rad) packed in 1 M acetic acid. Fractions of 4 ml were collected. The absorbance at 280 nm and the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: DME medium, Dulbecco's modified Eagle's medium.

growth inhibitory activity of the fractions were determined. Addition of 10 μg of crystalline bovine serum albumin per fraction may improve recovery of the inhibitors and does not interfere with subsequent purification.

Assay of the Kidney Epithelial Cell Growth Inhibitors. BSC-1 cells were plated at approximately 2×10^5 cells per 5-cm plate and were grown 2 or 3 days in DME medium supplemented with biotin at 0.4 μg/ml and 0.1% calf serum. The cells were changed to fresh medium of the same composition at the time the assay additions were made. Aliquots (20 μl) of the Bio-Gel P-60 column fractions were assayed (with the addition of 20 μl of 1 M sodium carbonate to neutralize the 1 M acetic acid). Incorporation of [*methyl*-^{3}H]thymidine was measured 20–25 hr after the assay additions. The cells were then washed, the acid-insoluble material was collected by filtration, and its radioactivity was determined. The results were calculated as percentage inhibition of the thymidine incorporation compared to control cells.

High-Performance Liquid Chromatography. The most active fractions of a peak of growth inhibitory activity were combined from three or four Bio-Gel P-60 columns. Typically, the fractions used were peak I (fractions 10–13 from the P-60 column) or, separately, peak II (fractions 21–25). The material from 20–25 liters of conditioned medium was used for each high-performance liquid chromatogram. The combined Bio-Gel P-60 column fractions were lyophilized and the residue was redissolved in approximately 0.5 ml of 1 M acetic acid. This solution was injected on a Waters μBondapak CN column (3.9 mm × 30 cm). Chromatography was with Altex gradient liquid chromatography equipment, with microprocessor, using a pH 4 pyridine/formic acid buffer (29 ml of pyridine and 19 ml of 88% formic acid per liter in water) with a stepwise gradient of *n*-propanol in this buffer (*n*-propanol replaces water in the buffer; the final solution contained 29 ml of pyridine, 19 ml of 88% formic acid, and 600 ml of *n*-propanol per liter). The column eluate was monitored with a fluorescamine stream sampling detector (24). The flow rate was 0.7 ml/min; 2.1-ml fractions were collected. Before assay, the fractions were lyophilized and residues were redissolved in 0.20 ml of 0.5 M acetic acid per fraction. A typical assay used 1 μl of a fraction.

RESULTS

Preparation of the Conditioned Medium. Medium conditioned by crowded BSC-1 cell cultures contains several growth inhibitors. Ultrafiltration of the conditioned medium, using an Amicon YM10 membrane, concentrated the high molecular weight inhibitors. Originally it was thought that the high molecular weight inhibitors were unstable to shaking and to heat because growth inhibitory activity was lost after these treatments (20). Eventually it became clear, however, that the growth inhibitors are stable but a "cryptic" growth factor for crowded BSC-1 cells is activated by shaking or heating the solution; the growth factor then overcomes the action of the growth inhibitors under some assay conditions.

Once the behavior of the high molecular weight inhibitors was understood, experiments were carried out to choose the optimal conditions for preparation of the inhibitors. Varying the length of exposure of serum-free DME medium to crowded BSC-1 cell cultures established that the high molecular weight inhibitors appear rapidly, within hours, and achieve approximately steady-state maximal concentrations in 24 hr. Serum-free DME medium can remain on the crowded BSC-1 cell cultures for as long as 3 days without deleterious effects, but this does not increase the yield of inhibitors. Increasing the volume of medium relative to the area of cells by as much as 6-fold leads to almost a proportional increase in yield of growth inhibitors because the concentrations of inhibitors attained in the medium remain approximately the same. A 6-fold increase in the volume of medium entails practical difficulties in handling 150 ml of medium in a 15-cm tissue culture dish, so the preparative procedure chosen used 100 ml (4 times the normal volume) of culture medium per 15-cm dish and the conditioned medium was collected after 1 day of exposure to the BSC-1 cells. The cells were returned to DME medium with 0.1% calf serum for at least 1 day before serum-free conditioned medium was prepared again. It is practical for one person to prepare the inhibitors from 20 liters of conditioned medium a week.

Concentration of the Inhibitors by Ultrafiltration. This was complicated by adsorption of the inhibitors to the ultrafiltration membrane. A YM10 membrane adsorbs less than a UM10 membrane, but the recovery of inhibitors was improved greatly by rinsing the surface of the YM10 membrane with 1 M acetic acid at the conclusion of the ultrafiltration. A final concentration step, to 1000-fold the original concentration, was carried out in 1 M acetic acid. The growth inhibitory activity is retained by the YM10 membrane even in 1 M acetic acid.

Gel Filtration of the Inhibitors. The results of gel filtration of the 1000-fold concentrate of the inhibitors through a Bio-Gel P-60 acrylamide gel column (exclusion limit, 60,000 daltons) are shown in Fig. 1. Two peaks of growth inhibitory activity were obtained. The first peak (peak I) chromatographed at a position corresponding to a size of approximately 15,000 daltons. The second peak (peak II) eluted from the column after the total column volume, indicating that the inhibitor adsorbs to the gel. The size of this inhibitor is uncertain, but it must be moderately high because it was retained by a YM10 ultrafiltration membrane in 1 M acetic acid.

High-Performance Liquid Chromatography. The results of high-performance liquid chromatography of peak I from the Bio-Gel P-60 column on a reverse phase μBondapak CN column are shown in Fig. 2. The major peak of growth inhibitory activity corresponds to a minor protein peak.

High-performance liquid chromatography of peak II from the Bio-Gel P-60 column gave a growth-inhibitory peak that was indistinguishable from that shown in Fig. 2 under the chromatographic conditions used. In this instance, most of the fluorescamine-positive material appeared in fractions 2 and 3. Again, the growth-inhibitory peak contained little protein.

Dose-response curves of the growth-inhibitory peaks isolated

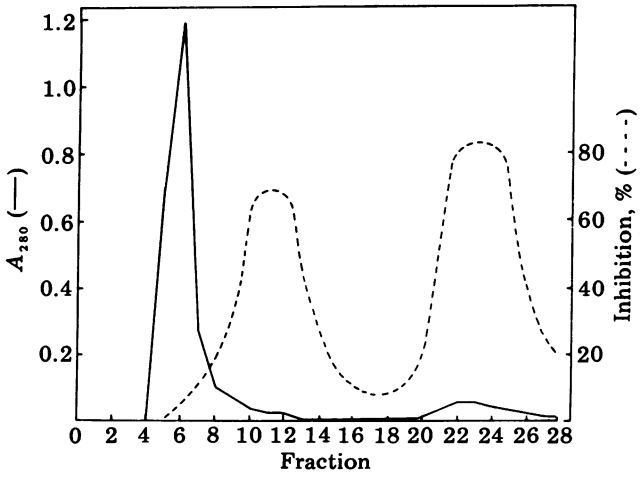

FIG. 1. Chromatography of 6 ml of a 1000-fold YM10 concentrate of conditioned medium on a 2 × 25-cm column of Bio-Gel P-60 polyacrylamide gel (100–200 mesh) in 1 M acetic acid; 4-ml fractions were collected.

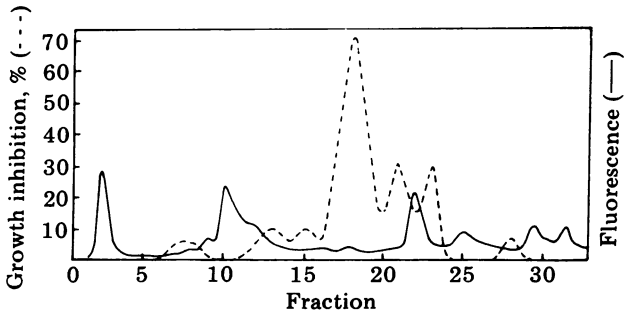

FIG. 2. High-performance liquid chromatography of peak I (fractions 10–13) from the Bio-Gel P-60 column (Fig. 1). The elution sequence of *n*-propanol was 0% for 4 min, 10% for 4 min, 20% for 20 min, 25% for 20 min, 30% for 20 min, 40% for 20 min, and 60% for 1 min.

by high-performance liquid chromatography are shown in Fig. 3. The inhibitors were active at approximately 0.1 nM. The amounts of protein present in the preparations of the growth inhibitors were estimated from the fluorescamine reaction and from absorbance of the fractions at 280 nm, with bovine serum albumin as the standard. A typical preparation from 20 liters of conditioned medium gives approximately 20 μg of protein in the peak fraction of growth-inhibitory activity from the high-performance liquid chromatogram.

Reversible Arrest of Cell Growth in G_1. Autoradiography (25) of growth-inhibited BSC-1 cell cultures showed that the inhibition of [^{3}H]thymidine incorporation by both purified inhibitors was paralleled by an inhibition of the labeling index of the cultures. That this represents a decrease in the fraction of the cells in the S phase of the cell cycle in the growth-inhibited cultures was confirmed by flow microfluorometric analyses: at 24 hr after addition of the inhibitor, growth-inhibited cultures had 93% of the cells in G_1, 3.5% in S, and 3.5% in G_2 + M, and the control cells had 78% in G_1, 13% in S, and 9% in G_2 + M. It takes approximately 24 hr to arrest the growth of a BSC-1 cell culture. After removal of the inhibitor, growth of the BSC-1 cell culture resumed (Fig. 4). (The shapes of the growth curves shown in Fig. 4 are distorted by the medium changes on days 0 and 2.)

Specificity of the Action of the Kidney Epithelial Cell Growth Inhibitors. The two purified kidney epithelial cell growth inhibitors, at concentrations that inhibited 70% of the thymidine incorporation into BSC-1 cells, did not inhibit thymidine incorporation by 3T3 mouse embryo fibroblasts or by secondary human skin fibroblasts. There were differences between the actions of the two growth inhibitors and both showed considerable selectivity in their action. At a concentration that inhibited 80% of the initiation of DNA synthesis in BSC-1 cells, the first inhibitor (purified as in Fig. 2) inhibited DNA synthesis in secondary human breast epithelial cell cultures 20% and in secondary rat lung epithelial cell cultures 0%. At a concentration that inhibited initiation of DNA synthesis in BSC-1 cells 85%, the second inhibitor (peak II of the Bio-Gel P-60 column purified by high-performance liquid chromatography) inhibited DNA synthesis in secondary rat lung epithelial cell cultures 20%.

The kidney epithelial cell growth inhibitors had no interferon activity in an assay of vesicular stomatitis virus infection of BSC-1 cells.

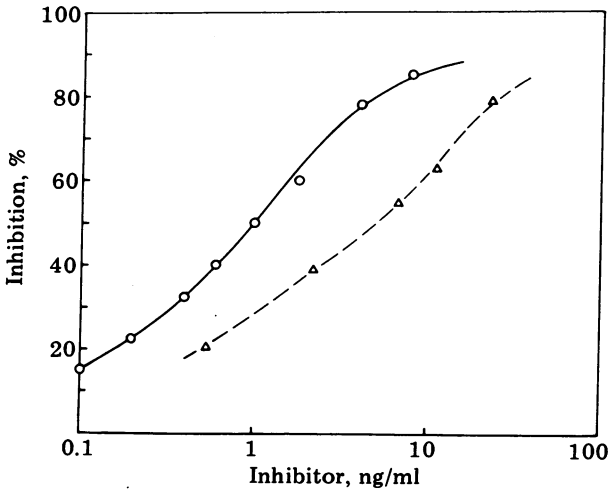

FIG. 3. Dose-response curves of the growth inhibitors after purification by high-performance liquid chromatography. Δ, Peak I, from fractions 10–13 of the Bio-Gel P-60 column; O, Peak II, from fractions 21–25 of the column.

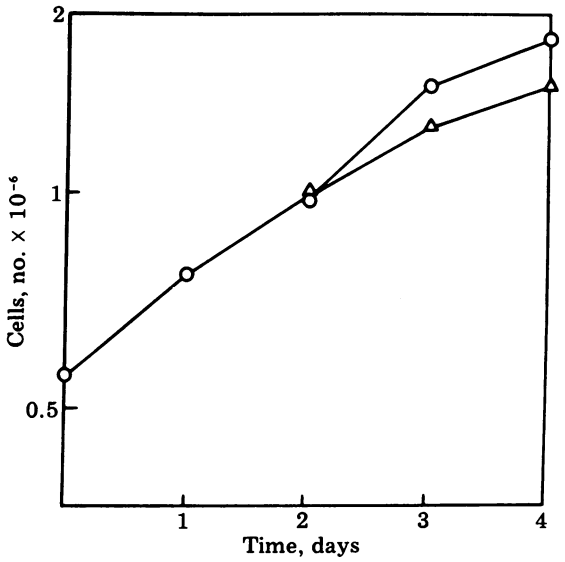

FIG. 4. Growth of BSC-1 cells in DME medium with 2% calf serum with (Δ) or without (O) added growth inhibitor. The medium was changed on all of the plates on days 0 and 2. Inhibitor (peak II), sufficient to inhibit DNA synthesis 80% in the standard assay, was added to half the dishes on day 1 and was removed by the medium change on day 2.

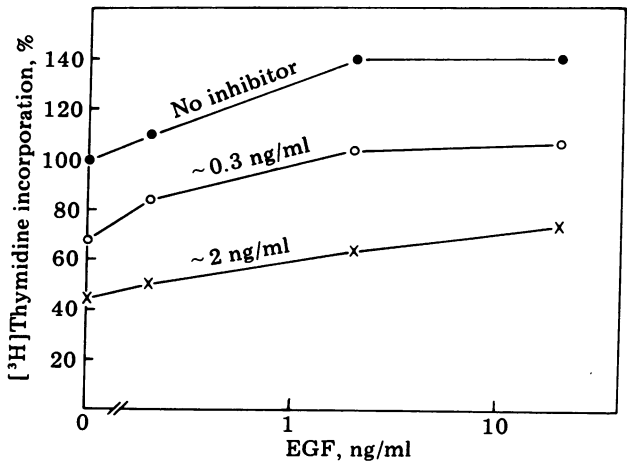

FIG. 5. Interaction between the growth-inhibiting effects of the inhibitor (peak II, after purification by high-performance liquid chromatography) and the growth stimulating effects of epidermal growth factor (EGF).

Interaction Between the Inhibitors and Epidermal Growth Factor. The growth inhibitory action of the kidney epithelial cell growth inhibitors was counteracted by adding epidermal growth factor to the cell cultures (Fig. 5). Similar results were obtained with calf serum.

DISCUSSION

The results presented here demonstrate that extremely active growth inhibitors can be recovered from the culture medium of BSC-1 cells. Two kidney epithelial cell growth inhibitors, peak I and peak II, have been purified sufficiently so that at approximately 5 ng and 1 ng of protein per ml, respectively, they inhibit growth 50% (Fig. 3). These specific activities approximate those of interferon and corticotropin, the most active natural growth inhibitors known for mammalian cells (18, 19).

The kidney epithelial cell growth inhibitors are assumed to be proteins because of their molecular weights, chromatographic properties, and tendencies to adsorb to surfaces and because their growth inhibitory activities are destroyed by pepsin. Because of limited amounts of material, the inhibitors have not been further purified, and presumably are not completely pure.

The peak I and peak II growth inhibitors are indistinguishable by high-performance liquid chromatography under the conditions used, suggesting that they may be structurally related, even though the second adsorbs more strongly to an acrylamide gel column.

Thus far it has not been possible to extract the inhibitors from BSC-1 cells or from kidney tissue, and no source of large amounts of the inhibitor has been found. The yield of inhibitor was increased 4-fold by increasing the volume of culture medium relative to the area of cells. Attempts to increase the scale of preparation by growing the BSC-1 cells in roller bottles have been unsuccessful because the cell layer is unstable on the bottles in serum-free medium.

The kidney epithelial cell growth inhibitors are selective in their action. They are most active in inhibiting the growth of BSC-1 cells, are less active in inhibiting the growth of secondary rat lung and mouse mammary epithelial cells, and have no growth inhibitory activity on fibroblasts.

The action of the kidney epithelial cell growth inhibitors is counteracted by epidermal growth factor (Fig. 5) or calf serum. It seems unlikely that the inhibitors interefere with epidermal growth factor receptors because the binding of ^{125}I-labeled epidermal growth factor to BSC-1 cells (21) is not affected by the presence of conditioned medium containing the inhibitors. It seems more likely that the interaction between the growth-depressing effects of the inhibitors and the growth-stimulating effects of epidermal growth factor takes place inside the cells. The mechanism of action of the inhibitors inside the cell is unknown.

The kidney epithelial cell growth inhibitors cause reversible arrest of the growth of BSC-1 cells in the G_1 phase of the cell cycle. This is the action expected of a natural growth regulator because most quiescent cells *in vivo* contain the G_1 DNA content.

The presence of extremely active growth inhibitors in the culture medium of BSC-1 cells strengthens the view that endogenous growth inhibitors are important in growth control. The roles of these kidney epithelial cell growth inhibitors *in vivo* and the effects that changes in the concentrations of inhibitors may have on epithelial cell growth *in vivo* remain to be determined.

We are indebted to Dr. Trudy Messmer and Dr. Susan Potter of The Salk Institute and Dr. Hans-Jürgen Ristow of the University of California at San Diego for the gifts of the secondary cell cultures. We thank Dr. Walter Eckhart (The Salk Institute) for the interferon assays and Drs. S. Udenfriend and S. Stein (Roche Institute of Molecular Biology, Nutley, NJ) for the gift of the high-performance liquid chromatography equipment. This work was supported in part by a grant from the Whitehall Foundation, by Grant CA11176 from the National Cancer Institute, and by Grant BC-304-F from the American Cancer Society. R.W.H. is an American Cancer Society Professor of Molecular Biology.

1. Szent-Gyorgyi, A., Hegyeli, A. & McLaughlin, J. A. (1963) *Proc. Natl. Acad. Sci. USA* **49,** 878–879.
2. Bullough, W. S., Laurence, E. B., Iverson, O. H. & Elgjo, K. (1967) *Nature (London)* **214,** 578–580.
3. Lozzio, B. B., Lozzio, C. B., Bamberger, E. G. & Lair, S. V. (1975) *Intl. Rev. Cytol.* **42,** 1–47.
4. Houck, J. C., ed. (1976) *Chalones* (North Holland/American Elsevier, New York).
5. Bullough, W. S. & Laurence, E. B. (1964) *Exp. Cell Res.* **33,** 176–194.
6. Rytomaa, T. & Kiviniemi, K. (1968) *Eur. J. Cancer* **4,** 595–606.
7. Kiger, N., Florentin, I. & Mathe, G. (1973) *Natl. Cancer Inst. Monogr.* **38,** 135–141.
8. Knecht, M. E. & Lipkin, G. (1977) *Exp. Cell Res.* **108,** 15–22.
9. Houck, J. C., Kanagalingam, K., Hunt, C., Attallah, A. & Chung, A. (1977) *Science* **196,** 896–897.
10. Richter, K. H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359,** 1435–1439.
11. Sassier, P. & Bergeron, M. (1978) *Cell Tissue Kinet.* **11,** 641–650.
12. Bertsch, S. & Marks, F. (1979) *Cancer Res.* **39,** 239–243.
13. Lehmann, W., Graetz, H., Schutt, M. & Langen, P. (1979) *Exp. Cell Res.* **119,** 396–399.
14. Sekas, G., Owen, W. G. & Cook, R. T. (1979) *Exp. Cell Res.* **122,** 47–54.
15. Holley, R. W. (1967) *Biochim. Biophys. Acta* **145,** 525–527.
16. Allen, J. C., Smith, C. J., Curry, M. C. & Gaugas, J. M. (1977) *Nature (London)* **267,** 623–625.
17. Lenfant, M., Garcia-Giralt, E., Thomas, M. & Di Giusto, L. (1978) *Cell Tissue Kinet.* **11,** 455–463.
18. Balkwill, F. & Taylor-Papadimitriou, J. (1978) *Nature (London)* **274,** 798–800.
19. Hornsby, P. J. & Gill, G. N. (1978) *Endocrinology* **100,** 926–936.
20. Holley, R. W., Armour, R. & Baldwin, J. H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1864–1866.
21. Holley, R. W., Armour, R., Baldwin, J. H., Brown, K. D. & Yeh, Y. C. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5046–5050.
22. Morton, H. J. (1970) *In Vitro* **6,** 89–108.
23. Smith, J. D., Freeman, G., Vogt, M. & Dulbecco, R. (1960) *Virology* **12,** 185–196.
24. Böhlen, P., Stein, S., Stone, J. & Udenfriend, S. (1975) *Anal. Biochem.* **67,** 438–445.
25. Holley, R. W. & Kiernan, J. A. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 2908–2911.