Purification and properties of a rat liver protein that specifically inhibits the proliferation of nonmalignant epithelial cells from rat liver

(hepatic proliferation inhibitor/high-performance liquid chromatography/isoelectric focusing)

JAMES B. McMAHON, JAMES G. FARRELLY, AND P. THOMAS IYPE*

Chemical Carcinogenesis Program, Frederick Cancer Research Center. Frederick, Maryland 21701

ABSTRACT   An inhibitor of cell proliferation was purified from rat liver by alcohol precipitation, ultrafiltration, and DEAE-cellulose chromatography. The hepatic proliferation inhibitor was shown to be pure by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, analytical isoelectric focusing, and high-performance liquid chromatography. The hepatic proliferation inhibitor was found to have a molecular weight of 26,000 and an isoelectric point of 4.65. This protein inhibited the proliferation of nonmalignant rat liver cells in culture, and removal of the protein reversed the inhibition produced by low doses. It exerted no effect on the proliferation of malignant rat liver cells.

Growth stimulatory factors, nutrients, and ions have been shown to be important in the control of cell proliferation (1–3). A number of factors that stimulate cell proliferation have been purified from various tissues and are well characterized. Indeed, many of these purified growth factors are commercially available and are being used extensively to investigate various aspects of cell biology. In contrast, little is known about the role of growth inhibitory factors, although they clearly are involved in the control of cell proliferation which itself is critical in the carcinogenic process as discussed in depth by Potter (4, 5). Considerable literature is available on the existence of inhibitors of cell proliferation in different cells and tissues (6–15), but such factors from normal tissues have not been purified.

Research in this laboratory has been directed toward understanding the mechanism of carcinogenesis, primarily in rat liver cells in vitro. Recently, we reported the partial purification of a growth inhibitory factor from rat liver that inhibited cell division in nonmalignant rat liver cells and activated cell division in a few malignant rat liver cell lines (9, 10). This preparation was not homogeneous and the presence of both growth stimulatory and inhibitory factors could not be ruled out. Having removed the growth stimulatory factor we now report the purification and properties of the hepatic proliferation inhibitor (HPI).

MATERIALS AND METHODS

Purification of HPI. Preparation of the partially purified extract from rat livers was described (9). This extraction procedure is a modification of that of Verly et al. (16) and involves homogenization, in distilled water, of livers from 50 adult (200–250 g) male Fischer rats. After centrifugation of this homogenate at 105,000 × g for 2 hr, the supernatant fluid was subjected to fractional precipitation with ethanol and the 70–87% ethanol precipitate was collected. After lyophilization, this material was redissolved in distilled water and filtered through an Amicon

PM-30 ultramembrane filter. The PM-30 filtrate was subjected to Amicon UM-10 filtration and the retained material (retentate) was then subjected to DEAE-cellulose chromatography. The total UM-10 retentate (80 mg) was applied to a column (1.5 × 90 cm of DEAE-cellulose (DE-23, Whatman) equilibrated with 5 mM sodium phosphate at pH 6.0. The column was washed extensively with the same buffer until the 280-nm absorbance of the eluate reached the baseline level. Elution of the material retained on the column was achieved with a linear 0–0.15 M NaCl gradient in the same buffer at a flow rate of 42 ml/hr. Ten-milliliter fractions were collected, the material was concentrated by using UM-10 ultrafiltration, and the retentate was diluted with distilled water; the concentration process was repeated three times. The final retentate was then lyophilized and used for the various analyses. In some cases, aliquots were treated with 0.1% trypsin (204 units/mg, Worthington) or 0.1% Pronase (45 units/mg, Calbiochem) for 30 min at 37°C in a 0.05 M sodium phosphate at pH 8.0 before the analysis.

Electrophoretic Methods. Analytical polyacrylamide gel electrophoresis in the presence of NaDodSO₄ was carried out by the method of Laemmli and Favre (17) on discontinuous 10% polyacrylamide gels. Each lane received a minimum of 50 μg of protein. Molecular weight determinations were made on the NaDodSO₄-containing gels by using soybean trypsin inhibitor, chymotrypsinogen A, carbonic anhydrase, and ovalbumin as markers. Analytical isoelectric focusing was performed on polyacrylamide gels (Ampholine PAG plates, pH 4.0–6.5, LKB with the LKB Multiphor system as described by Winter et al (18). For determination of the pH gradient, an unfixed gel was sliced into 5-mm sections, and each section was equilibrated overnight at 4°C in 0.5 ml of distilled water before the pH was measured. After separation by the above electrophoretic techniques the gels were fixed in 15% trichloroacetic acid for 16 hr. They were then stained for protein with Coomassie brilliant blue R 250 or for carbohydrates with periodic acid–Schiff reagent according to the method of Fairbanks et al. (19).

High-Performance Liquid Chromatography (HPLC). Separations were performed on a 0.46 × 25 cm Altex Ultrasphere ODS column (Alltech Associates, Arlington Heights, IL according to the method of Henderson et al. (20). A Waters HPLC system which included a model 450 variable wavelength detector, a M-660 solvent programmer, a UGK sample injector and two M-6000 solvent delivery pumps was used. One-milliliter samples consisting of 50–150 μg of protein dissolved in 0.05% trifluoroacetic acid in water were injected and chromatographed by using a linear (0–20%) gradient of 0.05% trifl...

...retic acid in acetonitrile over 30 min at a flow rate of 1.0 ml/...n. The absorbance of the column effluent was moni-...red at 206 nm. Peak fractions were lyophilized prior to further ...alyses.

Culture Methods and Assay Conditions. Nonmalignant liver ...ells were isolated from a 12-day-old male Fischer rat as de-...cribed (21). The malignant liver cell line was isolated from a ...ft-agar colony of spontaneously transformed liver epithelial ...ells (22). Both cell lines were maintained routinely as mono-...er cultures in Ham's F-10 medium supplemented with 10% ...tal bovine serum (K. C. Biologicals, Lenexa, KS) (control ...edium). The cells were grown on plastic Petri dishes (Falcon ...lastics) and incubated at 37°C in humidity cabinets with a gas ...hase of 5% CO_2 in air. Throughout the purification, biological ...ctivity was determined by a quantitative assay based on the ...eversible inhibition of cell proliferation as described in detail ...lsewhere (9, 10). In brief, this assay involved plating cells at ...low density (20 cells per cm^2) in 60-mm plastic Petri dishes ...nd after a 1-day attachment period, treating them with either ...ontrol medium or control medium plus different concentra-...ions of HPI. After a treatment period of 4 days, the medium ...as replaced with control medium or, in some dishes, medium ...lus HPI was replaced with fresh medium plus HPI. The cells ...ere maintained for an additional 5 days and then fixed in meth-...nol and stained with Giemsa. The total number and size dis-...ribution of the stained cell colonies were determined by using ...n Artek model 880 automatic colony counter (Artek Systems, ...armingdale, NY).

RESULTS

The various fractionation steps used in the purification proce-...dure (from 50 rat livers), the yields, and the specific activities ...of the material obtained at each step are summarized in Table ...1. A large amount of the material was removed by the ethanol ...precipitation step. The ED_{50} of the $105,000 \times g$ supernatant ...could not be assessed in the bioassay because the indicator liver ...cells were digested by this material. Therefore, the actual en-...richment of the specific activity at this step could not be de-...termined unequivocally. The initial ED_{50} of the ethanol-pre-...cipitated material (300 µg/ml) was decreased by a factor of 1/...6 by the Amicon ultrafiltration. When applied to a DEAE-cel-...lulose column, 6% of this Amicon fraction was retained after the
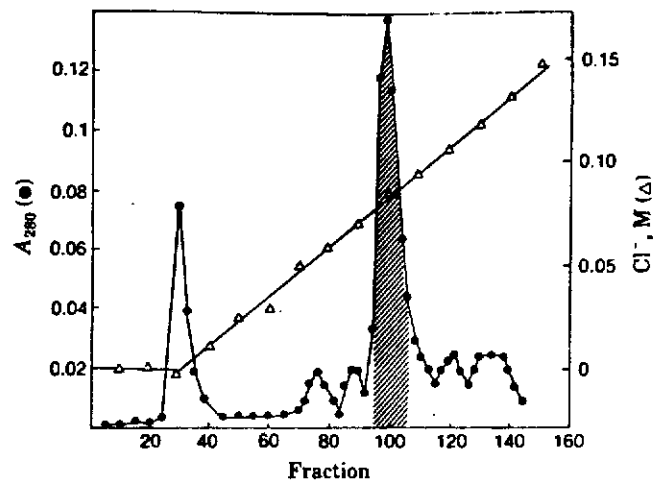

FIG. 1. DEAE-cellulose chromatogram of UM-10 retentate eluted at pH 6.0 with NaCl gradient. Hatched area, fractions that contained proliferation inhibitory activity.

extensive washing procedure used. The total material retained on the column (eluted completely by 0.2 M NaCl) showed a much higher specific activity. When the total retained material was eluted with the linear NaCl gradient, six peak fractions were obtained (Fig. 1). When these fractions were bioassayed, all the growth inhibitory activity was found in the peak indicated by the hatched area in Fig. 1. This represents 50% of the total material retained on the DEAE-cellulose column (Table 1). The active material from the DEAE-cellulose column (HPI, source 1) had the highest specific activity which represented a mini-mum of a 6000-fold purification. Any material that was frac-tionated away during the purification procedure (starting from the Amicon filtration) had no proliferation inhibitory activity.

When heat-denatured, reduced samples were subjected to electrophoresis in the presence of $NaDodSO_4$ (19), numerous protein bands were detectable from the ethanol-precipitated material (Fig. 2, lane a) and the UM-10 retentate (Fig. 2, lane b); only a single protein band was observed from the HPI, source 1 (Fig. 2, lane c). This band did not stain with periodic acid–Schiff reagent. Treatment of the purified material with trypsin or Pronase resulted in the loss of this band as well as the loss of its biological activity (data not shown). Electrophoresis of protein markers (Fig. 2, lane e) under these conditions re-vealed that the molecular weight of the protein was 26,000. Iso-electric focusing, with its high resolution capability, separated

Table 1.　Purification of HPI from 50 adult rat livers

Fractionation step	Total protein, mg	ED_{50}, µg/ml*	Purification, -fold†
105,000 × g supernatant	62,400	‡	—
70–87% EtOH cut	2,750	300	1
UM-10/PM 30	80	50	6
Total material retained on DEAE-cellulose column	5	2	150
Active fraction eluted from DEAE-cellulose (HPI, source 1)	2.5	0.05	6000

* ED_{50} is defined as the effective dose, in µg/ml, giving approximately 50% inhibition of cell proliferation as determined by cell number (Coulter Counter) 4 days after treatment with or without different doses of the materials. This assay is different from the colony assay used to determine the reversible inhibition of HPI shown in Table 2.

† Fold purification of the inhibitory activity based on ED_{50}.

‡ Not determined; crude supernatant fraction was too cytotoxic for evaluation.
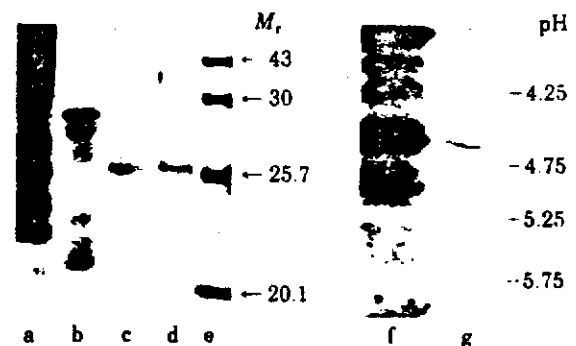

FIG. 2. Electrophoretic profiles of proteins isolated from rat liver. A minimum of 50 µg of protein was electrophoresed in each lane. Left: Polyacrylamide gel electrophoresis in 0.1% $NaDodSO_4$ (17) and protein staining by the method of Diezel *et al.* (23). Lanes a: ethanol-precipi-tated material; b. UM-10 retentate; c. HPI, source 1. d. HPI source 2. e. M_r markers × 10^{-3}. (Right: Isoelectric focusing. Lanes f. ethanol-precipitated fraction. g. HPI, source 1
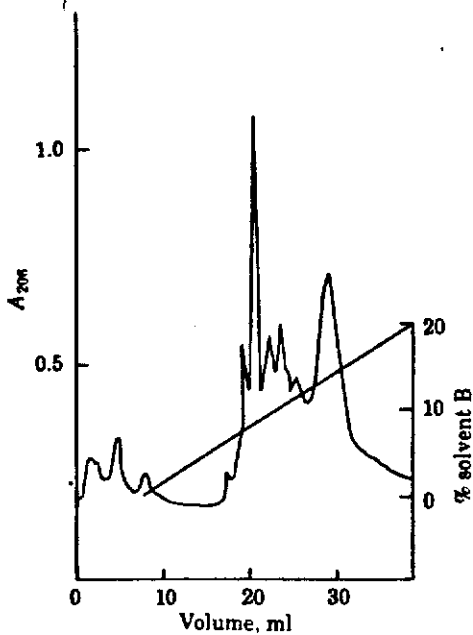
FIG. 3. HPLC of the UM-10 retentate. Solvent A, 0.05% trifluoroacetic acid in water; solvent B, 0.05% trifluoroacetic acid in acetonitrile; gradient, 0% solvent B to 20% solvent B over 30 min at a flow rate of 1.0 ml/min.

the ethanol-precipitated material into a great number of components but HPI, source 1, still consisted of a single protein band with an isoelectric point of 4.65 (Fig. 2 *Right*).

HPLC of the UM-10 retentate (Fig. 3) showed the presence of a number of components, whereas, under the same conditions, HPI, source 1, was eluted as a sharp single peak at approximately 8% acetonitrile (Fig. 4). This HPLC peak fraction (HPI, source 2) was lyophilized and analyzed further. When subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, it migrated as a single band (Fig. 2, lane d) identical in position to that of HPI, source 1 (Fig. 2, lane c).

The results of the bioassay are given in Table 2. At low concentrations (1–10 nM) the purified material isolated either by the DEAE-cellulose column alone (HPI, source 1) or by the additional step using HPLC (HPI, source 2) inhibited the proliferation of nonmalignant liver cell line as evidenced by the reduced number of large cell colonies (diameter >0.5 mm) but did not significantly affect the total number of colonies at the lower doses. At a dose of 0.1 µg/ml or higher, the inhibition was statistically highly significant. No countable colonies were formed when nonmalignant liver cells were maintained with HPI for the entire duration of the experiment. The inhibitor had no significant effect (inhibitory or activatory) on the proliferation of the malignant cell line NRL ST whether HPI was present for 4 days or 4 + 5 days.

DISCUSSION

Ever since the introduction of the chalone concept by Bullough (11), studies involving inhibitors of cell proliferation have been the subject of both controversy and criticism. Although some of this criticism was directed at the assay conditions and the very high concentrations of the inhibitors used, most dealt with the lack of purity of the preparations tested. In a recent review (24), Iudal emphasized the need for purified material as well as reliable assays. The material that was isolated in this study was considered to be pure because electrophoresis of this preparation by two different analytical procedures resulted in a single
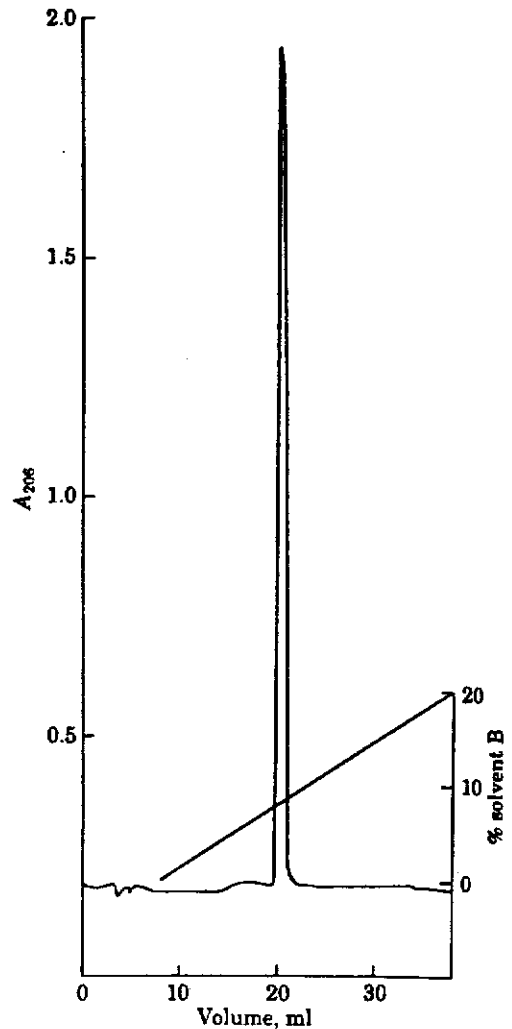

FIG. 4. HPLC of HPI, source 1. Conditions as in Fig. 3.

protein band. The possibility of a low molecular weight minor contaminant having the biological activity without actually being bound to the M_r 26,000 protein may be ruled out for the following reasons. (a) The biological activity was not lost or reduced after extensive dialysis (9). (b) The activity was retained by UM-10 ultrafiltration. (c) The active material eluted in the void volume from a Sephadex G-25 column (9), indicating that it was associated with a macromolecule larger than M_r 25,000. (d) Analysis of the HPI by reverse-phase HPLC was monitored at 206 nm, a condition that is highly sensitive. Under these conditions minor contaminants were not detected. (e) Proteolytic digestion of the HPI, source 1, resulted in the total loss of activity as well as the loss of the M_r 26,000 protein. If there was a contaminating substance, it could not be separated by any of the techniques used and would have been tightly bound to the M_r 26,000 protein. Thus, the HPI is a protein (degradable by proteolytic enzymes and with a A_{280}/A_{260} ratio of 1.65) but not a glycoprotein (not stainable by periodic acid–Schiff reagent). It consists of a single subunit as revealed by NaDodSO$_4$/polyacrylamide gel electrophoresis and shows no molecular heterogeneity by isoelectric focusing. As shown previously 9, HPI is not a thymidine-catabolizing enzyme or arginase.

This purified protein (HPI, source 1 or source 2) inhibits the proliferation of nonmalignant rat liver epithelial cells at low concentrations, but it had no effect on the malignant line. Many of the "hepatic chalone" preparations isolated heretoto (14, 15, 25–29) inhibited DNA synthesis in malignant

Table 2. Effect of HPI on proliferation of nonmalignant and malignant rat liver epithelial cells in culture

Target cells[*]	HPI		Colonies, no.[†]			Fraction of large colonies
	Source	μg/ml	>0.2 mm	>0.5 mm	P[‡]	
FNRL	1	0	214 ± 5 (100)	94 ± 5 (100)	—	0.44
		0.01	201 ± 10 (94)	84 ± 10 (89)	NS	0.42
		0.1[§]	196 ± 2 (92)	55 ± 4 (59)	<0.005	0.28
		1.0[§]	192 ± 5 (90)	25 ± 5 (27)	<0.001	0.13
		10.0	183 ± 8 (84)	11 ± 4 (12)	<0.001	0.06
FNRL	2	0	214 ± 5 (100)	94 ± 5 (100)	—	0.44
		0.01	205 ± 13 (96)	105 ± 12 (112)	NS	0.51
		0.1[§]	181 ± 4 (85)	60 ± 2 (64)	<0.005	0.33
		1.0[§]	182 ± 4 (85)	20 ± 1 (21)	<0.001	0.11
		10.0	160 ± 5 (75)	8 ± 2 (9)	<0.001	0.05
NRL ST	1	0	194 ± 9 (100)	166 ± 7 (100)	—	0.86
		0.5	181 ± 3 (93)	161 ± 3 (97)	NS	0.89
		1.0[§]	177 ± 3 (91)	156 ± 3 (94)	NS	0.88
		5.0	182 ± 6 (94)	158 ± 6 (95)	NS	0.87
		10.0	183 ± 4 (94)	154 ± 4 (93)	NS	0.84

[*] FNRL, nonmalignant; NRL ST, malignant.

[†] Mean ± SEM of the number of colonies per dish, from six dishes per dose. The values in parentheses are percentages of the respective control values.

[‡] Student's *t* test was used for comparing the means. NS, nonsignificant.

[§] When this concentration of HPI was maintained throughout the experimental period (4 + 5 days), no countable colonies were formed in FNRL but there was no reduction in the number or size of NRL ST colonies.

atoma) cells; in one case, a liver extract was also reported (15) to induce differentiation of the hepatoma cell line H-35. Inhibitory factors derived either from BSC 1, an epithelial cell line isolated from kidney of African green monkey (6), or from calf serum (7) have been shown to inhibit the proliferation of nonmalignant epithelial cells. The serum factor (7) did show slight inhibition of proliferation in different malignant cells also. We have shown earlier that partially purified proliferation inhibitors from liver not only inhibit nonmalignant liver cells but act as growth factors in some malignant liver cells (9, 10). HPI is now shown to have no growth-promoting activity toward one of the same malignant liver cell lines. The growth-promoting component(s) was recovered in another DEAE-cellulose chromatographic fraction and this material stimulated cell division of both the malignant and the nonmalignant liver cell lines. The separation of the growth-stimulatory factor(s) from the HPI during salt elution may account for the fact that there was a 40-fold increase in specificity activity of the inhibitor with only 50% decrease in protein (Table 1). The 6000-fold increase in specific activity observed during the purification procedure represents a minimal enhancement because the crude liver homogenate was cytotoxic and could not be evaluated by bioassay.

A major difficulty in studies with growth-inhibitory factors has been the problem of differentiating between reversible and irreversible inhibition. Short-term assays involving the use of [³H]thymidine incorporation into DNA as the sole indicator of cell proliferation have many apparent drawbacks and have been criticized (30, 31). Recently, we described a simple quantitative bioassay which takes into account the actual proliferative capacity of a cell (9, 10). This assay, which is based on the ability of cells in culture to proliferate and form colonies after treatment and removal of cytostatic inhibitors, distinguishes physiological inhibition of proliferation from acute cytotoxicity. When cells treated for 4 days with such a reversible inhibitory factor are released from a block of cell division by being fed normal culture medium and are maintained for an additional 5 days they would be expected to form more smaller colonies and fewer larger colonies compared with the untreated control cell population. After treatment with HPI, the fraction of the larger colonies was

decreased with a concurrent increase in the fraction of the smaller colonies. We have used a range of doses of HPI covering 4 orders of magnitude and found that, even when a low dose (0.1 μg/ml) was used, there was considerable inhibition of the larger colonies (reversible inhibition) and very little inhibition of the total colonies. However, when a dose of 1 or 10 μg/ml was used, inhibition was observed also in the total of number of colonies. This could be due to the incomplete removal of the HPI on day 5, and effective doses may have remained for the next 5 days, especially in the dishes receiving 10 μg/ml. This might explain the "toxicity" seen in such dishes. This is consistent with the fact that no cell colonies were formed when HPI at low concentrations was purposely left for 4 + 5 days with the nonmalignant cells but no effect of HPI was observed in the malignant cell line, NRL ST.

These results demonstrate that an endogenous protein can exert a differential effect on the proliferation of malignant and nonmalignant liver epithelial cells. Compared with the majority of the proliferation-inhibitory factors reported so far, HPI is effective at a considerably lower concentration with the exception of the kidney cell inhibitors purified by Holley *et al.* (6) which were tested in cells cultured in a low-serum medium. The differences in the dose–response compared with that of Holley *et al.* (6) may be because we have tested HPI in nonmalignant liver epithelial cells cultured with 10% serum, a condition reported (6) to counteract the inhibitory effect or because there is some minor active substance contaminating the HPI preparation. We have shown that, even in the presence of 10% serum, HPI is active at a relatively low concentration. The second possibility is unlikely as discussed above. However, a physiological association may exist between the HPI and some minor cofactors. It is also likely that there may be an intrinsic difference between different inhibitors isolated from different sources

1. Lipkin, G., Rosenberg, M. & Knecht, M. E. (1976) *Biochem. Pharmacol.* 25, 1333–1337.
2. Holley, R. W., Armour, R. & Baldwin, J. H. (1978) *Proc. Natl. Acad. Sci. USA* 75, 339–341.
3. Bowen-Pope, D. F., Vidair, C., Sanui, H. & Rubin, A. H. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1308–1312.
4. Potter, V. R. (1980) *Yale J. Biol. Med.* 53, 367–384.
5. Potter, V. R. (1981) *Oncodevelopmental Biology and Medicine* 2, 243–266.
6. Holley, R. W., Böhlen, P., Fava, R., Baldwin, J. H., Kleeman, G. & Armour, R. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5989–5992.
7. Harrington, W. N. & Godman, G. C. (1980) *Proc. Natl. Acad. Sci. USA* 77, 423–427.
8. Steck, P. A., Voss, P. G. & Wang, J. L. (1979) *J. Cell Biol.* 83, 562–575.
9. McMahon, J. B. & Iype, P. T. (1980) *Cancer Res.* 40, 1249–1254.
10. Iype, P. T. & McMahon, J. B. (1981) *Cancer Res.* 41, 3352–3354.
11. Bullough, W. S. (1965) *Cancer Res.* 25, 1683–1727.
12. Iversen, O. H. (1981) in *Handbook of Experimental Pharmacology*, ed. Baserga, R. (Springer, New York), Vol. 57, pp. 491–550.
13. Houck, J. C. & Hennings, H. (1973) *FEBS Lett.* 32, 1–8.
14. Kuo, C. Y. & Yoo, T. J. (1977) *J. Natl. Cancer Inst.* 59, 1691–1695.
15. dePaermentier, F., Barbason, H. & Bassleer, R. (1979) *Biol. Cell* 34, 205–211.
16. Verly, W. G., Deschamps, Y., Pushpathadam, J. & Desrosiers, M. (1971) *Can. J. Biochem.* 49, 1376–1383.
17. Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* 80, 575–599.
18. Winter, A., Ek, K. & Andersson, U. (1977) LKB Application Note 250.
19. Fairbanks, G., Steck, T. L. & Wallack, D. F. H. (1971) *Biochemistry* 10, 2606–2616.
20. Henderson, L. E., Sowder, R. & Oroszlan, S. (1981) in *Chemical Synthesis and Sequencing of Peptides and Proteins*, eds. Liu, T.-Y., Schechter, A. K., Heinrikson, R. L. & Condliffe, P. G. (Elsevier, Amsterdam), pp. 251–260.
21. Iype, P. T. (1971) *J. Cell Physiol.* 78, 281–288.
22. Iype, P. T. & Allen, T. D. (1979) *Cancer Lett.* 6, 27–32.
23. Diezel, W., Koppeischläger, G. & Hofmann, E. (1972) *Anal. Biochem.* 48, 617–620.
24. Nadal, C. (1979) *Arch. Toxicol. Suppl.* 2, 131–142.
25. Miyamoto, M. & Terayama, H. (1971) *Biochim. Biophys. Acta* 228, 324–330.
26. Molinard, R., Pieter, G., Chany, E., Trincal, G. & Frayssinet, C. (1975) *Biomedicine* 23, 434–437.
27. Vinet, B. & Verly, W. G. (1976) *Eur. J. Cancer* 12, 211–218.
28. Sekas, G. & Cook, R. T. (1976) *Exp. Cell Res.* 102, 422–425.
29. Barra, R., Parsons, J., Koch, M. R. & Lea, M. A. (1979) *Cancer Res.* 39, 1655–1660.
30. Allen, J. (1978) *Nature (London)* 276, 669.
31. Davison, P., Liu, S. & Karasek, M. (1979) *Cell Tissue Kinet.* 12, 605–614.