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The Rifamycin Derivative AF/013 Is Cytolytic

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SUMMARY

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When intact cells (HeLa S-3, mouse 3T3 fibroblasts, hen erythrocytes) are incubated with the rifamycin SV derivative AF/013, which is widely used as a potent inhibitor of mammalian DNA-dependent RNA polymerases, a rapid and extensive disintegration of the plasma membrane takes place. This cell disruption results, for example, in hemolysis of the erythrocytes at concentrations above 50 μg/ml, in the release of cytoplasmic components, including ribosomes, and in dramatic ultrastructural changes in the HeLa and mouse 3T3 cells. In HeLa cells incubated for 30 min in 100 μg/ml of the drug, about 70% of the RNA, 44% of the protein, and 26% of the phospholipids leak out into the medium. The permeability of the plasma membrane is markedly increased, even at relatively low AF/013 concentrations. In the presence of 20 μg/ml of the drug, for example, 70% of the trichloracetic acid-soluble radioactivity and 10% of the trichloracetic acid-precipitable RNA are released from [^{3}H]uridine-labeled HeLa cells. The membranolytic action of AF/013 is higher than that of the nonionic surfactant Triton X-100. This high toxicity indicates that AF/013 cannot be used as a specific inhibitor of RNA polymerases *in vivo*.

Rifamycin SV and several of its derivatives, the most common one being rifampicin, are important antibiotics in chemotherapy (1). They bind to free bacterial DNA-dependent RNA polymerases and modify these molecules, thus blocking the initiation processes before the onset of transcription (2, 3). Several semisynthetic derivatives of rifamycin SV, especially C-3-substituted molecules with large side chains, also inhibit the RNA-dependent DNA polymerase ("reverse transcriptase") of RNA tumor viruses and various types of mammalian RNA polymerases (4, 5). One of the most potent inhibitors of eukaryotic RNA polymerases is a rifamycin derivative with an aliphatic (octyl) side chain in position 3 of the naphthoquinone chromophor (trivial name, 3-formylrifamycin SV *O*-*n*-octyloxime, or AF/013). This compound was shown to inactivate different mammalian RNA polymerases nonselectively *in vitro* and primarily to prevent the initiation steps of transcription, whereas elongation of the nascent chains was not affected (e.g., refs. 6 and 7) or was only partially (8, 9) impaired. As to its application to living cells, however, it is possible that AF/013 might interfere with several other metabolic processes. For example, it has been reported that this drug almost totally inhibits the uptake of exogenous nucleosides into HeLa cells (10). In the

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course of experiments with various inhibitors of transcriptional events the present author made observations that raise the question whether the inhibition of RNA synthesis seen *in vivo*, for example in normal and leukemic human blood cells (11) and in explanted salivary glands of *Chironomus* (12), can be explained by the specific molecular mechanism that has been hypothesized from enzyme assays *in vitro*.

HeLa S-3 cells grown in suspension, mouse 3T3 fibroblasts grown in monolayers, and freshly prepared hen erythrocytes were used. The rifamycin SV derivative AF/013 was a gift of Dr. Marco Crippa (Université de Genève, Switzerland). Mass spectroscopic analysis (Varian 711) confirmed the purity of the compound, with a molecular weight of 852 ($C_{46}H_{64}N_2O_{13}$). AF/013 was freshly dissolved before each experiment in DMSO[1] at a concentration of 20 mg/ml and was further diluted with the appropriate incubation medium.

HeLa S-3 cells grown in Eagle's basal medium supplemented with 10% fetal calf serum were washed 5 times at room temperature in medium 1 (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 154 mM NaCl, and 1 mM $MgCl_2$, pH 7.2). In some experiments the cells had been labeled for 20 hr by adding 2 μCi/ml of [^{3}H]uridine (43 Ci/mmole; Radiochemical Centre, Amersham, England) to the growth medium. The washed cell pellet was suspended in medium 1 at a density of about 1×10^6 cells/ml. Some aliquots were incubated with 10–100 μg/ml of AF/013; to the others was added the same amount of the pure solvent (0.05–0.5% DMSO, final concentration). Incubation was carried out for 30 min at 37° under continuous gentle shaking.

The growth medium of an almost confluent monolayer of 3T3 cells was removed, mixed with AF/013 to give a concentration of 100 μg/ml, and immediately added back to the cells. Controls were treated identically with the DMSO solvent (final concentration, 0.5%).

Blood was obtained from a decapitated hen and diluted with 0.9% NaCl solution containing 2% of the anticoagulate Liquemin (Hoffman–La Roche AG, Grenzach, Federal Republic of Germany). Erythrocytes were enriched by centrifugation at 300 × *g* for 10 min and resuspension in 0.9% NaCl solution to a final density of 9×10^7 erythrocytes/ml. To 5-ml aliquots increasing amounts of AF/013 (0.1–160 μg/ml) were added; controls were incubated with similar quantities of DMSO. Incubation was performed at room temperature for 10 min.

[^{3}H]Uridine-labeled HeLa S-3 cells and hen erythrocytes were incubated under the conditions described above with the detergent Triton X-100 at concentrations ranging from 10 to 1000 μg/ml. After incubation the HeLa cells were centrifuged at 2000 × *g* for 10 min at 25°, the pellets were homogenized in ice-cold 5% TCA using a motor-driven Potter-Elvehjem homogenizer, and the supernatants were adjusted to 5% TCA in the cold. After 30 min the TCA-precipitated material was sedimented (3000 × *g*, 15 min, 4°) and washed twice with 5% cold TCA. Protein was determined by the method of Lowry *et al.* (13) and/or by a modified Nessler reaction (14). DNA, RNA, and phospholipids were determined as described (15).

Labeled HeLa cells were harvested after incubation with AF/013 and the solvent DMSO, with DMSO alone, or with Triton X-100 by centrifugation at 2000 × *g* for 10 min. Aliquots were taken from the supernatants in order to determine the radioactivity released from the cells. The cell pellets were then washed three times in medium 1 at room temperature and finally homogenized in ice-cold 10% TCA. After 30 min at 4°, the solutions were centrifuged at 3000 × *g* for 15 min. TCA-soluble radioactivity was determined in the supernatants. The pellets were resuspended in 10% TCA and applied to Whatman GF/C glass fiber filters. After thorough washing with cold 10% TCA the filters were immersed in 2 ml of "NCS" (Nuclear-Chicago solubilizer; Amersham/Searle) and incubated overnight at 40°. Toluene-based scintillation fluid was added, and radioactivity was determined in a scintillation counter (Betaszint 5000; Berthold Friesecke, Wildbad, Federal Republic of Germany). Counting efficiency was corrected for quenching.

[1] The abbreviations used are: DMSO, dimethyl sulfoxide; TCA, trichloracetic acid.

After incubation, the erythrocytes were centrifuged for 5 min at 3000 × *g*. Hemolysis could readily be judged from the color that remained in the supernatant as compared to the control.

Light micrographs of unfixed 3T3 cells grown on Falcon dishes were taken with an inverted phase contrast microscope (Leitz Diavert). HeLa S-3 cells were photographed in phase contrast using a Zeiss photomicroscope III. For electron microscopy, the cells were fixed at room temperature in 2.5% glutaraldehyde buffered to pH 7.2 with 0.05 M sodium cacodylate containing 70 mM KCl and 2 mM $MgCl_2$. After 1 hr the cells were washed several times in cold cacodylate buffer and incubated in 1% OsO_4 (same buffer) for 2 hr in the cold. Dehydration was carried out in graded ethanol solutions and propylene oxide, and the cells were embedded in Epon 812. Ultrathin sections were cut on a Reichert ultramicrotome OmU_3. Electron micrographs were taken with a Siemens Elmiskop 101.

The rifamycin derivative AF/013 at a concentration higher than 50 μg/ml (approximately 60 μM) causes immediate disruption of the plasma membrane, as indicated by the complete hemolysis of hen erythrocytes. When erythrocytes are incubated at the same cell density with 50 μg/ml of Triton X-100, no leakage of hemoglobin occurs. Hemolysis starts at a detergent concentration of about 180–200 μg/ml (approximately 300 μM). When HeLa or mouse 3T3 cells are treated with 100 μg/ml of AF/013, striking cytopathic effects are observed. Under the light microscope the cells appear more refractile and the plasma membrane is no longer a distinct boundary but reveals the formation of numerous large, bleblike evaginations (arrows in Fig. 1b and inset of Fig. 2d). The nuclei stand out against the cytoplasmic zones with enhanced contrast as compared to the controls (compare Fig. 1a with 1b and the inset in Fig. 2b with that in 2d). Electron micrographs demonstrate extensive disintegration not only of the plasma membrane, which in most cases is totally lost, but also of the endoplasmic reticulum (Fig. 2c and d). Cytoplasmic organelles, like mitochondria, no longer show their

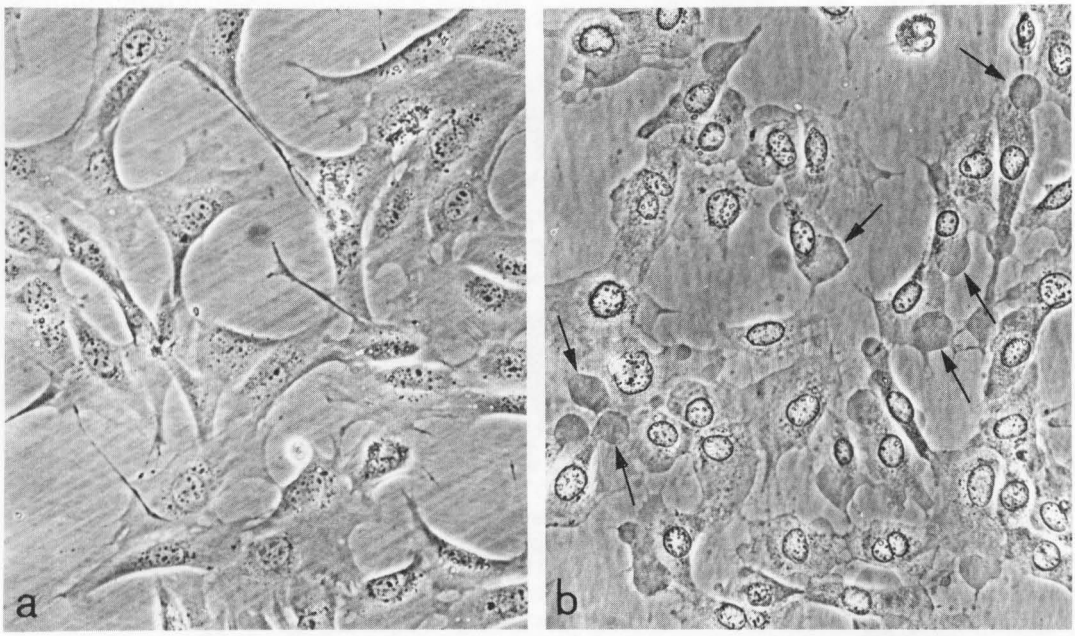
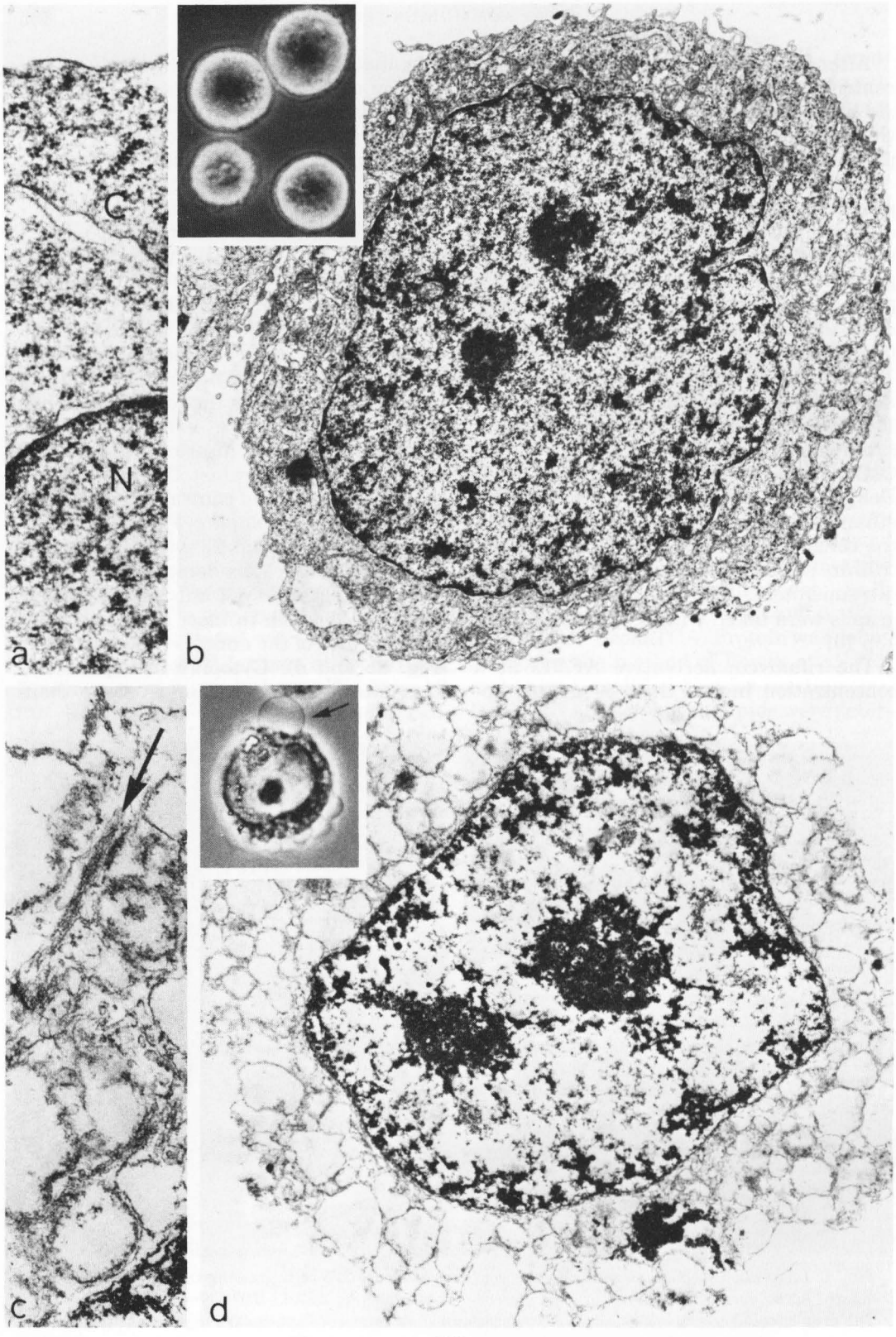


FIG. 1. *Light microscopic appearance (phase contrast) of mouse 3T3 cells growing in Falcon dishes when incubated for 30 min in the absence (a) and presence (b) of rifamycin AF/013 (100 μg/ml)*

The drug-exposed cells have rounded-off surfaces and show numerous spherical evaginations (e.g., at the arrows in Fig. 1b). Note also the enhanced contrast of their nuclei. × 175.


C
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a
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typical internal architecture. In addition, ribosomes and polyribosomes, which are abundant in control cells (Fig. 2a and b), cannot be distinguished in the cytoplasm of the drug-exposed cells (Fig. 2c and d). Characteristically, the nuclear envelope is somewhat better preserved, as is also the case for the chromatin and the nucleoli. The interchromatinic regions, however, appear to be less electron-dense, indicating leakage of nuclear material. The chemical determinations (Table 1) are in agreement with these observations. The DNA content per cell is unaltered, whereas most of the acid-precipitable RNA and nearly half the protein is lost. The total phospholipid content is reduced by about 25%. All this released material can be recovered from the supernatant obtained after centrifugation of the cells. Low concentrations of AF/013 cause a pronounced leakage of charged solutes through the plasma membrane, as demonstrated for the [^{3}H]uridine-labeled nucleosides, nucleotides, and low molecular weight RNA (Fig. 3). At a drug concentration of 10 μg/ml 25% of the TCA-soluble radioactivity is released, but almost all the high molecular weight, TCA-precipitable RNA is retained within the cells. At an AF/013 concentration of 20 μg/ml 70% of the TCA-soluble material is lost from the cells, compared to only 10% of the high molecular weight RNA. A representative nonionic surfactant, Triton X-100, causes a similar leakage only at much higher concentrations, i.e., 70 and 100 μg/ml (Fig. 3).

The rifamycin SV derivative AF/013, when added at concentrations necessary for significant inhibition of mammalian RNA polymerases *in vitro* (50–100 μg/ml; e.g., refs. 8–10), has a strong membranolytic activity and lyses eukaryotic cells. Immediately after exposure to the drug

TABLE 1

Gross composition of HeLa S-3 cells with and without treatment with rifamycin AF/013 (100 μg/ml, 30 min)

	Control	AF/013-treated	Loss after AF/013 treatment
	pg/cell	*pg/cell*	*%*
DNA	14.7	14.8	0
RNA	26.7	8.6	68.0
Protein	447.5	253.3	43.5
Phospholipids	35.6	26.3	26.1

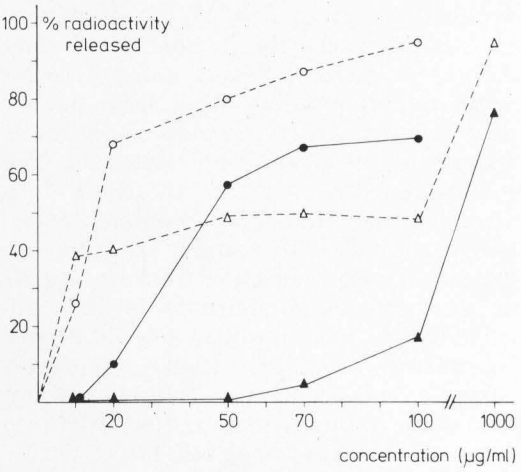

FIG. 3. *Leakage of radioactivity contained in TCA-soluble (– – –) and TCA-insoluble (——) fractions of [^{3}H]uridine-labeled HeLa S-3 cells after exposure to various concentrations of AF/013 (○, ●) and the nonionic detergent Triton X-100 (△, ▲)*

HeLa S-3 cells were grown for 20 h in the presence of 2 μCi/ml of [^{3}H]uridine, washed several times in medium 1, and then incubated in the same buffer at a density of about 1×10^6 cells/ml for 30 min in the presence of the specific substance. The solvent DMSO alone did not affect the permeability of the plasma membrane at the concentrations used for the AF/013 experiments.

FIG. 2. *Light and electron micrographs of untreated (a, b) and AF/013-treated (c, d) HeLa S-3 cells*

Most of the plasma membrane of the cells that have been incubated for 30 min with 100 μg/ml of AF/013 is lost or appears in isolated blebs (inset in Fig. 2d, arrow); the cytoplasmic region is recognized only as an aggregate of large membrane vesicles and filaments (Fig. 2c and d; some microfilaments are denoted by the arrow in Fig. 2c). In contrast to the controls (Fig. 2a and b), ribosomes and polyribosomes can no longer be recognized in the drug-treated cells. Mitochondria appear destroyed. Note the low electron density of the interchromatinic regions in the drug-treated cells, indicative of the loss of nuclear material. N, nucleus; C, cytoplasm. a. × 40,000. b. × 8400 (inset, × 750). c. × 47,000. d. × 8000 (inset, × 750).

the cells are no longer viable, since the plasma membrane is destroyed and the internal compartmentalization appears to be lost. Most of the cellular RNA and half the protein are released into the medium. It seems reasonable to assume that the AF/013 molecule, owing to its hydrophobic side chain, strongly interacts with membrane constituents, by phase partitioning or by hydrophobic association, thus causing the destabilization and, finally, the breakdown of the membrane. This membranolytic effect might also explain the inhibited nucleoside uptake observed in the presence of AF/013 and other rifamycin derivatives (10), and the blockade of DNA replication seen *in vivo* (11, 16). Moreover, it has been clearly demonstrated that AF/013 *in vitro* interacts nonspecifically with several proteins other than nucleic acid polymerases (17, 18) and also inhibits translation in a cell-free system (10). The membranolytic effect of AF/013 is even stronger than that of the nonionic detergent Triton X-100. In order to achieve a release of macromolecules from HeLa cells or erythrocytes comparable to that obtained with AF/013, about a 4-fold higher concentration of the surfactant is necessary, i.e., a concentration that is known to transform cultured mammalian cells into "ghost monolayers" (see ref. 19).

From the above results it is concluded that the rifamycin derivative AF/013 (and probably also other derivatives with hydrophobic side chains) is highly toxic to living animal cells and therefore cannot be used as a specific inhibitor of DNA-dependent RNA polymerases *in vivo* (12). Other compounds of the rifamycin series, including the potent inhibitor of prokaryotic RNA polymerases, rifampicin, also appear to exert toxic effects on eukaryotic cells, as has been concluded from studies on the suppressed focus formation in chick fibroblasts after infection with Rous sarcoma virus (20, 21).

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REFERENCES

1. Binda, G., Domenichini, E., Gottardi, A., Orlandi, B., Ortelli, E., Pacini, B. & Fowst, G. (1971) *Arzneim. Forsch.*, **21**, 1907–1968.
2. Wehrli, W. & Staehelin, M. (1969) *Biochim. Biophys. Acta*, **182**, 24–29.
3. Lill, U. I. & Hartmann, G. R. (1973) *Eur. J. Biochem.*, **38**, 336–345.
4. Riva, S. & Silvestri, L. G. (1972) *Annu. Rev. Microbiol.*, **26**, 199–224.
5. Green, M., Gurgo, C., Gerard, G., Grandgenett, D. & Shimada, K. (1974) in *Molecular Studies in Viral Neoplasia*, pp. 258–289, Williams & Wilkins, Baltimore.
6. Butterworth, P. H. W., Cox, R. F. & Chesterton, C. J. (1971) *Eur. J. Biochem.*, **23**, 229–241.
7. Meilhac, M., Tysper, Z. & Chambon, P. (1972) *Eur. J. Biochem.*, **28**, 291–300.
8. Juhasz, P. P., Benecke, B. J. & Seifart, K. H. (1972) *FEBS Lett.*, **27**, 30–34.
9. Tsai, M.-J. & Saunders, G. F. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 2072–2076.
10. Busiello, E., Di Girolamo, A., Di Girolamo, M., Fischer-Fantuzzi, L. & Vesco, C. (1973) *Eur. J. Biochem.*, **35**, 251–258.
11. Smith, R. G., Whang-Peng, J., Gallo, R. C., Levine, P. & Ting, R. C. (1972) *Nat. New Biol.*, **236**, 166–171.
12. Egyhazi, E. (1975) *Biochim. Biophys. Acta*, **378**, 205–215.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
14. Strauch, L. (1965) *Z. Klin. Chem.*, **3**, 165–167.
15. Zentgraf, H., Deumling, B., Jarasch, E.-D. & Franke, W. W. (1971) *J. Biol. Chem.*, **246**, 2986–2995.
16. Binkerd, P. & Toliver, A. (1974) *Mol. Cell. Biochem.*, **5**, 177–183.
17. Riva, S., Fietta, A. & Silvestri, L. G. (1972) *Biochem. Biophys. Res. Commun.*, **49**, 1263–1271.
18. Gerard, G. F., Gurgo, C., Grandgenett, D. P. & Green, M. (1973) *Biochem. Biophys. Res. Commun.*, **53**, 194–201.
19. Tsai, R. L. & Green, H. (1973) *Nat. New Biol.*, **243**, 168–170.
20. Robinson, H. L. & Robinson, W. S. (1971) *J. Natl. Cancer Inst.*, **46**, 785–788.
21. Vaheri, A. & Hanafusa, H. (1971) *Cancer Res.*, **31**, 2032–2036.