

1-1-1981

Erythroglycan biosynthesis in K-562 cells. Inhibition of synthesis by tunicamycin and lack of attachment to the G-protein of vesicular-stomatitis virus

J. S. Rush
University of Kentucky

S. J. Turco
University of Kentucky

R. A. Laine
University of Kentucky

Follow this and additional works at: https://digitalcommons.lsu.edu/biosci_pubs

Recommended Citation

Rush, J., Turco, S., & Laine, R. (1981). Erythroglycan biosynthesis in K-562 cells. Inhibition of synthesis by tunicamycin and lack of attachment to the G-protein of vesicular-stomatitis virus. *Biochemical Journal*, 193 (1), 361-365. <https://doi.org/10.1042/bj1930361>

This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Digital Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Digital Commons. For more information, please contact ir@lsu.edu.

Erythroglycan biosynthesis in K-562 cells

Inhibition of synthesis by tunicamycin and lack of attachment to the G-protein of vesicular-stomatitis virus

Jeffrey S. RUSH, S. J. TURCO* and R. A. LAINE*
Department of Biochemistry, College of Medicine, University of Kentucky,
Lexington, KY 40536, U.S.A.

(Received 14 May 1980/Accepted 28 August 1980)

K-562 cells, which express foetal erythroglycan, are shown to synthesize the lipid-linked oligosaccharide intermediates commonly found in tissues and cultured fibroblasts. The addition of tunicamycin, which blocks the formation of these intermediates and thus of asparagine-linked oligosaccharides, inhibits the synthesis of erythroglycan (M_r 7000–11 000). Vesicular-stomatitis-virus infection of K-562 cells results in the glycosylation of the G-protein with the transferrin-type oligosaccharide (M_r 3000), but not with the larger erythroglycan. These results suggest that, in K-562 cells, the early stages of erythroglycan biosynthesis are the same as those of the transferrin-type oligosaccharides. However, maturation of the oligosaccharide is influenced by protein structure such that erythroglycan is only expressed on specific glycoproteins.

K-562 cells, originating from the pleural effusion of a chronic-myelogenous-leukaemia patient, have been shown to express a long-chain *N*-linked oligosaccharide (M_r 7000–11 000; 'erythroglycan') on specific cell-surface glycoproteins (Turco *et al.*, 1980). The distinct feature of erythroglycan is the presence of keratan-like repeating Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 3 sequences attached to the polypeptide via a mannose-containing core (Järnefelt *et al.*, 1978). This cell line also synthesizes the common bi- and tri-antennary types of *N*-linked 'complex' glycopeptides such as those that can be isolated from bovine transferrin (M_r 3000) and that differ from erythroglycan by the absence of repeating *N*-acetyl-lactosamine units. The common complex chains have already been shown to be synthesized via lipid-linked oligosaccharide intermediates (Parodi & Leloir, 1979), and the present study, with the use of the antibiotic tunicamycin, provides evidence that the larger complex chains are synthesized in a similar manner.

In addition, we have examined vesicular-stomatitis-virus-infected K-562 cells in order to evaluate the selectivity of *N*-glycosylation of the viral glycoprotein in a cell line known to produce several kinds of *N*-linked saccharides. Vesicular-stomatitis virus is a lipid-enveloped RNA virus containing one glycoprotein (G-protein) in its membrane. It is an appropriate model system to address this aspect, since normal host protein, as well as

* To either of whom correspondence and requests for reprints should be addressed.

RNA synthesis, is inhibited during viral infection (Etchison *et al.*, 1977). The G-protein is reported to be glycosylated with two transferrin-type oligosaccharides by host enzymes in fibroblastic cell lines such as BHK (baby-hamster kidney) (Reading *et al.*, 1978). Thus we decided to determine whether the G-protein could be glycosylated with the erythroglycan chain in K-562 cells.

In the present paper we show that the G-protein is glycosylated in K-562 cells only with oligosaccharides of approx. $M_r \approx 3000$ and not with the longer-chain erythroglycan. The results indicate that there are at least two pathways of synthesis via lipid-linked intermediates of *N*-linked glycopeptides of the complex type in K-562 cells. The G-protein of vesicular-stomatitis virus appears to be selectively glycosylated by the transferrin-type pathway, leading to typical bi- and tri-antennary oligosaccharide structures (Kornfeld & Kornfeld, 1976).

Materials and methods

Materials

Endo- β -galactosidase from *Escherichia freundii* was generously given by Professor Y.-T. Li (Tulane University, New Orleans, LA, U.S.A.). Other materials were as follows: [6- 3 H]glucosamine (18.8 Ci/mmol), [2- 3 H]mannose (18.4 Ci/mmol) and [3 H]leucine (51.6 Ci/mmol) from New England Nuclear Corp.; Bio-Gel P-4 from Bio-Rad Laboratories; Sephadex G-50 from Sigma. Tunicamycin

was obtained from Dr. Robert Hamill of Eli Lilly Co.

Cell culture

K-562 cells were obtained from Professor C. B. Lozzio (University of Tennessee, Knoxville, TN, U.S.A.) as passage no. 211. The cells were maintained in RPM1-1640 medium (Gibco) supplemented with 10% (v/v) foetal-calf serum (Gibco). Cell cultures were determined to be free of mycoplasma as described by Livingston *et al.* (1975). All experiments were performed on cells below passage no. 240.

Metabolic labelling and extraction

Exponentially growing cells (10^6 cells/ml) were centrifuged (1000 g) for 5 min to remove the medium and washed with 10 ml of phosphate-buffered saline (0.8% NaCl/0.02% KCl/0.115% Na_2HPO_4 /0.2% KH_2PO_4 /0.01% CaCl_2 /0.01% MgCl_2). The cells (10^7) were incubated in 1 ml of Dulbecco's modified Eagle's medium containing 2% of the normal concentration of glucose (20 $\mu\text{g}/\text{ml}$) and [^3H]glucosamine (20 $\mu\text{Ci}/\text{ml}$) or [^3H]mannose (20 $\mu\text{Ci}/\text{ml}$) and supplemented with 10% dialysed foetal-calf serum and glutathione (1 $\mu\text{g}/\text{ml}$). After 18 h of incubation at 37°C, the cells were separated from the medium by centrifugation, washed with 10 ml of phosphate-buffered saline and the labelled proteins extracted with chloroform/methanol/water (3:2:1, by volume). The insoluble residue obtained by extraction of the [^3H]mannose-labelled cells was washed with water and extracted with chloroform/methanol/water (10:10:3, by volume), which solubilized the oligosaccharide-lipid fraction (Turco *et al.*, 1977).

Preparation of protein-derived oligosaccharides

The oligosaccharides were released from the lipid-extracted pellet by hydrazinolysis with freshly distilled anhydrous hydrazine at 100°C for 20 h (Bayard & Roux, 1975). The hydrazine was removed by rotary evaporation under reduced pressure and by drying, under a stream of N_2 , with toluene three to five times. The amino sugars in the oligosaccharides were re-*N*-acetylated by treatment with pyridine/acetic anhydride (1:1, v/v) for 1 h at 100°C, followed by de-*O*-acetylation with 0.1 M-KOH in toluene/methanol (1:3, v/v) at room temperature for 4 h. The oligosaccharides were then reduced with 1 M- NH_3 /0.1 M- NaBH_4 , neutralized with acetic acid and chromatographed on Sephadex G-50.

Vesicular-stomatitis-virus infection of cells

Exponentially growing K-562 (2×10^5 – 20×10^5 cells/ml) or BHK cells (2×10^5 – 20×10^5 cells/60 mm-diam. plate) were infected with

vesicular-stomatitis virus (multiplicity of infection = 5–10). At 5 h after infection the medium was removed and the cells were incubated for 60 min in Dulbecco's modified Eagle's medium supplemented with dialysed 10% foetal-calf serum and the appropriate radioisotopically labelled amino acid or sugar. Then the medium was removed and the cells were washed twice with phosphate-buffered saline. The cells were precipitated with 5% trichloroacetic acid and washed with ice-cold acetone.

The radiolabelled proteins were dissolved and separated by polyacrylamide-gel electrophoresis as described by Laemmli (1970). The separating gel was 10% acrylamide and the stacking gel was 3.6% acrylamide. Electrophoresis was carried out for 5 h at 25 mA. After electrophoresis, the gels were subjected to fluorography as described by Bonner & Laskey (1974), Kodak XR-5 film being used.

Results

The pathway of biosynthesis of erythroglycan is unknown; however, preliminary information concerning its expression in K-562 cells was obtained by examining the distribution of lipid-linked oligo-

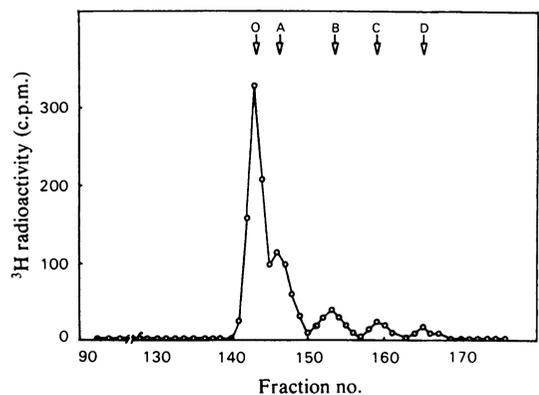


Fig. 1. Bio-Gel P-4 chromatogram of K-562-cell-derived [^3H]mannose-labelled oligosaccharides released from lipid by mild acid hydrolysis

The [^3H -Man]oligosaccharide-lipid fraction, obtained as described in the Materials and methods section, was subjected to mild acid hydrolysis (Turco *et al.*, 1977), treated with endo- β -*N*-acetylglucosaminidase H (Robbins *et al.*, 1977), mixed with [^{14}C -Man]oligosaccharide standards (not plotted) and applied to a column (1 cm \times 240 cm) of Bio-Gel P-4 (400 mesh); 0.5 ml fractions were collected. Letters above the peaks refer to the elution positions of standards (Liu *et al.*, 1979): O, $\text{Glc}_3\text{Man}_9\text{GlcNAc}$; A, $\text{Glc}_2\text{Man}_9\text{GlcNAc}$; B, $\text{Glc}_1\text{Man}_9\text{GlcNAc}$; C, $\text{Man}_9\text{GlcNAc}$; D, $\text{Man}_9\text{GlcNAc}$.

saccharide intermediates synthesized in these cells and by determining the effect of tunicamycin on metabolic carbohydrate labelling of high-molecular-weight oligosaccharides. As shown in Fig. 1, the profile of radioactivity from [^3H]mannose labelling *in vivo* of lipid-linked oligosaccharides is similar to that reported in numerous cell systems (Robbins *et al.*, 1977). Thus the largest labelled lipid-derived oligosaccharide is believed to be $\text{Glc}_3\text{Man}_2\text{GlcNAc}_2$, with lesser amounts of smaller lipid-derived oligosaccharides thought to be precursors to $\text{Glc}_3\text{Man}_2\text{GlcNAc}_2$ -lipid intermediate. With variable labelling times, oligosaccharides of unusual size were not observed.

Tunicamycin inhibits the glycosylation of glycoproteins with *N*-linked oligosaccharides by block-

ing the formation of dolichyl pyrophosphate *N*-acetylglucosamine and consequently all *N*-acetylglucosamine-lipid intermediates (Hickman *et al.*, 1977). As previously reported (Turco *et al.*, 1980), metabolic labelling of K-562 cells with [^3H]mannose results in the incorporation of label into glycoproteins whose glycopeptides can be separated into two major size classes by gel filtration on Sephadex G-50; 'erythroglycan' (M_r 10000), and bi- and tri-antennary structures of the transferrin-type (M_r 3000). As shown in Fig. 2, tunicamycin inhibits the incorporation of [^3H]mannose into both of these size classes to comparable extents. At a tunicamycin concentration of $4\ \mu\text{g}/\text{ml}$, specific radioactivity (c.p.m./ μg of cell protein) found in erythroglycan decreased by 75% and in transferrin-type structures by 82%. At this concentration of tunicamycin, total cellular protein was only 14% less than that present in control cultures, indicating that the primary effect of the antibiotic is on glycoprotein glycosylation. Increasing the tunicamycin concentration to $10\ \mu\text{g}/\text{ml}$ had no further significant effect on synthesis of either erythroglycan or the 3000- M_r saccharides. The incorporation of [^3H]glucosamine into erythroglycan was decreased by 66% at $4\ \mu\text{g}$ of tunicamycin/ml; no further decrease in synthesis was

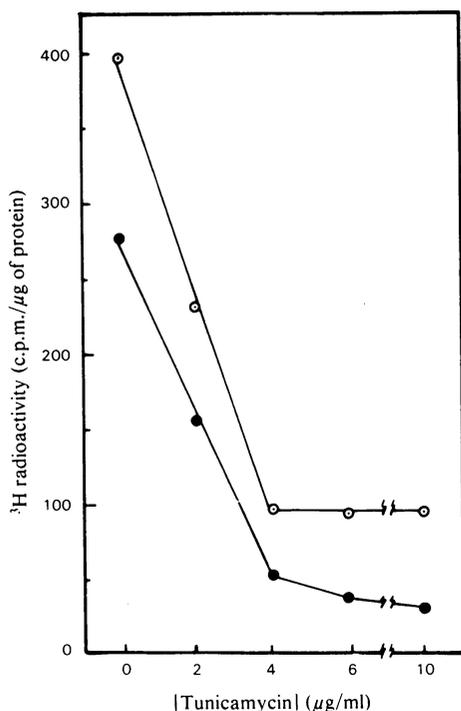


Fig. 2. Inhibition of synthesis of protein-derived oligosaccharides by tunicamycin

K-562 cells (2×10^6 cells) were preincubated with tunicamycin for 2 h and labelled with [^3H]glucosamine ($20\ \mu\text{Ci}$) for 16 h in the presence of tunicamycin. After labelling, protein-derived [^3H]mannose-labelled oligosaccharides were prepared as described in the Materials and methods section and subsequently chromatographed on Sephadex G-50 (Turco *et al.*, 1980). Protein was determined from duplicate plates of cells. The radioactivity (c.p.m./ μg of protein) in oligosaccharides of M_r 7000–11000 (○) and 3000 (●) is shown.

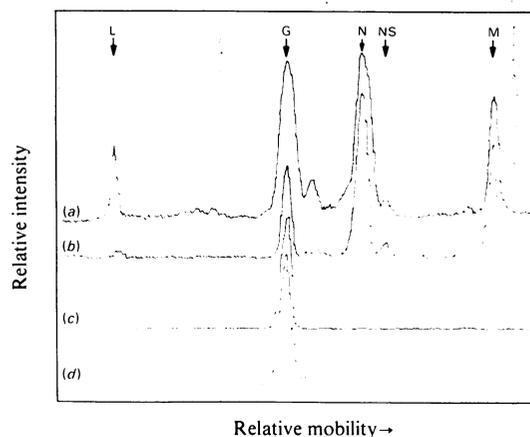


Fig. 3. Gel profiles of radiolabelled proteins of vesicular-stomatitis-virus-infected K-562 and BHK cells

Radiolabelled proteins were obtained as described in the Materials and methods section. Shown are soft-laser-densitometer tracings of a fluorogram (7 days exposure). L, G, N, NS and M indicate the proteins of vesicular-stomatitis virus, as described by Wagner *et al.* (1972). (a) [^3H]Leucine-labelled vesicular-stomatitis-virus-infected K-562 cells; (b) [^3H]leucine-labelled vesicular-stomatitis-virus-infected BHK cells; (c) [^3H]mannose-labelled vesicular-stomatitis-virus-infected K-562 cells; (d) [^3H]glucosamine-labelled vesicular-stomatitis-virus-infected K-562 cells.

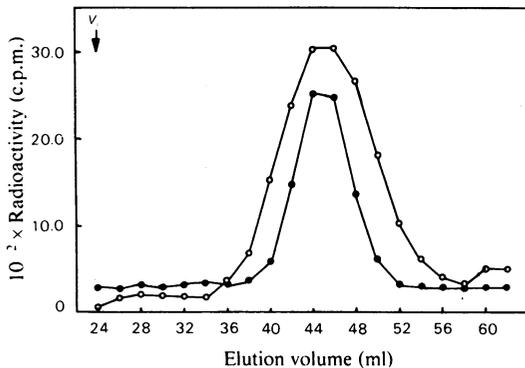


Fig. 4. Gel filtration of G-protein glycopeptides on Sephadex G-50

[³H]Mannose- and [³H]glucosamine-labelled G-protein from vesicular-stomatitis-virus-infected K-562 cells were excised from a polyacrylamide gel (Fig. 3), freed of the polyacrylamide by electrophoresis, precipitated with 5% (w/v) trichloroacetic acid, washed twice with ice-cold acetone, and the glycopeptides prepared by Pronase digestion. The Sephadex G-50 column (1.5 cm × 40 cm) was eluted with 0.1 M-pyridine/acetic acid, pH 5.0. Fractions of 2.0 ml were collected and radioactivity was monitored by liquid-scintillation counting of appropriate samples. O, [³H]Mannose-labelled G-protein glycopeptides; ●, [³H]glucosamine-labelled G-protein glycopeptides.

seen up to 10 μg/ml of tunicamycin. These results strongly suggest that the tunicamycin-sensitive biosynthetic steps leading to the common N-linked glycopeptides (transferrin-type) are the same as the initial stages of erythroglycan synthesis.

As discussed in the introduction, we decided to determine whether erythroglycan could be attached to the G-protein of vesicular-stomatitis-virus-infected K-562 cells, since these cells potentially could glycosylate the G-protein with the smaller transferrin-type, as is the case with BHK cells or with the long-chain versions of 'complex' units (erythroglycan). As seen in the densitometer scans of radioautograms of sodium dodecyl sulphate/polyacrylamide gels in Fig. 3, 5.5 h after infection of K-562 cells (a) with vesicular-stomatitis virus the same proteins are radiolabelled with [³H]leucine as in vesicular-stomatitis-virus-infected BHK cells (b). Fig. 3 further reveals that only the G-protein of vesicular-stomatitis-virus-infected K-562 cells is labelled when either [³H]mannose (c) or [³H]glucosamine (d) is incubated with the cells 5.5 h after infection. Fig. 4 shows the profile of the glycopeptides derived from Pronase-digested [³H]glucosamine and [³H]mannose-labelled G-protein from vesicular-stomatitis-virus-infected K-562 cells. All of the resultant glycopeptides are co-eluted with stan-

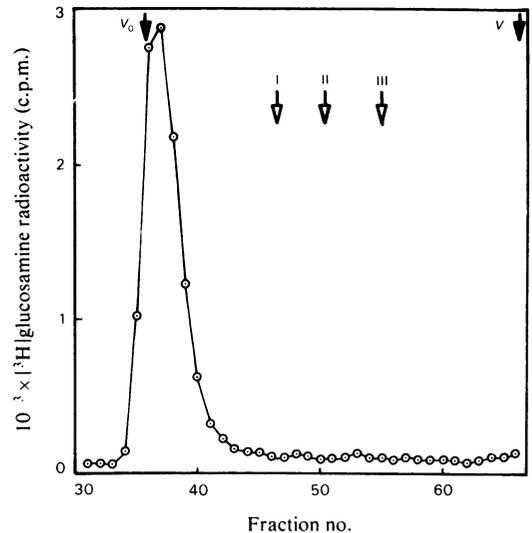


Fig. 5. Gel filtration of endo-β-galactosidase-digested [³H]glucosamine-labelled G-protein glycopeptides on Bio-Gel P-2

[³H]Glucosamine-labelled G-protein glycopeptides from vesicular-stomatitis-virus-infected K-562 cells were treated with 200 munits of endo-β-galactosidase in 0.05 M-sodium acetate, pH 5.8, for 18 h. The sample was then applied to a column (1.5 cm × 100 cm) of Bio-Gel P-2 and eluted with 15% (v/v) acetic acid. Fractions of 1.5 ml were collected. I, II and III indicate the elution volumes of authentic tetrasaccharide (αAcNeu1→3,6βGal1→4βGlcNAc1→3-βGal), trisaccharide (βGal1→4βGlcNAc1→3-βGal), and disaccharide (βGlcNAc1→3βGal) isolated previously (Turco *et al.*, 1980).

dard glycopeptides of M_r 3000 isolated from bovine transferrin. No glycopeptides of $M_r = 7000-11000$ (the size of erythroglycan) were obtained. Consistent with this result, the [³H]glucosamine-labelled G-protein glycopeptides were found to be resistant to degradation by endo-β-galactosidase from *E. freundii* as shown in Fig. 5. This enzyme is specific for keratan-like molecules with a repeating N-acetyl-lactosamine structure of Galβ1→4GlcNAcβ1→3. No di-, tri- or tetra-saccharide products were obtained as for foetal erythroglycan (Fukuda *et al.*, 1979; Turco *et al.*, 1980), indicating the absence of the GlcNAcGal repeating unit. From these data we conclude that K-562 cells do not glycosylate the G-protein with erythroglycan N-acetyl-lactosamine repeating units.

Discussion

In the biosynthetic scheme for glycosylation of protein with N-linked saccharides [see Parodi &

Leloir (1979) for review], the initial glycosylation is achieved by the transfer of the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide from a lipid intermediate to an asparagine residue on the protein. The pathways leading to the formation of this lipid-linked oligosaccharide can be blocked by tunicamycin (Hickman *et al.*, 1977).

In our experiments, the synthesis of both erythroglycan and transferrin-type glycopeptides is inhibited by tunicamycin to the same extent, suggesting that both proceed via similar pathways using lipid-linked intermediates. The observation that specific glycoproteins of K-562 cells and erythrocyte membranes are not randomly glycosylated with all available *N*-linked chains, but specifically with erythroglycan, suggests that a strictly controlled branch point in the synthetic mechanisms must exist. In the accepted scheme for the synthesis of the transferrin-type oligosaccharides, glucose and mannose residues are removed sequentially from the non-reducing ends of the protein-bound $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide, and the mannose core is then elaborated with two to four disaccharides consisting of $\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}$. Each of these disaccharides is then normally terminated by an *N*-acetylneuraminic acid residue (Schachter *et al.*, 1970) before leaving the Golgi apparatus. In the case of erythroglycan, the repeating chain of $\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 3$ is presumably added to the mannose core region in a series of additions to produce the higher oligomers found in erythrocyte membranes (Järnefelt *et al.*, 1978; Krusius *et al.*, 1978). Information encoded in the nascent protein must signal whether the processed oligosaccharide chain will be elongated to produce erythroglycan or terminated by *N*-acetylneuraminic acid. The glycosyltransferase systems of the endoplasmic reticulum must somehow be sensitive to this information in order to accomplish the observed selective glycosylation.

This investigation was supported in part by grants from the American Cancer Society (grant no. BC-337) and Biomedical Research Support Grant no. RR05374 to S. J. T. and by National Institutes of Health Research Grant no. 1R01AM25101 to R. A. L.

References

- Bayard, B. & Roux, D. (1975) *FEBS Lett.* **55**, 206–211
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Etchison, J. R., Robertson, J. S. & Summers, D. F. (1977) *Virology* **78**, 375–392
- Fukuda, M. N., Fukuda, M. & Hakomori, S.-I. (1979) *J. Biol. Chem.* **254**, 5458–5465
- Hickman, S., Kulczycki, A., Lynch, R. G. & Kornfeld, S. (1977) *J. Biol. Chem.* **252**, 4402–4408
- Järnefelt, J., Rush, J., Li, Y. T. & Laine, R. A. (1978) *J. Biol. Chem.* **253**, 8006–8009
- Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* **45**, 217–237
- Krusius, T., Finne, J. & Rauvala, H. (1978) *Eur. J. Biochem.* **92**, 289–300
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C. & Robbins, P. W. (1979) *J. Biol. Chem.* **254**, 4554–4559
- Livingston, D. M., Hinkle, D. C. & Richardson, C. C. (1975) *J. Biol. Chem.* **250**, 461–469
- Parodi, A. J. & Leloir, L. F. (1979) *Biochim. Biophys. Acta* **559**, 1–37
- Reading, C. L., Penhoet, E. E. & Ballou, C. E. (1978) *J. Biol. Chem.* **253**, 5600–5612
- Robbins, P. W., Hubbard, S. C., Turco, S. J. & Wirth, D. F. (1977) *Cell* **12**, 893–900
- Schachter, H., Jabbal, I., Hudgin, R. L., Pinteric, L., McGuire, E. J. & Roseman, S. (1970) *J. Biol. Chem.* **245**, 1090–1100
- Turco, S. J., Stetson, B. & Robbins, P. W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4411–4414
- Turco, S. J., Rush, J. S. & Laine, R. A. (1980) *J. Biol. Chem.* **255**, 3266–3269
- Wagner, R. R., Preric, L., Brown, F., Summers, D. F., Sokol, F. & MacLeod, R. (1972) *J. Virol.* **10**, 1228–1230