Synthesis of Ribonucleic Acid in Baby-Hamster Kidney Cells in the Presence of Actinomycin D

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1. RNA molecules with sedimentation values in sucrose gradients of 12–20s are synthesized in baby-hamster kidney cells even after prolonged incubation in medium containing 1 μ g. of actinomycin D/ml. 2. The rate of formation of this RNA is dependent on the age of the cultures and is greatest during the exponential phase of growth. 3. Growth of cells on nutritionally poor medium causes degradation and inhibits the synthesis of these RNA fractions. 4. Replacement of the nutritionally poor medium with a rich medium stimulates the synthesis of actinomycin-resistant RNA. This stimulation is blocked by cycloheximide. 5. The base composition of this RNA is characterized by low cytidine and high guanosine values.

The antibiotic actinomycin D has been useful in the investigation of the metabolism of RNA and the replication of RNA viruses (Reich, Franklin, Shatkin & Tatum, 1962; Franklin, 1963). Two experimental systems, using either low or high concentrations of actinomycin, have been studied. It is generally accepted that these produce partial or complete inhibition of RNA synthesis respectively. The use of low concentrations of actinomycin D in metabolic studies was stimulated by the observations of Perry (1962) that under appropriate conditions the synthesis of ribosomal RNA in HeLa cells could be selectively inhibited. A number of authors have now shown that RNA formed in the presence of low concentrations of actinomycin D has a base composition similar to the cell DNA and it has been suggested that this represents cellular messenger RNA (Georgiev, Samarina, Lerman, Smirnov & Severtzov, 1963; Harel, Harel, Boer, Imbenotte & Carpeni, 1964; Perry, Srinivasan & Kelley, 1964; Martin & Leslie, 1964; Roberts & Newman, 1966).

Actinomycin D acts through the formation of a complex between the antibiotic and the guanine residues in DNA (Goldberg, Rabinowitz & Reich, 1962). Hence some degree of differential inhibition, depending on the guanine content of the various DNA molecules present, would be expected to occur. Pollock (1963) has provided an example of this by growing *Bacillus subtilis* and *Bacillus cereus* with very low concentrations of actinomycin D (0.01–0.05 μ g./ml.). Under these conditions the induction of α -glucosidase was greatly inhibited whereas penicillinase induction was unaffected.

In current studies of the replication of virus

RNA in animal cells the technique of inhibiting cellular RNA by relatively high concentrations of actinomycin D (1.0–5.0 μ g./ml.) is generally used. This permits the detection of virus-specific RNA in the absence of cellular RNA synthesis. As a result of such studies on the replication of foot-and-mouth-disease virus RNA (Brown & Cartwright, 1964; Brown & Martin, 1965a), it was found that under some conditions synthesis of high-molecular-weight RNA occurred in non-infected cells even in the presence of actinomycin at 1 μ g./ml. Similar RNA fractions were detected in HeLa and L cells grown under the same conditions (F. Brown, unpublished work). The present paper is concerned with defining the conditions under which RNA is formed in BHK cells* in the presence of actinomycin D.

METHODS AND MATERIALS

Culture conditions. The BHK cells, designated BHK 21 (clone no. 13), were isolated in the Institute of Virology, Glasgow, and have been described by Macpherson & Stoker (1962). The cells were grown as monolayers in either Roux bottles or 4 oz. bottles in Eagle's medium containing 10% (v/v) of ox serum, 0.2% (w/v) of tryptose-phosphate broth and antibiotics (penicillin, streptomycin and polymyxin at 100 units/ml. each). Roux bottles containing approx. 10^8 cells are issued to our Laboratory 4–5 days after culture in fresh medium by our Institute's Tissue Culture Unit. The 4 oz. bottles are set up with an initial inoculum of approx. 5×10^6 cells/bottle.

32 P-labelling of cells. Monolayers were washed twice with a modified Earle's medium containing 10 mM-tris buffer,

* Abbreviation: BHK cells, baby-hamster kidney cells.

pH 7.6, instead of phosphate buffer (this medium is referred to below as phosphate-deficient medium). The cells were incubated with 5 ml. of phosphate-deficient medium for 5–10 min. before the addition of 200 μ C of [32 P]phosphate in 1 ml. of medium. In some experiments actinomycin or cycloheximide was added to the medium as indicated in the Results section.

Preparation of RNA. The cultures were rinsed with phosphate-buffered saline (Dulbecco & Vogt, 1954) and the cells removed from the glass with phenol equilibrated with 40 mM-phosphate buffer, pH 7.6, and then shaken with an equal volume of buffer at 4° for 5 min. Similar results were obtained when the extraction was carried out at room temperature. The aqueous phase containing the RNA was extracted a second time with phenol and then mixed with 2 vol. of cold ethanol and stored at –20°. The resulting precipitate of RNA and sodium phosphate crystals was dissolved in 5 ml. of 0.1 M-acetate buffer, pH 5.0, and once again precipitated with 2 vol. of ethanol. This precipitate was dissolved in 1 ml. of 0.1 M-acetate buffer, pH 5.0, and passed through a Sephadex G-200 column (1 cm. $\times$ 48 cm.) that had been equilibrated with 0.1 M-acetate buffer, pH 5.0. Elution with acetate buffer separated the ribosomal RNA from the soluble RNA and small molecules.

Sucrose-gradient centrifugation. A sample (2 ml.) of the RNA solution collected from the Sephadex G-200 column was centrifuged in a 25 ml. gradient of 5–25% (w/v) sucrose in 0.1 M-sodium acetate buffer, pH 5.0, in the SW 25 rotor of a Spinco model L ultracentrifuge. Centrifugation was continued at 4° for 14 hr. at 20000 rev./min. (42000g). At the end of this period 25 1 ml. fractions were collected from the bottom of the tube. Extinction measurements showed that the 4s RNA fraction had been eliminated by the Sephadex G-200 treatment. Within a given experiment the data are presented as relative specific radioactivities, since the radioactivities from different gradients have been correlated with the E_{260} values.

Analysis of RNA. Fractions from the gradients were pooled and the RNA was precipitated with 2 vol. of ethanol and 1 mg. of yeast RNA. The RNA was hydrolysed in 0.3 N-KOH at 37° for 18 hr., and the nucleotide composition was determined by paper electrophoresis (Davidson & Smellie, 1952).

Materials. [32 P]Phosphate was obtained from The Radiochemical Centre, Amersham, Bucks., and cycloheximide (Actidione C) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. We are grateful to Dr A. Zeissig of Merck, Sharp and Dohme, Inc. Rahway, N.J., U.S.A., for his gift of actinomycin D.

RESULTS

Effect of actinomycin D on [32 P]phosphate incorporation into RNA of BHK cells. Monolayers of BHK cells that had been grown in Roux bottles for 4–5 days were washed twice with phosphate-deficient medium and then incubated at 37° in this medium with or without actinomycin D at 1 μ g./ml. for a 5 min. period before the addition of [32 P]-phosphate. After 2 hr. or 12 hr. the radioactive medium was removed and the cells were extracted with phenol. The RNA was then filtered through

Fig. 1. Sucrose-gradient centrifugation of the RNA from cells incubated in the presence or absence of actinomycin. The RNA was eluted from Sephadex G-200 columns and centrifuged in gradients (5–25% sucrose in 0.1 M-acetate buffer, pH 5.0) for 14 hr. at 4° in a SW 25 rotor of a Spinco model L ultracentrifuge at 20000 rev./min. —, E_{260} ; ----, 32 P counts/min. after labelling for 2 hr. in phosphate-deficient medium; -.-.-, 32 P counts/min. after labelling for 2 hr. in phosphate-deficient medium plus actinomycin D (1 μ g./ml.).

Sephadex G-200 to remove small molecules and the 4s RNA.

Sucrose-gradient analysis of RNA from cells not treated with actinomycin D (Fig. 1) shows that 32 P is incorporated during the first 2 hr. into both the 28s and 16s ribosomal RNA. When 1 μ g. of actinomycin D/ml. was present the amount of 32 P associated with high-molecular-weight RNA was negligible. Estimation of total radioactivity by summation of the radioactivity after sedimentation in sucrose gradients indicates that incorporation of 32 P into the RNA fraction in the presence of actinomycin is less than 4% of that in the inhibited cultures. After a 12 hr. labelling period incorporation in the presence of actinomycin D was less than 0.01% of the untreated cultures.

Effect of age of monolayer cultures on the synthesis of RNA in the presence of actinomycin D. For our experiments on the synthesis of virus-induced

RNA, cells are generally transferred from the Roux bottles to 4 oz. bottles and grown for approx. 48 hr. on fresh supplemented Eagle's medium. In these cells it was frequently found that ^{32}P incorporation into the RNA fraction was not completely inhibited in the presence of actinomycin. The radioactivity was shown by analysis to be associated with the 2',3'-nucleotides (see Table 1). Sucrose-gradient analysis showed that the actinomycin-resistant RNA had a heterogeneous profile with a peak predominantly in the 12–20s region. Preliminary observations suggested that the resistance to actinomycin D may depend on the rate of growth of the cells. The following experiment was carried out to investigate the effect of actinomycin D on the incorporation of ^{32}P into RNA at different times after passage of the cells into fresh medium.

Cells were transferred from Roux bottles to 4 oz. bottles, and grown on supplemented Eagle's medium. At 24 hr. intervals replicate cultures were rinsed with phosphate-deficient medium and 5 ml. of this medium plus actinomycin D ($1\text{ }\mu\text{g./ml.}$) was added. Then 5 min. later [^{32}P]phosphate was added, and after a further 2 hr. incubation at 37° the tissue was extracted with phenol, and the RNA was purified by passing through a column of Sephadex G-200 before analysis by sucrose-gradient sedimentation.

The state of the tissue at the various periods was examined visually. At 24 hr. the cells had settled out on the glass but had not multiplied significantly. At 48 hr., however, the sheets were confluent and by 72 hr. the medium had become slightly acid (pH approx. 6.8) and the sheets had become overgrown and begun to degenerate.

Fig. 2 shows the ^{32}P profiles obtained by sucrose-gradient sedimentation of RNA from both control and actinomycin D-treated cultures. During a 2 hr. period of labelling of control cultures, the 48 hr. culture showed a more rapid incorporation into both the ribosomal RNA peaks than did the 24 hr. or 72 hr. cultures. In the actinomycin-treated cells there was significantly more ^{32}P incorporated into RNA in the 48 hr. cultures than in the 72 hr. cultures. In the 48 hr. culture the percentage incorporation in the presence of actinomycin was approx. 25% compared with about 5% at 72 hr.

Stability of actinomycin D-resistant RNA. The stability of the RNA synthesized in the presence of actinomycin was studied by labelling cells for various times after the addition of the drug and then analysing the RNA by sucrose-gradient sedimentation. The 48 hr. cultures in 4 oz. bottles were washed with phosphate-deficient medium and then 5 ml. of the same medium containing actinomycin D ($1\text{ }\mu\text{g./ml.}$) was added. After 5 min. [^{32}P]phosphate was added and the RNA was extracted from replicate cultures after 105 min.,

Fig. 2. Sucrose-gradient centrifugation of RNA extracted from cells at various times after subculturing. The RNA was eluted from Sephadex G-200 columns and centrifuged under conditions described in Fig. 1. (a) RNA from 24 hr. cultures; (b) RNA from 48 hr. cultures; (c) RNA from 72 hr. cultures. —, ^{32}P counts/min. after labelling for 2 hr. in phosphate-deficient medium; ----, ^{32}P counts/ml. after labelling for 2 hr. in phosphate-deficient medium plus actinomycin D.

135 min. and 165 min. Fig. 3 shows that after 105 min. the pattern is heterogeneous with radioactivity predominantly in the 20–12s region of the gradient. Approx. 30% of the radioactivity does, however, sediment with values greater than 20s. After 135 min. the profile has become more heterogeneous with 35% of the counts corresponding to values greater than 20s. Also, the total amount of radioactivity incorporated has decreased. This trend continues and by 165 min. after addition of the drug 43% of the ^{32}P radioactivity sediments with values higher than 20s.

It is emphasized that in these experiments the radioactive medium is present throughout and therefore the decrease in amount of ^{32}P incorporated during the longer periods of labelling shows

Fig. 3. Sucrose-gradient centrifugation of RNA extracted from cells after labelling for various times in the presence of actinomycin and phosphate-deficient medium. The RNA was eluted from Sephadex G-200 columns and centrifuged under conditions described in Fig. 1., E_{260} ; —, ^{32}P counts/min. after labelling for 105 min. in the presence of actinomycin; ----, ^{32}P counts/min. after labelling for 135 min. in the presence of actinomycin; -.-.-, ^{32}P counts/min. after labelling for 165 min. in the presence of actinomycin.

that the rapidly formed RNA is being degraded. Both these features, the decrease in amount of ^{32}P incorporated during the continuous labelling periods and the increase in heterogeneity of the RNA, support the contention that actinomycin D may cause the degradation of rapidly formed RNA (Acs, Reich & Valanju, 1963).

Effect of cycloheximide on the stability of the actinomycin D-resistant RNA. Cycloheximide is a potent inhibitor of protein synthesis in mammalian cells (Bennett, Smithers & Ward, 1964). Incorporation of ^{14}C -labelled amino acids into protein of BHK cells is inhibited 97% by 100 μg . of cycloheximide/ml. (S. J. Martin & B. Underwood, unpublished work). The stability of actinomycin-resistant RNA in the absence of protein synthesis was studied in the following experiment.

Six monolayers (48 hr. cultures) of BHK cells were analysed in duplicate. The cells were treated

Fig. 4. Sucrose-gradient centrifugation of RNA extracted from cells treated with cycloheximide and actinomycin in phosphate-deficient medium. The RNA was eluted from Sephadex G-200 columns and centrifuged under conditions described in Fig. 1., E_{260} ; —, ^{32}P counts/min. after labelling for 2 hr. in phosphate-deficient medium plus actinomycin; ----, ^{32}P counts/min. after labelling for 6 hr. in phosphate-deficient medium plus actinomycin; -.-.-, ^{32}P counts/min. after labelling for 2 hr. in phosphate-deficient medium plus actinomycin followed by the addition of cycloheximide (100 μg /ml.) for a further 4 hr.

with phosphate-deficient medium plus actinomycin D for 5 min., followed by addition of [^{32}P]phosphate for 2 hr. Two monolayers were then extracted with phenol. To two of the remaining monolayers cycloheximide was added to a final concentration of 100 μg /ml., and incubation was continued for a further 4 hr. before extraction of the RNA. Fig. 4 shows the sucrose-gradient sedimentation profiles of the RNA from these cells. As found in previous experiments, when ^{32}P labelling continues for 6 hr. there is a marked decrease in the radioactivity incorporated compared with that found in RNA after a 2 hr. labelling period. The presence of cycloheximide during the last 4 hr. period of the experiment did not prevent or enhance this tendency.

Effect of composition of medium on the synthesis of RNA. In the experiments described so far the labelling of cells was carried out in a phosphate-deficient medium based on Earle's saline with 0.01 M-tris replacing the phosphate buffer. Compared with the medium used for growing the cells, that is supplemented Eagle's medium containing tryptose-phosphate broth and serum, this phos-

phate-deficient medium is a nutritionally poor medium. In fact, applying this medium to cells is essentially a 'step-down' culture (Hayashi & Spiegelman, 1961). Such a change in nutritional conditions could also affect the stability of the RNA formed. An experiment was carried out to test the effect of preincubating the cells with a nutrient-poor medium before the addition of [32 P]-phosphate. For this purpose, Earle's saline was used rather than the phosphate-deficient medium as this allowed various periods of preincubation to be given without depletion of the cellular phosphate pools.

Replicate 48hr.-old monolayers of BHK cells in

4oz. bottles were treated with Earle's saline plus actinomycin D (1 μ g./ml.) for 1, 2 or 3hr. The sheets were then rinsed with phosphate-deficient medium and incubation with 5ml. of this medium plus actinomycin D was continued for 2hr. after the addition of [32 P]phosphate. Two monolayers were incubated in Earle's saline alone for 2hr. before the addition of the phosphate-deficient medium plus actinomycin D and [32 P]phosphate.

Fig. 5 compares the sedimentation profiles obtained for the RNA from these cultures. As the labelling period is constant throughout the experiment, the amount of incorporation of 32 P into the RNA can be compared directly. Preincubation with Earle's saline plus actinomycin D for 1, 2 or 3hr. before the labelling resulted in an increasing inhibition of 32 P incorporation. However, preincubation with Earle's saline without actinomycin D causes the same decrease in the amount of 32 P incorporated as did the pretreatment with Earle's saline plus actinomycin D.

This experiment demonstrates that the decrease in incorporation of 32 P in RNA during prolonged exposure to actinomycin is not caused by the drug but is directly related to the time of exposure to the nutritionally poor medium.

Effect of cycloheximide on the synthesis of actinomycin-resistant RNA. The 72hr.-cultured monolayers in 4oz. bottles were treated with phosphate-deficient medium plus actinomycin D and [32 P]-phosphate for 2hr. The medium was then removed and Eagle's medium plus tryptose-phosphate broth, 10% of ox serum and actinomycin was added. Cycloheximide was added to two monolayers to a final concentration 100 μ g./ml. Incubation was continued for 2hr. and the RNA extracted by phenol.

As these cells were from a 72hr. culture there was little incorporation of 32 P into RNA during the initial 2hr. incubation (cf. Fig. 2). Addition of the nutrient-rich Eagle's medium stimulated the formation of RNA. The sucrose-gradient profile shown in Fig. 6 is heterogeneous, indicating the presence of a peak at 28s, but a predominant fraction was present in the 12–20s region of the gradient. When cycloheximide is present during the second phase of the experiment, studies of the incorporation of 14 C-labelled amino acids indicated that protein synthesis had been inhibited by 96%. The sucrose-gradient profile of the RNA from the cycloheximide-treated cells shows that no stimulation of the actinomycin-resistant RNA occurs in the absence of protein synthesis. Although this result demonstrates the dependence of RNA synthesis on protein synthesis, in these experiments the cycloheximide may inhibit the initiation of RNA synthesis by blocking the ability of the cells to adapt to the nutrient-rich medium.

Fig. 5. Sucrose-gradient centrifugation of RNA extracted from cells pretreated with Earle's saline with and without actinomycin before a 2hr. labelling period. The RNA was eluted from Sephadex G-200 columns and centrifuged under conditions described in Fig. 1. All samples were given a 2hr. labelling period with [32 P]phosphate in phosphate-deficient medium plus actinomycin after pretreatment in Earle's saline as follows: —, 1hr. pretreatment with Earle's saline plus actinomycin; ----, 2hr. pretreatment with Earle's saline plus actinomycin; - · - · - ·, 3hr. pretreatment with Earle's saline plus actinomycin; - - - - -, 2hr. pretreatment with Earle's saline (no actinomycin); ····, E_{260} .

Base composition of RNA. RNA from ^{32}P -labelled cells has been analysed for the distribution of radioactivity in the four nucleotides adenosine,

Fig. 6. Sucrose-gradient centrifugation of RNA extracted from cells that had been labelled with ^{32}P in the presence of actinomycin followed by a 2 hr. period in Eagle's medium with and without cycloheximide. The RNA was eluted from Sephadex G-200 columns and centrifuged under conditions described in Fig. 1. The 72 hr.-old cultures were treated with [^{32}P]phosphate in phosphate-deficient medium plus actinomycin for 2 hr. The radioactive medium was then replaced by Eagle's medium for a further 2 hr. period, as follows: —, Eagle's medium plus actinomycin for 2 hr. post-labelling period; ----, Eagle's medium plus actinomycin and cycloheximide (100 $\mu\text{g./ml.}$) for 2 hr. post-labelling period;, E_{260} .

cytidine, guanosine and uridine. Table 1 shows the composition of the actinomycin-resistant RNA. As reported (Brown & Martin, 1965b) the 28s and 16s ribosomal RNA fractions are characterized by higher cytidine + guanosine/adenosine + uridine ratios. The 16s fraction, however, is significantly different from the 28s in having relatively lower cytidine + guanosine/adenosine + uridine ratios.

The RNA formed in the presence of actinomycin D during a 2 hr. labelling period has a high guanosine content with lower amounts of cytidine and uridine. These ratios are in contrast with reports that DNA-like ratios are found at low concentrations of actinomycin D (Roberts & Newman, 1966). One consistent feature is the low percentage of cytidine.

The possibility that these ratios are caused by non-equilibration of the nucleotide phosphate-labelled pools during the 2 hr. labelling period is unlikely, as similar ratios have been found in cells labelled for 12 hr. under appropriate conditions (Martin & Brown, 1967). It is known that other compounds labelled with ^{32}P often remain associated with nucleotides during electrophoresis (Davidson & Smellie, 1952). In the present experiments the distribution of ^{32}P along the electrophoretograms has been determined by scanning and radioactivity was found associated only with the u.v.-absorbing regions of the four nucleotides. No evidence of the presence of other labelled compounds has been found when RNA is prepared by the technique used here.

DISCUSSION

The present experiments have shown that, in the presence of 1 $\mu\text{g.}$ of actinomycin D/ml., RNA with a sedimentation coefficient approx. 12–20s is synthesized in BHK cells. Interpretation of the function or origin of these RNA fractions is limited by our incomplete understanding of the process of labelling of RNA with [^{32}P]phosphate. It is possible, though unlikely, that terminal addition of labelled nucleotides to preformed high-molecular-weight RNA molecules occurs under these experimental conditions, as in transfer RNA (Merits,

Table 1. *Base composition of ribosomal RNA and actinomycin-resistant RNA fractions*

The RNA was prepared and fractionated as described in the text. The composition of the 28s and 16s ribosomal RNA are quoted from Brown & Martin (1965b). The other values are the means of eight analyses of the actinomycin-resistant RNA.

RNA fraction	Base composition (% of ^{32}P radioactivity)			
	Adenosine	Uridine	Guanosine	Cytidine
Ribosomal (28s)	18.4	17.7	34.4	29.6
Ribosomal (16s)	22.1	21.5	30.5	25.9
Actinomycin-resistant (12–20s)	25.8	22.5	30.1	21.5

1963; Harel *et al.* 1964). In the present experiments the use of Sephadex G-200 eliminates the contamination of the high-molecular-weight RNA by transfer RNA molecules. The present results could also be explained by the presence of latent viruses in these cell cultures. Since, however, similar RNA fractions can be isolated from both HeLa and L cells (F. Brown, unpublished work) such an interpretation would suggest that the presence of latent viruses is a common feature of cultured cells. A more plausible explanation is that this actinomycin-resistant RNA represents a normal fraction that is not inhibited by the drug under these experimental conditions (Roberts & Newman, 1966).

Although the present evidence does not eliminate the possibility that the fraction represents artifacts due to the presence of actinomycin (Acs *et al.* 1963), four points may be made indicating that this is a fraction that is metabolically active in cells. First, more of this RNA is formed in rapidly growing cultures compared with older cultures. Secondly, the fraction is unstable when the cells are maintained on nutritionally deficient medium. This suggests that the degradation is not caused by the presence of actinomycin but is related to the metabolic state of the cultures. Thirdly, the effect of cycloheximide on the stability and synthesis of these fractions suggests that they are involved in protein synthesis. Finally, the base composition of the RNA, characterized by a low cytidine content, is consistent with the concept that DNA primers with a low deoxyguanosine content would be less likely to be inhibited by actinomycin than DNA containing a high percentage of deoxyguanosine residues. These observations could be interpreted as indicating that the actinomycin-resistant RNA represents a fraction or fractions of metabolically active cellular RNA (Georgiev *et al.* 1963; Harel *et al.* 1964; Perry, 1962; Roberts & Newman, 1966).

These results demonstrate the importance of defining precisely the nutritional conditions under which experiments involving the use of actinomycin are conducted, and are especially relevant to the study of the replication of viral RNA in animal cells. It seems to be the practice in several Laboratories to infect cells with virus that has been grown in a nutrient-rich medium. Infection of cells in the presence of actinomycin may stabilize these cellular RNA components and hence make it

difficult to assess whether or not all the RNA fractions obtained after virus infection are in fact specified by the virus (Brown & Cartwright, 1964; Sonnabend, Dalgarno, Friedman & Martin, 1964; Sreevalsan & Lockart, 1966; Friedman, Levy & Carter, 1966; Martin, 1966).

The following paper (Martin & Brown, 1967) studies the effect of foot-and-mouth-disease virus on the stability and synthesis of these actinomycin D-resistant RNA fractions.

REFERENCES

- Acs, G., Reich, E. & Valanju, S. (1963). *Biochim. biophys. Acta*, **76**, 68.
- Bennett, L. L., jun., Smithers, S. & Ward, C. T. (1964). *Biochim. biophys. Acta*, **87**, 60.
- Brown, F. & Cartwright, B. (1964). *Nature, Lond.*, **204**, 955.
- Brown, F. & Martin, S. J. (1965a). *Nature, Lond.*, **208**, 861.
- Brown, F. & Martin, S. J. (1965b). *Biochem. J.* **97**, 20c.
- Davidson, J. N. & Smellie, R. M. S. (1952). *Biochem. J.* **52**, 599.
- Dulbecco, R. & Vogt, M. (1954). *J. exp. Med.* **99**, 167.
- Franklin, R. M. (1963). *Biochim. biophys. Acta*, **72**, 555.
- Friedman, R. M., Levy, H. B. & Carter, W. B. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 440.
- Georgiev, G. P., Samarina, O. P., Lerman, M. I., Smirnov, M. N. & Severtzov, A. N. (1963). *Nature, Lond.*, **200**, 1291.
- Goldberg, I. H., Rabinowitz, M. & Reich, E. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2094.
- Harel, L., Harel, J., Boer, A., Imbenotte, J. & Carpeni, N. (1964). *Biochim. biophys. Acta*, **87**, 212.
- Hayashi, M. & Spiegelman, S. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1564.
- Macpherson, I. A. & Stoker, M. G. P. (1962). *Virology*, **16**, 147.
- Martin, E. M. (1966). *Abstr. 3rd Meet. Fed. Europ. biochem. Soc., Warsaw*, p. 117.
- Martin, S. J. & Brown, F. (1967). *Biochem. J.* **105**, 987.
- Martin, S. J. & Leslie, I. (1964). *Abstr. 1st Meet. Fed. Europ. biochem. Soc., London*, p. 37.
- Merits, I. (1963). *Biochem. biophys. Res. Commun.* **10**, 254.
- Perry, R. P. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2178.
- Perry, R. P., Srinivasan, P. R. & Kelley, D. E. (1964). *Science*, **145**, 504.
- Pollock, M. R. (1963). *Biochim. biophys. Acta*, **76**, 80.
- Reich, E., Franklin, R. M., Shatkin, A. J. & Tatum, E. L. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1238.
- Roberts, W. K. & Newman, J. F. E. (1966). *J. molec. Biol.* **20**, 63.
- Sonnabend, J., Dalgarno, L., Friedman, R. M. & Martin, E. M. (1964). *Biochem. biophys. Res. Commun.* **17**, 455.
- Sreevalsan, T. & Lockart, R. Z., jun. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 974.