

Studies on the Reverse Transcriptase of RNA Tumor Viruses. Structural Relatedness of Two Subunits of Avian RNA Tumor Viruses*

(polyacrylamide gel electrophoresis/*in vitro* radiolabeling with ¹²⁵I/
two-dimensional peptide analysis)

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ABSTRACT The structural relationship of the small (α) and large (β) subunits of reverse transcriptase isolated from two avian RNA tumor viruses has been examined by tryptic peptide analysis. Comparison of the tryptic hydrolysates of the isolated subunits by two-dimensional separation on thin-layer cellulose plates indicates that (i) the α subunit of reverse transcriptase of avian myeloblastosis virus is structurally related to the β subunit; (ii) the α and β subunits of the enzyme of Rous sarcoma virus also appear to be related; and (iii) there appears to be an extensive amino-acid sequence homology between reverse transcriptases of avian myeloblastosis virus and Rous sarcoma virus. Evidence is also presented that both α and β subunits can be identified in purified avian myeloblastosis virions.

RNA tumor viruses contain a DNA polymerase that can utilize both ribopolymers and deoxyribopolymers as templates to synthesize complementary deoxyribopolymers (1-4). Because of its ability to transcribe polyribonucleotides into complementary polydeoxyribonucleotides, this enzyme has been referred to as reverse transcriptase. During the life cycle of the RNA tumor viruses, this enzyme is required to synthesize DNA complementary to viral RNA (5, 6).

Reverse transcriptase isolated from avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV) has been extensively characterized (7-12). Purified enzyme preparations from these sources exhibit at least three characteristic enzymatic activities: RNA-directed DNA synthesis, DNA-directed DNA synthesis, and ribonuclease H (which selectively degrades the RNA moiety of DNA-RNA hybrids) (5, 6). The enzymes of both AMV and RSV are composed of one small subunit (α) and one large subunit (β) with apparent molecular weights of 60,000 and 100,000, respectively (7, 10-12). A molecular species containing only α subunit has been isolated and shown to possess all three of the enzymatic activities exhibited by the native enzyme, $\alpha\beta$ (11). Furthermore, it has been shown that these activities of the α subunit isolated from a temperature-sensitive mutant of RSV (*tsL1337* PR-C) are 5- to 7-fold more thermolabile than those of the α subunit isolated from the wild-type parent†. Thus, it appears that at least the lower-molecular-weight subunit is coded for by the viral genome (12, †). Although there is evidence that the β subunit serves

to increase the affinity of the native enzyme for template-primer (13, 14), neither the source of the genetic information for the large-molecular-weight subunit nor its function is known.

We report here tryptic peptide comparisons of the α and β subunits of reverse transcriptases of AMV and RSV. The following experimental approach was used: Purified reverse transcriptase was radiolabeled *in vitro* with ¹²⁵I. The two radiolabeled subunits were then separated electrophoretically in polyacrylamide gels and processed for tryptic peptide analyses. Results of these analyses indicate that (i) the α and β subunits from AMV are structurally related, (ii) the α and β subunits from RSV also appear to be related to each other, and (iii) there may be an extensive amino-acid sequence homology between reverse transcriptases of AMV and RSV. Evidence is also presented that both the α and β subunits are present in purified avian myeloblastosis virions.

MATERIALS AND METHODS

Materials. Avian myeloblastosis virus (BAI, strain A) (15) and Rous sarcoma virus (Prague strain, subgroup C) were provided by the Office of Program Resources and Logistics of the Virus Cancer Program of the National Cancer Institute. Other materials were carrier-free [¹²⁵I]sodium iodide (NEZ-033H, 400-600 mCi/ml) from New England Nuclear Corp., Boston, Mass.; Nonidet P-40, a gift from Shell Oil Co., New York, N.Y.; *N,N'*-diallyltartardiamide from Aldrich Chem. Corp., San Leandro, Calif.; L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (lot 3BA, 219 units/mg) from Worthington Biochemical Corp., Freehold, N.J.; 20 × 20-cm thin-layer cellulose plates (0.01-mm layer thickness, no. 5757/0001) from E. M. Laboratories, Elmsford, N.Y.; and chloramine-T from J. T. Baker Chem. Co., Phillipsburg, N.J.

Purification of Reverse Transcriptase from AMV and RSV. Purified virions of AMV and RSV were lysed with nonionic detergent (Nonidet P-40). The viral DNA polymerase was then purified from the lysate by sequential column chromatography on DEAE-Sephadex and phosphocellulose, as described (10). A minor peak of activity was eluted from the phosphocellulose column at a salt concentration of 0.1 M, followed by a major peak of enzymatic activity eluting at a salt concentration of 0.25 M KCl (10). Only material from the major peak of enzymatic activity was used in the experiments described below.

In Vitro Radiolabeling of Reverse Transcriptase. Purified reverse transcriptase, isolated from either AMV or RSV, was

Abbreviations: AMV, avian myeloblastosis virus; RSV, Rous sarcoma virus.

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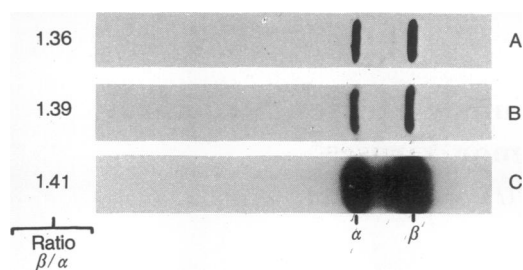


FIG. 1. Polyacrylamide gel analysis of reverse transcriptase of AMV. Photographs are shown here of the Coomassie brilliant blue-stained reverse transcriptase subunits of AMV before (A) and after (B) iodination, and an autoradiogram of the gel containing ^{125}I -labeled subunits (C). The ratio of the densitometric absorbance (measured with an EC 910 scanning densitometer, E. C. Apparatus Corp., St. Petersburg, Fla.) of the α and β subunits is presented at the end of each panel. The direction of the protein's electrophoretic migration was from the left to the right in these figures.

precipitated from solution with trichloroacetic acid and extracted with acetone as described (16). The dried protein was then resuspended in 30 μl of 0.05 M Tris buffer (pH 7.5), containing 2% sodium dodecyl sulfate and 4 M urea, and iodinated by the addition of 4 μl (about 2 mCi) of ^{125}I sodium iodide and 5 μl of chloramine-T (5 mg/ml in H_2O). After 15 min at 23 $^\circ$, the reaction was stopped by the addition of 100 μl of 0.05 M Tris buffer (pH 7.0), 2% sodium dodecyl sulfate, 20 mM dithiothreitol, or 5% 2-mercaptoethanol, 20% glycerol, and 0.005% bromophenol blue (solubilization buffer). The radiolabeled sample was then subjected to electrophoresis in a preparative polyacrylamide gel.

Polyacrylamide Gel Electrophoresis Techniques. Samples in solubilization buffer (described above) were subjected to electrophoresis in polyacrylamide slab gels using a discontinuous buffer system (17, 18) containing sodium dodecyl sulfate (19). Preparative gels were cross-linked with *N,N'*-diallyltartardiamide (20, 21); analytical gels were cross-linked with methylene-bisacrylamide. After electrophoresis, the protein bands were located in the gel by staining with Coomassie brilliant blue (22) and/or by autoradiography (23). Details of these procedures have been described (21, 24).

Peptide Analysis. The procedures used for peptide analysis have been described in detail elsewhere (24). Briefly, radiolabeled proteins were removed from the preparative gel, oxidized with performic acid, and digested with TPCK-treated trypsin. Tryptic hydrolysates were then subjected to two-dimensional separations on thin-layer cellulose plates, and the distribution of the ^{125}I -labeled peptides (primarily those peptides containing tyrosine residues) determined by autoradiography.

RESULTS

In Vitro Radiolabeling of Reverse Transcriptase of AMV. About 100 μg of purified reverse transcriptase of AMV was radiolabeled with ^{125}I , solubilized, and subjected to electrophoresis in a 14% preparative polyacrylamide gel, as described above. For purposes of comparison, a noniodinated portion (10–20 μg) of the same enzyme preparation was subjected to electrophoresis adjacent to the radiolabeled enzyme.

As shown in Fig. 1, the iodination procedure had no apparent effect on the electrophoretic mobility of either sub-

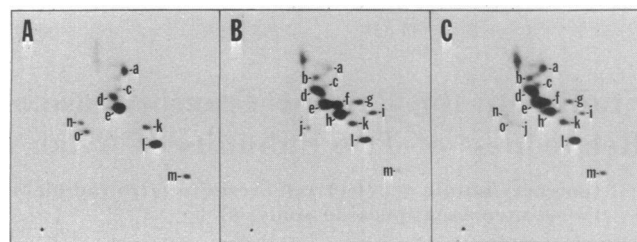


FIG. 2. Autoradiograms prepared from two-dimensional separations of tryptic hydrolysates of the α and β subunits of reverse transcriptase of AMV. These photographs show the autoradiograms of the α subunit (A), the β subunit (B), and a mixture of the two (C). Electrophoretic and chromatographic separations were from the left to the right, and from the bottom to the top, respectively. The origin of sample application appears as a spot in the lower left-hand corner of these photographs.

unit (compare panels A and B). It did, however, render even the native enzyme functionally inactive. This inactivation was not a consequence of iodination itself, but of the procedure used, since we have recently found that reverse transcriptase iodinated by use of lactoperoxidase (25) retains its enzymatic activity (data not shown). Estimates based on densitometric measurements indicate that (i) the relative amounts of α and β were also unchanged by the iodination procedure (compare ratios at bottom of panels A and B), and (ii) greater than 95% of the total radioactivity in the gel was contained in the two subunit bands in a ratio of 1.4, β to α (panel C). The apparent molecular weights of the α and β subunits in this polyacrylamide gel system were 62,000 and 95,000, respectively. Small amounts of a third protein (molecular weight 84,000), detected both by staining and autoradiography, were present between α and β . This minor band is a contaminant of the enzyme preparation, since it is present in variable amounts from preparation to preparation, and can be removed almost completely by sedimenting the enzyme through a glycerol gradient or by chromatography on an oligo(dT)-cellulose column (data not shown).

Comparison of the α and β Subunits of Reverse Transcriptase, Isolated from AMV and RSV, by Peptide Analysis. After iodination and separation by electrophoresis, the α and β subunit bands of the reverse transcriptases of AMV and RSV were sectioned from the gel and processed for tryptic peptide analysis. The respective hydrolysates were compared by two-dimensional separation on thin-layer cellulose plates, as described in *Materials and Methods*. Photographs of the resulting autoradiograms are presented in Figs. 2 and 3.

These data can be summarized as follows. (i) The general distribution of radioactive spots was strikingly similar in all preparations (compare Figs. 2 and 3). (ii) With the possible exception of several ^{125}I -labeled peptides in the region of spots n and o, all of the ^{125}I -labeled peptides of the small subunit of AMV are also present in the large subunit (Figs. 2 and 3). It is not yet clear whether the apparent differences in the region of spots n and o, observed between the large and small subunits and between AMV and RSV preparations (see Figs. 2 and 3), are reproducible, since some variability has been noted in this region from preparation to preparation. (iii) Although some quantitative variation was observed between the two AMV preparations shown here, there were no detectable qualitative differences (compare panels A and B, Figs. 2 and 3). (iv) There were few differences observed between the re-

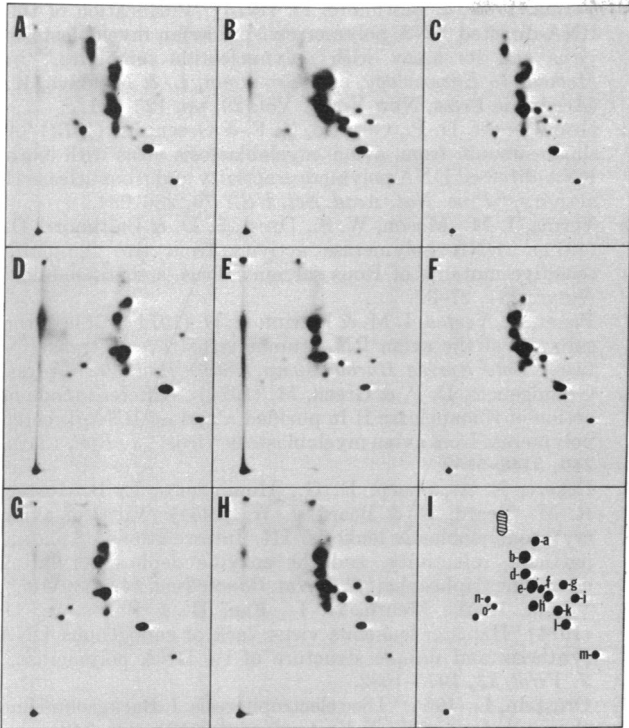


FIG. 3. Autoradiograms prepared from two-dimensional separations of tryptic hydrolysates of reverse transcriptase subunits of AMV and RSV. The photographs shown here are of the ^{125}I -labeled peptide distributions of AMV α (A), AMV β (B), a mixture of AMV α and AMV β (C), RSV α (D), RSV β (E), a mixture of RSV α and RSV β (F), a mixture of AMV α and RSV α (G), a mixture of AMV β and RSV β (H), and a diagram indicating the positions of the spots identified in Fig. 2. Three undesigned spots are also shown in the diagram. One of these, below and to the left of spot *h*, is present in the AMV and RSV preparations shown in this figure, but was not detected in the AMV preparation shown in Fig. 2. The second, below and to the left of spot *o*, was detected only in the RSV preparations. The third, above and to the left of spot *a*, was detected in AMV but not in RSV preparations. Some spot "doubling" occurred in the mixture shown in panel F. The reason for this is not known, but the similarities between the α and β subunits of RSV (panels D and E, respectively) are supported by the mixtures shown in panels G and H. Electrophoretic and chromatographic separations were from left to right, and from bottom to top, respectively. The origin of sample application appears as a spot in the lower left-hand corner of the photographs.

verse transcriptase preparations of AMV and RSV. As mentioned above, there are apparent differences in the region of spots *n* and *o* and all AMV preparations examined contain a series of spots to the left and above the spots *a* and *b*, which are not observed in RSV preparations (Figs. 2 and 3).

Detection of Reverse Transcriptase in Purified Virions. To determine whether the α and β subunits are both present in the virions, a preparation of purified AMV was solubilized and subjected to electrophoresis in an analytical polyacrylamide slab gel. As shown in Fig. 4, panel A, five major and several minor protein bands were detected in the virion preparation. Two of the minor bands had electrophoretic mobilities indistinguishable from those of the α and β subunits of purified reverse transcriptase of AMV (compare panels A and B). Densitometric quantitation of the relative amounts of these two virion proteins showed them to be present in a ratio

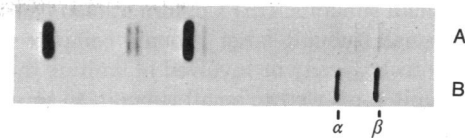


FIG. 4. Comparison of the electrophoretic mobilities of avian myeloblastosis virion proteins with those of the α and β subunits of purified reverse transcriptase of AMV. About 50 μg of virion protein and 10 μg of reverse transcriptase were solubilized and subjected to electrophoresis side-by-side in sodium dodecyl sulfate-containing polyacrylamide slab gel. The photograph shows avian myeloblastosis virion proteins (A) and reverse transcriptase subunits of AMV (B) stained with Coomassie brilliant blue.

of approximately 2:1 (β to α), and together, account for approximately 2% of the total protein mass of the AMV preparation. Although similar observations have been reported by Stromberg *et al.* (26), it remains to be established by other criteria that these virion proteins are in fact the two subunits of reverse transcriptase.

DISCUSSION

The purpose of the experiments reported here was to investigate the structural relatedness of the two subunits of avian RNA tumor virus reverse transcriptase. This was done by comparing tryptic hydrolysates of each subunit, radiolabeled *in vitro* with ^{125}I , by two-dimensional separation on thin layer cellulose plates. Two observations emerged from these experiments. First, the large subunit of reverse transcriptase isolated from either AMV or RSV contains most of the ^{125}I -radiolabeled peptide present in the small subunit (Figs. 2 and 3). Second, the two-dimensional tryptic peptide distributions of the reverse transcriptase subunits of AMV and RSV resemble each other closely (Fig. 3).

The observed similarities between the large subunit and small subunit ^{125}I -labeled peptide distributions suggest a close structural relationship between these two proteins. One interpretation of these peptide similarities is that the small subunit of reverse transcriptase is derived from the large subunit by proteolytic cleavage. The observation that the small subunit may contain two ^{125}I -labeled peptides (e.g., spots *n* and *o*) that are absent from the large subunit (Fig. 2) is not incompatible with this interpretation, since cleavage at the NH_2 -terminal or COOH -terminal or both ends could generate one or two new peptides. If the small subunit is derived from the large subunit and the native enzyme is the $\alpha\beta$ complex (13, 14), then reverse transcriptase from avian RNA tumor viruses may be an example of an enzyme that requires close association between the product and precursor for maximal enzymatic activity.

A number of questions concerning the synthesis and putative processing of avian reverse transcriptase arise from the suggestion that the small subunit may be derived from the large subunit. For example, (i) At what point during the life cycle of the virus is the small subunit produced? (ii) What is the function of each of the subunits? (iii) Is there a mechanism that regulates the amount of large subunit converted to small subunit? We propose the following possible chain of events leading to the synthesis of enzymatically active reverse transcriptase. (i) The large subunit is the molecular species of reverse transcriptase synthesized in infected cells and packaged into the virions. (ii) After or during maturation of the virions some of the large subunit molecules are cleaved

to produce small subunits. (iii) Conformational changes that render the small subunit-large subunit complex more resistant to proteolysis may be involved in limiting the amount of large subunit converted to small subunit, to an equimolar ratio.

The close structural relatedness of α and β subunits can also be explained on the basis that the two proteins are independently coded for by the same gene (e.g., more than one initiation and/or termination site within the same gene), or that each subunit is coded for by separate but related genes. Although neither of these possibilities can be ruled out by the data presented here, the last one at least seems unlikely, since together, these proteins would require nearly 50% of the potential coding capacity of the avian RNA tumor virus genome (27, 28).

Finally, the observed similarities of reverse transcriptases of ¹²⁵I-labeled peptide distributions of AMV and RSV (Fig. 3) suggest that these enzymes may share extensive amino-acid sequence homology. This is consistent with the observation that antisera to reverse transcriptase of AMV inhibit the enzymatic activity of reverse transcriptase of RSV (29). Although the technique used in the experiments reported here allows detection of only those peptides containing tyrosine residues (30), it can be used to make a limited comparative study of reverse transcriptase from other sources to determine whether some portions of its amino-acid sequence may have been conserved during the evolution of the RNA tumor viruses.

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- Baltimore, D. (1970) "RNA directed DNA polymerase in virions of RNA tumor viruses," *Nature* **226**, 1209-1211.
- Temin, H. & Mizutani, S. (1970) "RNA dependent DNA polymerase in virions of Rous sarcoma virus," *Nature* **226**, 1211-1213.
- Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M. & Watson, K. (1970) "Synthetic DNA-RNA hybrids and RNA-RNA duplexes as templates for the polymerase of oncogenic RNA viruses," *Nature* **228**, 430-432.
- Baltimore, D. & Smoler, D. F. (1971) "Primer requirement and template specificity of the DNA polymerase of RNA tumor viruses," *Proc. Nat. Acad. Sci. USA* **68**, 1507-1511.
- Temin, H. M. & Baltimore, D. (1972) "RNA-directed DNA synthesis of RNA tumor viruses," in *Advances in Virus Research* eds. Smith, K. M. & Lauffer, M. A. (Academic Press, New York), Vol. 17, pp. 129-186.
- The Molecular Biology of Tumour Viruses*, ed. Tooze, J. (1973) (Cold Spring Harbor Laboratory, New York), chap. 11.
- Kacian, D. L., Watson, K. F., Burny, A. & Spiegelman, S. (1971) "Purification of the DNA polymerase of avian myeloblastosis virus," *Biochim. Biophys. Acta* **246**, 365-383.
- Hurwitz, J. & Leis, J. P. (1972) "RNA dependent DNA polymerase activity of RNA tumor viruses. I. Directing influence of DNA in the reaction," *J. Virol.* **9**, 116-129.
- Faras, A. J., Taylor, J. M., McDonald, J. P., Levinson, W. E. & Bishop, J. M. (1972) "Purification and characterization of the deoxyribonucleic acid polymerase associated with Rous sarcoma virus," *Biochemistry* **11**, 2334-2342.
- Verma, I. M. & Baltimore, D. (1973) "Purification of the RNA-directed DNA polymerase from avian myeloblastosis virus and its assay with polynucleotide templates," in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 29, pp. 125-131.
- Grandgenett, D. P., Gerrard, G. F. & Green, M. (1973) "A single-subunit from avian myeloblastosis virus with both RNA-directed DNA polymerase activity and ribonuclease H activity," *Proc. Nat. Acad. Sci. USA* **70**, 230-234.
- Verma, I. M., Mason, W. S., Drost, S. D. & Baltimore, D. (1974) "DNA polymerase activity from two template-sensitive mutants of Rous sarcoma virus is thermolabile," *Nature* **251**, 27-31.
- Panet, A., Verma, I. M. & Baltimore, D. (1974) "Role of the subunits of the avian RNA tumor virus reverse transcriptase," *Cold Spring Harbor Symp. Quant. Biol.* **39**, in press.
- Grandgenett, D. P. & Green, M. (1974) "Different mode of action of ribonuclease H in purified α and $\alpha\beta$ RNA-directed polymerase from avian myeloblastosis virus," *J. Biol. Chem.* **249**, 5148-5152.
- Eckert, E. A., Sharp, D. G., Mommaerts, E. B., Reeve, R. M., Beard, D. & Beard, J. W. (1954) "Virus of avian erythromyeloblastic leukosis. III. Interrelations of plasma particles, infectivity, and the enzyme dephosphorylating adenosine triphosphate," *J. Nat. Cancer Inst.* **14**, 1039-1053.
- Verma, I. M., Meuth, N. L., Fan, H. & Baltimore, D. (1974) "Hamster leukemia virus: lack of endogenous DNA synthesis and unique structure of its DNA polymerase," *J. Virol.* **13**, 1075-1082.
- Ornstein, L. (1964) "Disc electrophoresis. I. Background and theory," *Ann. of the N.Y. Acad. of Sci.* **121**, 321-349.
- Davis, B. J. (1964) "Disc electrophoresis. II. Methods and application to human serum proteins," *Ann. of the N.Y. Acad. of Sci.* **121**, 404-427.
- Laemmli, U. K. (1970) "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* **227**, 680-685.
- Anker, M. S. (1970) "A solubilizable acrylamide gel for electrophoresis," *FEBS Lett.* **7**, 293.
- Gibson, W. & Roizman, B. (1974) "Proteins specified by herpes simplex virus: staining and radiolabelling properties of B capsid and virion proteins in polyacrylamide gels," *J. Virol.* **13**, 155-165.
- Fairbanks, G., Levinthal, C. & Reede, R. H. (1965) "Analysis of ¹⁴C-labeled proteins by disc electrophoresis," *Biochem. Biophys. Res. Commun.* **20**, 393-399.
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) "Electrophoretic analysis of the major polypeptides of human erythrocyte membrane," *Biochemistry* **10**, 2606-2617.
- Gibson, W. (1974) "Polyoma virus proteins: a description of the structural proteins of the virion based on polyacrylamide gel electrophoresis and peptide analysis," *Virology* **62**, in press.
- David, G. & Reisfeld, R. A. (1974) "Protein iodination with solid state lactoperoxidase," *Biochemistry* **13**, 1014-1021.
- Stromberg, K., Hurley, N. E., Davis, N. L., Rueckert, R. R. & Fleissner, E. (1974) "Structural studies of avian myeloblastosis virus: comparison of polypeptides in virion and core component by sodium dodecyl sulfate polyacrylamide gel electrophoresis," *J. Virol.* **13**, 513-528.
- Billeter, M. A., Parsons, J. T., & Coffin, J. M. (1974) "The nucleotide sequence complexity of avian tumor virus RNA," *Proc. Nat. Acad. Sci. USA* **71**, 3560-3564.
- Beemon, K., Duesberg, P. & Vogt, P. K. (1974) "Evidence for crossingover between avian tumor viruses based on analysis of viral RNA," *Proc. Nat. Acad. Sci. USA* **71**, 4254-4258.
- Nowinski, R. C., Watson, K. F., Yaniv, A. & Spiegelman, S. (1972) "Serological analysis of the deoxyribonucleic acid polymerase of avian oncornaviruses. II. Comparison of avian deoxyribonucleic acid polymerase," *J. Virol.* **10**, 959-964.
- Marchalonis, J. J., Cone, R. E. & Santer, V. (1971) "Enzyme iodination: A probe for accessible surface proteins of normal and neoplastic lymphocytes," *Biochem. J.* **124**, 921-927.