Proc. Nat. Acad. Sci. USA
Vol. 70, No. 1, pp. 36–40, January 1973

Structural Characteristics of Some Murine RNA Tumor Viruses Studied by Lactoperoxidase Iodination

(radiation leukemia virus/Moloney leukemia virus/mouse mammary tumor virus/gel electrophoresis)

OWEN N. WITTE, IRVING L. WEISSMAN, AND HENRY S. KAPLAN

Departments of Pathology and Radiology, Stanford University School of Medicine, Stanford, California 94305

Contributed by Henry S. Kaplan, October 15, 1972

ABSTRACT **Iodination by the noninvasive enzymatic lactoperoxidase technique has been used to study the enzyme-accessible and enzyme-inaccessible proteins of three oncorna viruses (radiation leukemia virus, Moloney leukemia virus, and mouse mammary tumor virus).**

The number and relative molecular weight of proteins associated with virion preparations purified on sucrose gradients were characterized by scans of Coomassie blue-stained bands after dodecyl sulfate–polyacrylamide gel electrophoresis. Gel scans from the leukemia viruses are similar, each showing six distinct major protein bands on stained gels. The mammary tumor virus proteins by this analysis are not similar to those of the leukemia viruses.

Enzymatic iodination of intact virion preparations led to the solitary labeling of one of the major proteins of each virus—the 80,000-dalton protein of the leukemia viruses and the 52,500-dalton protein of the mammary tumor virus. These are tentatively positioned as surface (enzyme-accessible) moieties in the virions. Disruption of each virus with a nonionic detergent before enzymatic iodination led to the labeling of the remaining stainable bands. The four lower molecular weight bands of the leukemia viruses are tentatively positioned as internal (enzyme-inaccessible) components.

The structural proteins of RNA oncogenic viruses of mice have been studied by various techniques, including gel filtration, isoelectric focusing, and polyacrylamide gel electrophoresis (1–3). Major proteins commonly defined include a high molecular weight (80,000) glycoprotein, presumably present on the viral membrane, and a lower molecular weight (30,000) protein that may possess both intraspecies and interspecies antigenic reactivities (4). The relationship between individual proteins and their location in the virion is of interest, both for the study of viral assembly and to elucidate interrelationships of tumor cell-surface antigens and virion antigens. Electron microscopic analysis has been combined with the isolation of subviral particles to generate tentative models of virion structure (5, 6).

The purposes of this study were:

(*i*) To determine the virion-associated proteins of radiation leukemia virus (RadLV), (a naturally occurring C-type virus consistently recoverable from radiation-induced leukemias and lymphomas of strain C57BL mice (7, 8), now propagated in tissue culture), and to compare it to Moloney leukemia virus (MLV) and mouse mammary tumor virus (MTV).

(*ii*) To use the noninvasive and nondestructive lactoperoxidase iodination technique to label intact virions in order to distinguish enzyme-accessible (external) proteins from enzyme-inaccessible (internal) ones.

Abbreviations; RadLV, Radiation leukemia virus: MLV, Moloney leukemia virus: MTV, mouse mammary tumor virus: PBS, phosphate-buffered saline, pH 7.4.

MATERIALS AND METHODS

RadLV. Supernatant fluids from a RadLV-infected C57BL/Ka embryo fibroblast line, BL-5 (RadLV), were provided by Mr. O. Niwa and Dr. A. Declève of the Department of Radiology. Supernatant fluid from the uninfected BL-5 line of C57BL/Ka fibroblasts was used as a control. All of the BL-5 (RadLV) cells carry RadLV, as determined by immunofluorescence for group-specific antigen and by infectious center counts by the XC plaque assay (9). BL-5 (RadLV) supernatant fluid contains about 5×10^7 focus-forming units/ml, as monitored by the XC assay with newly infected mouse-embryo fibroblasts. BL-5 cells are completely virus-negative when examined by either assay (O. Niwa, A. Declève, and H. S. Kaplan, manuscript in preparation).

MLV was purified from the ascitic fluid of Balb/c mice carrying the transplantable LSTRA Moloney lymphoma (10). These cell-free ascitic supernatants (at dilutions up to 1:1000) were capable of inducing infectious-center plaques on XC cells with primarily infected mouse-embryo fibroblasts (assayed by Dr. A. Declève). Because this source of MLV might well be contaminated with other macromolecular aggregates of similar density, we felt this would be an interesting test of the resolving power of dodecyl sulfate–gel electrophoresis.

MTV was purified from chelated milk of lactating Balb/c mice fostered on strain C3H, and was a gift from Dr. P. Blair, Cancer Research and Genetics Laboratory, University of California, Berkeley.

Virus Purification—RadLV. After clarification of supernatants at 4° (Sorvall RC2B centrifuge, GSA rotor, 11,000 rpm, 15 min) virus was pelleted in a Beckman L3-50 ultracentrifuge, T-15 rotor (14,000 rpm, 2.5 hr). Virus pellets were resuspended in phosphate-buffered saline, 0.15 M NaCl–15 mM NaH_2PO_4–K_2HPO_4 containing 1 mM EDTA (pH 7.4) and spun through discontinuous 45–25% (w/w) sucrose (Swartz–Mann, ultrapure) gradients in PBS–EDTA in a Beckman SW27 rotor at 26,000 rpm for 2 hr. Material sedimenting to the interphase was slowly diluted and layered over 10-ml preformed (45–25%) continuous sucrose gradients and spun for 17 hr at 26,000 rpm in a SW27 rotor. A Hoffer gradient-collecting device was used to separate 0.4-ml fractions. Sucrose densities were measured with a Bausch

and Lomb Abbe 3L refractometer. Protein was monitored by its adsorbance at 280 nm with a Zeiss PMQ II spectrophotometer. Ratios of *A* 280/260 were taken for each gradient fraction to monitor the relative concentrations of protein and nucleic acids.

MLV. After cell removal and clarification, virus was purified by the two sucrose gradient steps described above.

MTV. Infected milk was chelated and skimmed as described by Nowinski *et al.* (11), then processed in sucrose gradients as above.

Concentrates containing each of these viruses shall hereafter be referred to as RadLV, MLV, and MTV, although there is no evidence that these preparations consist exclusively of biologically homogeneous virus particles.

Iodination. Lactoperoxidase iodination (12) was done at room temperature. About 0.4 ml of a sucrose-banded virus preparation was labeled either intact or with prior disruption with 0.2% (v/v) Triton X-100 (Rohm and Haas Chemical Corp.) at 37° for 30 min. $Na^{125}I$ in 0.1 N NaOH (New England Nuclear) was added, to a final concentration of 70–100 μCi/sample. Lactoperoxidase grade B (Calbiochem) was used at 15 μg/ml. Three to four additions of freshly prepared H_2O_2 (10 μl of a 6 μM solution in deionized–distilled H_2O) were made at 10-min intervals. The reaction mixture was extensively dialyzed at 4° to remove free iodide.

Gel Electrophoresis was performed as described by Hung *et al.* (13). Samples were pelleted and resuspended or dialyzed into 0.01 M NaH_2PO_4–Na_2HPO_4 (pH 7.2) buffer containing 1% dodecyl sulfate, 1% 2-mercaptoethanol, and 1 mM EDTA. 50-μl Aliquots were made 6 M with urea (Swartz-Mann, Ultrapure), heated at 37° for 0.5 hr, and then at 100° for 5 min before they were loaded onto gels. 3 μl of 0.05% Bromphenol blue was used as a tracking dye.

Gels were 5-mm diameter, 7% acrylamide (Biorad, electrophoresis purity grade) crosslinked with *N,N'*-methylene bisacrylamide (Eastman Kodak, recrystallized from chloroform), *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate. The gel buffer, as well as the upper and lower tank buffer, was 0.1 M NaH_2PO_4–Na_2HPO_4 (pH 7.2) with 0.1% dodecyl sulfate. In two experiments, 10% acrylamide gels with proportional crosslinking were used.

Samples were electrophoresed at 10 mA/gel until the tracking dye had migrated about 8 cm. Gels were stained with Coomassie Brilliant Blue R (Sigma Chemicals), as described by Weber and Osborn (14), and destained at 37° with shaking against 7.5% acetic acid–5% methanol. Gel patterns were recorded with a Gilford model 2000 spectrophotometer equipped with a linear gel-transport system.

Gels containing iodinated proteins were frozen and cut into 1-mm slices with a Hoffer gel fractionator and counted in a Packard model 578 Autogamma spectrophotometer.

Incorporation of [^{3}H]Uridine into RadLV. Both BL-5 and BL-5(RadLV) cell lines were seeded at 2 × 10^6 cells per 75-cm^2 flask (Falcon Plastics), and fed with 1 mCi of [^{3}H]-uridine (New England Nuclear) in 20 ml of Minimal Essential Medium (Gibco) with added glutamine and antibiotics. Supernatants were harvested after 3 days and clarified; 5-ml aliquots were analyzed directly on 10-ml preformed linear 60–20% (w/w) sucrose–PBS–EDTA gradients in a SW27 rotor at 26,000 rpm for 3 hr. 10 μl of 0.3-ml fractions were dried on Whatman GF/C filter papers and counted in a toluene-based scintillation fluid.

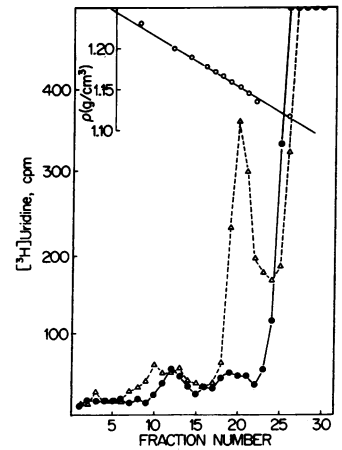

FIG. 1. 5-ml Aliquots of BL-5(RadLV) (△) and BL-5 line (●) culture supernatants labeled with [^{3}H]uridine were centrifuged onto 60–20% sucrose–PBS–EDTA gradients (SW 27 rotor, 26,000, rpm, 3 hr). 10 μl of each 0.3-ml fraction was counted. Sucrose densities were measured with a Bausch and Lomb refractometer.

RESULTS

Internal labeling of RadLV

Labeling with [^{3}H]uridine of a particulate fraction with a density of 1.14–1.15 g/cm^3 was demonstrated in supernatant fluids from the BL-5(RadLV) cell line, but not in those from the control BL-5 line, analyzed on continuous sucrose gradients (Fig. 1). Although incorporation is evident, the total number of counts available were not sufficient to use such internally labeled preparations for radioimmune assays or *in vivo* localization studies. Instead, the labeled BL-5(RadLV) supernatant was used as a tracer in the development of a purification scheme for RadLV from large volumes of BL-5(RadLV) culture supernatants and from the *in vivo* virus sources.

Virus purification

Viruses pelleted from large volumes of tissue culture fluid and viruses from *in vivo* sources were first banded on discontinuous sucrose gradients. They were further purified on 45–25% (w/w) continuous sucrose gradients with isopycnic banding to select out a very narrow density range for further structural studies. The results of three such gradients are shown in Fig. 2*a* for RadLV, MLV, and MTV. Sucrose density determinations for all three gradients were the same. Peak densities were: RadLV = 1.145 g/cm^3, MLV = 1.154 g/cm^3, and MTV = 1.174 g/cm^3. Only material from such peak fractions was used for iodination procedures or gel analysis.

Iodination of standard proteins, and both intact and disrupted virus preparations, was performed at room temperature. It was not necessary to remove sucrose or Triton X-100 from reaction mixtures before labeling. Concentrations of up to 1% Triton X-100 did not significantly change the efficiency of labeling of serum albumin by lactoperoxidase iodination. The reaction is specific for tyrosine and histidine residues (12). Self-labeling by lactoperoxidase was not detectable in the presence of substrate protein (unpublished

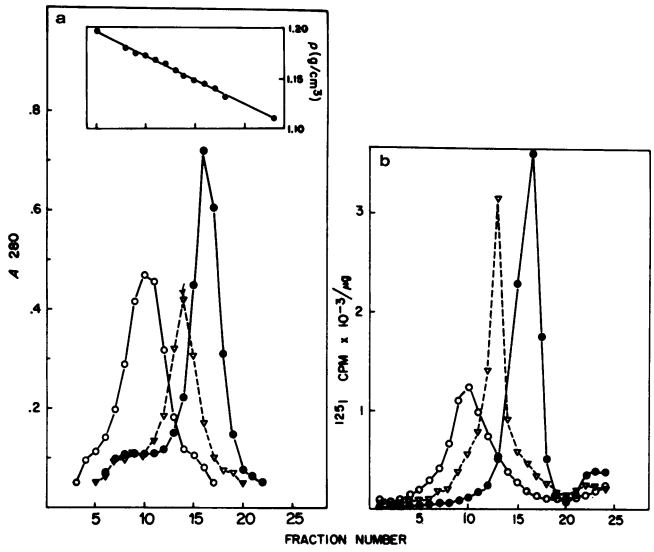

FIG. 2. (*a*) MTV (○), MLV (▽), and RadLV (●) purified on discontinuous 45–25% sucrose gradients were slowly diluted and centrifuged onto linear 45–25% continuous gradients for 17 hr (SW27 rotor, 26,000 rpm). 0.4-ml Fractions were monitored for A_{280}, with no correction for the effects of light scattering. Sucrose density determinations were nearly identical for all six gradients. Average values are shown. (*b*) Aliquots of MTV, MLV, and RadLV from peak fractions (shown above) were labeled with ^{125}I by the lactoperoxidase method, then rebanded as in (*a*). cpm normalized per μg of protein, estimated from *A* 280/260 ratio, with correction for light scattering.

observations, and ref. 15). The reaction is sensitive to changes in available tyrosine and histidine residues, free ^{125}I, enzyme concentration, and—especially—to the concentration of peroxide. To prevent the deleterious oxidizing effects of excess

TABLE 1. *Properties of iodinated protein standards and viral preparations*

	Substrate	μg*	Total cpm-($\times 10^{-6}$) after dialysis	cpm-($\times 10^{-6}$) per 100 μg
1.	Cytochrome *c*	800	7.2	0.9
2.	Bovine 7S Globulin	3000	41.0	1.4
3.	Ovalbumin	250	6.1	2.4
4.	Human serum albumin	770	63.0	8.2
5.	RadLV (intact)	175	2.5	1.4
6.	RadLV (disrupted)	175	6.6	3.8
7.	MLV (intact)	100	3.1	3.1
8.	MLV (disrupted)	100	4.3	4.3
9.	MTV (intact)	200	1.5	0.8
10.	MTV (disrupted)	200	12.3	6.1

The noted amounts of protein standards and virus preparations were reacted with 70 μCi of ^{125}I for protein standards and 100 μCi for each virus preparation, in 0.4 ml of PBS–EDTA buffer with 6 μg of lactoperoxidase. All reaction mixtures received three additions of 10 μl of 6 μM H_2O_2 at 10-min intervals, except MLV preparations, which received four.

* 1–4 by weight, 5–10 from *A* 280/260 after correction for light scattering (24).

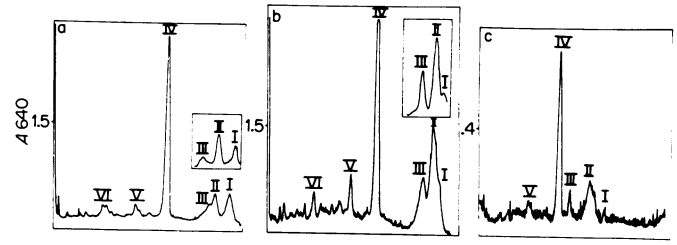

FIG. 3. (*a*) RadLV from peak fraction of a gradient as shown in Fig. 2 disrupted with dodecyl sulfate–urea–mercaptoethanol, then analyzed on a 7% acrylamide gel, stained with Coomassie blue, and scanned with a Gilford model 2000 spectrophotometer with a linear gel transport. Molecular weight estimates for the numbered peaks are given in Table 2. Electrophoresis is from *left* to *right*. *Inset:* scan of the low molecular weight region analyzed on a 10% acrylamide gel. (*b*) MLV prepared as above. (*c*) MTV prepared as above.

H_2O_2, several additions of a dilute solution were used (see *Methods*). The conditions of reaction and specific activities of protein standards and virus preparations are shown in Table 1.

Both RadLV and MLV could be labeled to high specific activities by the lactoperoxidase method and still remain intact. Such preparations, rebanded on sucrose gradients, retain the density characteristics of untreated virus (Fig. 2*b*). Rebanded iodinated virus fixed on carbon-coated grids and stained with uranyl acetate (5) showed dense nucleoids enclosed by membrane-like structures. These images were indistinguishable from unlabeled virus (unpublished observations). Prior disruption with a nonionic detergent exposed additional proteins to the labeling process and increased the relative specific activity. MTV was not labeled as well as the other two viruses while it was intact (see below).

Dodecyl sulfate–acrylamide gel electrophoresis

Preparations of RadLV, MLV, and MTV showed distinctive protein spectra when analyzed on 7% acrylamide gels stained with Coomassie blue (Fig. 3). 10% gels were needed to resolve the low molecular weight bands (10,000–15,000) found in the leukemia viruses. Molecular weight estimates for the major proteins shown in Table 2 are the average of at least three determinations, relative to the standard curve plotted in Fig. 4. Many very faint bands, that do not appear on the densitometer scans, were present on all gels, especially in the high molecular weight range above 50,000. These bands may represent aggregates of lower molecular weight forms or specific macromolecules present in virus preparations in very low concentrations.

RadLV and MLV were similar in that most of the Coomassie blue-positive material present was found in four lower molecular weight (10,000–30,000) proteins, generally referred to as those bearing the group-specific antigens (1). However, the relative concentrations and molecular weights of these four macromolecules from RadLV and MLV differ significantly. In the higher molecular weight range, MLV has two major bands, of molecular weights 44,000 and 80,000. These correspond to the glycoproteins reported by other workers (2). The higher molecular weight glycoprotein is believed to be the major protein of the viral membrane (16). RadLV

TABLE 2. *Molecular weight estimates of major viral proteins*

Band no.	MW × 10^{-3} RadLV	MLV	MTV
I	10.5	10.7	26.0
II	13.0	12.0	32.0
III	15.0	14.0	43.0
IV	29.5	29.5	52.5
V	47.0–51.0*	44.0	85.0
VI	77.0–89.0†	80.0	—

* Two close bands.
† Three close bands.

has a doublet in the range 47,000–51,000, and a triplet between 77,000 and 89,000. Preparations of MTV showed one large peak at 52,500, other major bands at 26,000, 32,000, 43,000, and 85,000, and many minor bands in the higher molecular weight region.

Differential enzymatic iodination

A striking difference is seen in the iodination patterns of intact and disrupted virus preparations. Both leukemia viruses label in a very restricted fashion. MLV labels primarily over peak VI, while RadLV labels over the area of the three stained bands at peak VI.

When MLV and RadLV are disrupted before enzymatic iodination with a nonionic detergent known to release internal proteins (17), the iodine label is found on the low molecular weight (10,000–30,000) proteins, as well as the high molecular weight proteins labeled under intact conditions (Fig. 5). The coincidence of radioiodinated peaks and Coomassie blue peaks strengthens the interpretation that these are, in fact, structural proteins; however, Coomassie blue stains molecules other than proteins under certain conditions (18).

Intact preparations of MTV did not label as well as intact leukemia viruses. A comparison of iodinated intact MTV with iodinated disrupted MTV indicates that there may be few available tyrosine residues on the membrane protein. Labeling of the major polypeptide of MTV (52,500) generally

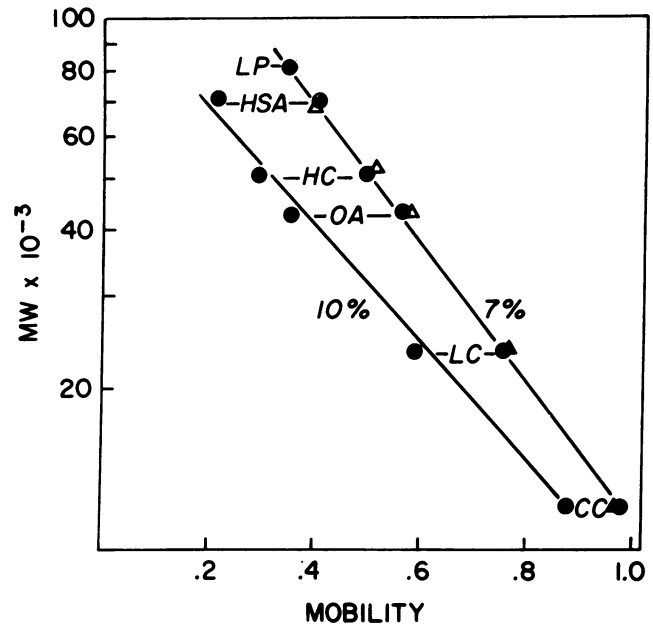

FIG. 4. Semilogarithmic plot of molecular weight (*MW*) against mobility of protein standards on 7 and 10% acrylamide gels, calculated relative to bromphenol blue by the method of Weber and Osborn (14), where:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

Abbreviations; Cytochrome *c* (*CC*), Bovine 7S gammaglobulin, light and heavy chains (*LC*, *HC*), ovalbumin (*OA*), human serum albumin (*HSA*), and lactoperoxidase (*LP*). Analyzed from stained gels (●). Analyzed with iodinated proteins (△).

associated with the nucleoid (11) under intact conditions was unexpected, and the presence of disrupted virions was excluded by first rebanding the iodinated virus, followed by analysis of the peak fraction by gel electrophoresis. The result was the same as shown in Fig. 5*c*. Other proteins of

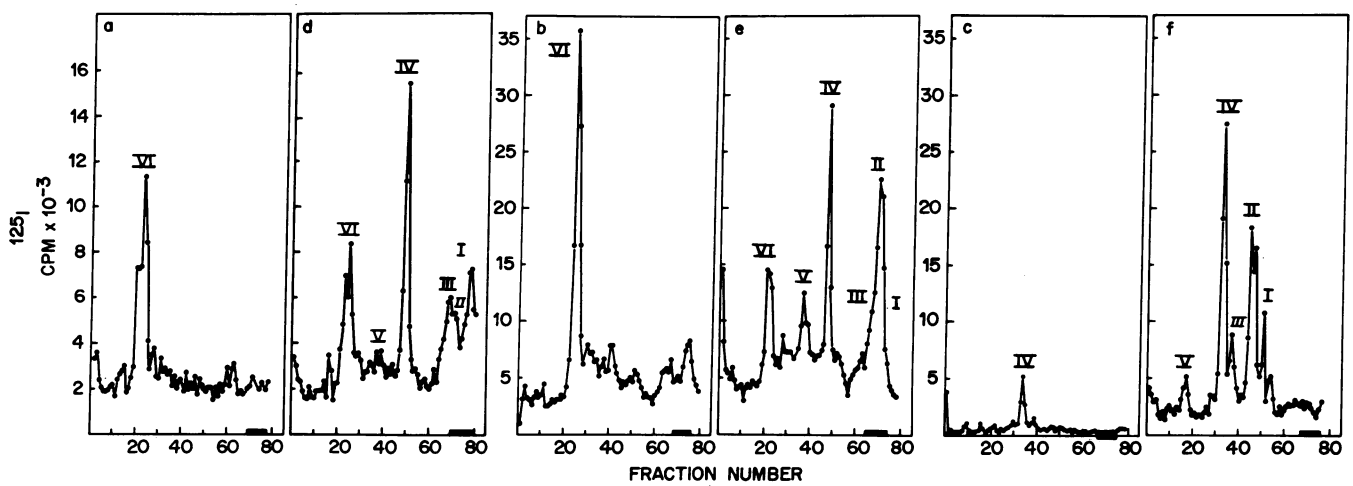

FIG. 5. Isopycnically-banded RadLV iodinated intact (*a*) or after disruption with Triton X-100 (*d*) analyzed on 7% gels after treatment with dodecyl sulfate, mercaptoethanol, and urea. Frozen gels were sliced into 1-mm pieces and counted in a Packard autogamma spectrophotometer. Numbered positions I to VI correspond to those in Fig. 3. Position of bromphenol blue tracking dye is indicated by the *bar*. (*b* and *e*) MLV treated as above. (*c* and *f*) MTV treated as above.

intact MTV (III and V) may be exposed to the iodination reaction, but were not clearly demonstrated in our experiments due to the lower specific activities of these preparations. Disrupted MTV labels comparably to the leukemia viruses, and iodinated peaks correspond well with stained preparations.

DISCUSSION

When studying viruses isolated from *in vivo* sources or from tissue culture, where high precursor labeling efficiency is not possible, one must use an exogenous label. Although various reagents are available (19), enzymatic iodination has many advantages. Reactions may be performed in neutral buffers in the presence of sucrose and nonionic detergents. ^{125}I may be counted in a gamma-ray counter or by liquid scintillation, and is useful as a marker in autoradiographic experiments (20). Due to the large size of lactoperoxidase and its site-specific catalysis, this method is noninvasive and may be used to study topographical characteristics of cell surfaces (21). Stanley and Haslam (22) have also used iodination techniques to study influenza virus.

Preparations of RadLV (tissue culture), MLV (ascitic fluid), and MTV (milk) all contained a limited number of major polypeptides, as evidenced by gels stained with Coomassie blue or iodinated, after disruption with Triton X-100. In all stained preparations there were numerous faint bands, especially in the higher molecular weight region. These may represent aggregates of lower molecular weight forms, since large amounts of virus (25–50 μg per gel) must be analyzed due to the preponderance of one major polypeptide in all three viruses (see Fig. 3). Gel electrophoresis of iodinated viruses showed a significant level of counts throughout the length of the gel (Fig. 5) when viruses were labeled under disrupted, as well as intact, conditions. This uniform distribution of label may represent contamination carried through the purification scheme, host-cell proteins carried by the virus, or virus-specific proteins present in low concentrations (such as viral enzymes).

Enzymatic iodination of intact MTV labeled the major polypeptide (molecular weight 52,500), which has been suggested to be a component of the viral nucleoid (11). This result strongly suggests that portions of at least some copies of this protein are at or near the surface of the virion. The failure to label the putative membrane protein of intact MTV (11) could relate to a lack of available tyrosine residues. Other aminoacid-specific reagents may prove more useful.

Although many similarities among the murine C-type viruses have been described (23), differences within the group have not been extensively studied. RadLV and MLV have quite distinctive protein distributions on stained gels and on unstained gels analyzed for iodinated proteins (see Figs. 3 and 5). Slight differences in the reported number and size of virion-associated proteins reported here and elsewhere are most likely due to differences in viral sources and separation techniques. The solitary labeling of the high molecular weight species (80,000), which coincides with the major glycoprotein reported by other workers (2), gives physical-chemical data to support its electron-microscopic and antigenic role as the major surface protein in the leukemia viruses. The inability to label low molecular weight proteins (10,000–30,000) of RadLV and MLV unless the virions are first disrupted with Triton X-100 presumably places them at internal (core) positions.

We thank O. Niwa and Dr. A. Declève for their performance of virus assays, their help with internal labeling of tissue-culture virus, and for their supplying tissue-culture supernatants for analysis. We thank Drs. D. Sedwick, G. Stark, and W. Robinson for helpful discussions during the course of this study. Supported by Grants IC-63A and IN-32M from the American Cancer Society, CA-03352 and CA-10372 from the National Cancer Institute, and AI-09072 from the National Institutes of Health. O.N.W. is a predoctoral fellow, Medical Scientist Training Program, National Institutes of Health-GM1922. I.L.W. is a Senior Dernham Fellow in Oncology, California Division, American Cancer Society.

1. Nowinski, R. C., Fleissner, E., Sarkar, N. H. & Aoki, T. (1972) *J. Virol.* **9**, 359–366.
2. Moroni, C. (1972) *Virology* **47**, 1–7.
3. Oroszlan, S., Foreman, C., Kelloff, G. & Gilden, R. V. (1971) *Virology* **43**, 665–674.
4. Parks, W. P. & Scolnick, E. M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1766–1770.
5. Luftig, R. B. & Kilham, S. S. (1971) *Virology* **46**, 277–297.
6. Sarkar, N. H., Nowinski, R. C. & Moore, D. H. (1971) *J. Virol.* **8**, 564–572.
7. Lieberman, M. & Kaplan, H. S. (1959) *Science* **130**, 387–388.
8. Kaplan, H. S. (1967) *Cancer Res.* **27**, 1325–1340.
9. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42**, 1136–1139.
10. Fefer, A., McCoy, J. L. & Glynn, J. P. (1967) *Cancer Res.* **27**, 962–967.
11. Nowinski, R. C., Sarkar, N. H., Old, L. J., Moore, D. H., Scheer, D. I. & Hilgers, J. (1971) *Virology* **46**, 21–38.
12. Morrison, M., Bayse, G. & Danner, D. J. (1970) in *Biochemistry of the phagocytic process*, ed. Schultz, J. (North-Holland Publ. Co., Amsterdam), pp. 51–66.
13. Hung, P. P., Robinson, H. L. & Robinson, W. S. (1971) *Virology* **43**, 251–266.
14. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
15. Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299–305.
16. Duesberg, P. H., Martin, G. S. & Vogt, P. K. (1970) *Virology* **41**, 631–646.
17. Gerwin, B. I., Todaro, G. J., Zeve, V., Scolnick, E. M. & Aaronson, S. A. (1970) *Nature* **228**, 435–438.
18. Dreyer, W. J., Papermaster, D. S. & Kühn, H. (1972) *Ann N.Y. Acad Sci.* **195**, 61–74.
19. Hoelzl Wallach, D. F. (1972) *Biochim. Biophys. Acta* **265**, 61–83.
20. Perkins, W. D., Karnovsky, M. J. & Unanue, E. R. (1972) *J. Exp. Med.* **135**, 267–276.
21. Poduslo, J. F., Greenberg, C. S. & Glick, M. C. (1972) *Biochemistry* **11**, 2616–2621.
22. Stanley, P. & Haslam, E. (1971) *Virology* **46**, 764–773.
23. Nowinski, R. C., Old, L. J., Sarkar, N. H. & Moore, D. H. (1970) *Virology* **42**, 764–773.
24. Layne, E. (1957) in *Methods in Enzymology*, eds. Colowick, S. & Kaplan, N. O. (Academic Press, New York), Vol. III, pp. 447–553.