SEPARATION AND ANALYSIS OF PROTEINS FROM FELINE LEUKEMIA VIRUS

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ABSTRACT

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by

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The need to learn more about RNA tumor viruses has increased recently since several C-type particles have been implicated as the etiological agents in human sarcomas. Since the feline leukemia virus (FeLV) can infect human cells and can be passed horizontally in the feline species, these are compelling reasons to study the virus thoroughly. Furthermore, Rickard's strain of virus is a good model system to work with because: (1) a high yield of virus (approximately 3 to 5 mg of viral protein per liter of cultural media) can be obtained from the F-422 suspension cell culture; and (2) it can be used for comparison with viruses of other species, especially the human candidate RNA tumor viruses.

Little was known about the number, size, location and detailed structure of the proteins of RNA tumor viruses, probably due to the lack of adequate methods for separating immunologically reactive viral proteins in quantities large enough to be used in further analysis. This

investigation was initiated to separate the structural proteins from the FeLV and to partially analyze them physically, chemically, and immunologically so that a better understanding of the molecular biology of the virus could be obtained.

Six major and one minor protein components were resolved from purified FeLV by agarose gel filtration in the presence of 6 M guanidine hydrochloride (GuHCl) and a reducing agent. Analysis of virus labeled with a mixture of ^{14}C-amino acids and ^{3}H-glucosamine revealed 2 glycoproteins (gp1 & gp2) and 5 nonglycoproteins (p1-p5). In order of elution the molecular weights (MW) of the proteins are: gp1≧100,000 (eluted with the void volume); gp2=70,000; p1=30,000; p5=21,000; p2=15,000; p3=11,200; and p4=10,000. In polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), p1 and p5 had lower MWs of 27,000 and 18,000, respectively. Protein p2 maintains its 15,000 MW whereas proteins p3 and p4 migrated identically in the MW region of 12,000. Proteins gp1 and gp2, which rechromatographed on 6% agarose as single peaks, appeared to be heterogeneous when analyzed by SDS-PAGE; gp1 gave several polypeptides in the 110-115,000 MW region; with gp2 six bands were observed in the MW region of 40-80,000. Proteins p3 and p4 which were poorly separated on 6% agarose and not resolved by SDS-PAGE with 10, 12.5 or 15% gels, were well separated on 8% agarose.

Protein p5 represents a component that has not been previously reported as a constituent of the mammalian RNA tumor viruses. It migrated between p1 and p2 on 6% agarose in a similar position to that of a seventh major protein component from the avian RNA tumor viruses. P1 was the major protein component making up 31% of the total viral proteins; p2, 17%; p3, 8%; p4, 5%, p5, 0.5%; gp1, 17%; and gp2, 10%. Isoelectric points for p1-p4 were: p1, 5.6; p2, 8.7; p3, 4.2; and p4, 3.8. Sedimentation coefficients (determined by sucrose gradient analysis) were: p1, 2.5; p2, 1.4; p3, 1.17; and p4, 1.14. The isoelectric point and sedimentation coefficient for p5 were not determined.

Efficient renaturation of p1, p2, and p3 can be achieved by dialysis to remove GuHCl. Antisera prepared against these three proteins revealed single, nonidentical precipitin bands by the Ouchterlony immunodiffusion assay. Similar preliminary studies with renatured p4 and p5 did not reveal antigenic activity. The interspecies gs-3 antigenic activity was shown to be associated with p1. Thus it appears that gel filtration in 6% agarose followed by 8% agarose is an effective technique for obtaining large amounts of the low molecular weight viral proteins for physical, chemical and immunological analysis.

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DEDICATION

This thesis is dedicated to my beloved wife, Cherie. Without her sacrifice, patience and encouragement, this may never have been possible; and to my two sons, Timothy and Todd.

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INTRODUCTION

A complete understanding of how viruses cause cancer, including those containing ribonucleic acid (RNA), is one of the primary goals of cancer research today. Since RNA containing viruses are responsible for many naturally occurring tumors in animals, this group is most likely to be the cause of some human neoplasms. Although no virus has yet been isolated and proven to induce human cancer, it would be unreasonable to consider man to be an exception.

The feline leukemia virus (FeLV) serves as an ideal and important model for study because of the following reaons: (1) it includes a chronically infected lymphoid cell suspension culture which produces relatively large amounts of virus that will be useful for virus purification and biochemical studies; (2) the virus shares immunological reactivity with other mammalian RNA tumor viruses which makes it an important candidate for comparative studies; (3) it can infect other mammalian cells including those of man; and (4) it has been shown to be transmitted horizontally.

Previous studies on the proteins and antigens of oncogenic RNA viruses have identified the glycoproteins in

the virion as type-specific antigens and non-glycoproteins with group-specific antigens. Discrepancies exist, however, on the number, size, and location of proteins and antigens in each class, which is probably due to difficulties encountered with extraction and separation procedures. Furthermore, little is known about the detailed structure of the proteins or their arrangement and functional roles. Thus, the purpose of this study was to gain a better understanding of FeLV by analyzing its structural proteins and comparing them with the proteins of other RNA tumor viruses including those of human candidate viruses. More specifically, the objectives were to isolate the structural proteins from the FeLV in homogeneous form; to begin chemical and immunological characterization of these proteins; and to produce monospecific sera against them.

To accomplish these objectives, it was first necessary to find a method which could be used to separate the proteins from the virus in relatively large quantities, in homogeneous form, and with recovery of their original antigenic activity. Originally the procedure most generally used for separation of proteins was polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). This method offered a fairly good resolution but had the disadvantage of causing a decrease in the original antigenic activity of the proteins. More recently an alternative method was shown to

be effective for separation of viral polypeptides: gel filtration in the presence of 6 M guanidine hydrochloride (GuHCl) and a reducing agent. By this method a greater degree of polypeptide separation is obtained especially for the polypeptides of low molecular weights. Furthermore, antigenic activity of the proteins can be recovered by dialysis to remove GuHCl; this will permit correlation of specific polypeptides with the principal antigens of the virion.

Proteins obtained by this method were used for physical, chemical, and immunological analysis. Antiserum was produced against several of the proteins after GuHCl was removed. These antisera can be used for subsequent analysis of viral protein synthesis, location and function(s) of the proteins within the virion, and comparison of proteins from other RNA tumor viruses.

The biochemical study of RNA tumor viruses from animals may lead to a better understanding of the fundamental mechanisms of oncogenesis. This in turn may give insight into methods of prevention, treatment, or control of cancer in man and domestic animals.

LITERATURE REVIEW

Oncogenic RNA Containing Viruses

Until recently there were three groups of oncogenic viruses known to possess RNA as their nucleic acid: the avian leukosis-sarcoma viruses, the murine leukemia-sarcoma viruses, and the mouse mammary tumor viruses. Now a variety of other RNA tumor viruses have been discovered including the leukemia-sarcoma viruses from the monkey (14, 94), cat (70, 94), hamster (42), rat (95), and the snake (29). The RNA tumor viruses of different species share several biological, biochemical, and structural properties although specific differences among groups have been established. These properties have distinguished them from other RNA viruses and therefore they have been called oncornaviruses (oncogenic RNA viruses) (60).

Structural Properties

Ultrastructural studies indicate that the feline oncornaviruses are nearly spherical in shape with a diameter of approximately 90 to 110 mu (60). They have a complex structure consisting of an outer-lipid containing envelope and an electron dense spherical core which consists of the RNA complexed with protein (RNP) surrounded by an outer membrane. The outer envelope has the structure of the

so-called "unit membrane"--two peripheral layers of protein with a central layer of lipid (5). Like other RNA tumor viruses, the FeLV obtains the outer envelope during the process of budding from the host membrane. Projections are seen emanating from the viral surface, but little is known about their role or chemical nature.

A membrane intermediate between the outer envelope and the central electron dense RNP has been seen in the electron microscope prior to or shortly after budding, but it has not been characterized chemically. Sarkar et al. (75) have shown that the intermediate membrane of the virion becomes the outer membrane of the RNP immediately following the budding process which is termed the shell and together they make up the core. This is probably due to the osmotic properties of the medium outside the cell.

The FeLV is classified as a C-type particle with a central core as compared to a B-type particle with an eccentric core when viewed in thin section. It has been postulated that the viral nucleic acid complexed with protein is coiled into a compact helical structure within the core shell (60). Further study is needed for confirmation of the structure and chemical nature of the complex core.

Biochemical and Biophysical Properties

The buoyant density of the FeLV is about 1.15 g/cc as determined in sucrose density gradients (40).

Lipid, protein, and RNA are the major constituents of these viruses. In addition, small amounts of carbohydrates have been found. Lipid is found mainly in the envelope and constitutes about 30-35% of the dry weight of the virus which probably accounts for the low density as compared to other viruses. Although not studied extensively, lipid components have been shown to confer upon the virus the property of being ether and detergent sensitive (5). Protein makes up about 60% of the dry weight of the virus; RNA about 2%; and small amounts of carbohydrate moities have been found associated with the virus (5). In addition, deoxyribonucleic acid (DNA) of small size have been found in the virus (36, 90).

The FeLV contains a major component of single-stranded RNA species with a sedimentation coefficient near 70S and a molecular weight of approximately 1.2×10^7 daltons (41). In addition, 35S and 4S RNA species have been found (40, 41). The 70S sedimenting RNA can be converted into 35S components by heating or by treating with dimethylsulfoxide which may suggest that the major RNA is a relatively specific aggregate of 3 or 4 pieces of 35S RNA held together by hydrogen bonds (90, 41). The 4S RNA species of the virus has not been thoroughly studied, but it is thought to be 4S transfer RNA of the cell (41). The 70S molecule has sufficient nucleotides to code for over 20 proteins assuming nonredundancy of

genes. However, the extent to which the viral genome is expressed in infected cells is yet unknown.

Proteins are found in both the outer envelope and the internal core. Thus far six major and several minor protein species have been separated from purified FeLV. These proteins are important for determining biological and structural properties of the virus which will be discussed in another section.

Biological Properties

The feline oncornaviruses are not only oncogenic in their natural host, but they have also been shown to induce malignancies in dogs, monkeys, and rabbits (40, 95). At present, however, there is no evidence to implicate FeLV as a cause of human malignancies (20, 40, 78). Epidemiological studies have failed to reveal any correlation between natural occurrence of leukemia in man and pets in the same household (20). However, the feline viruses have a wide range of biologic activity and have been shown to have the ability to replicate in human cells (78). Furthermore, once the viruses are passed through human cells, they have a greater ability to grow on human cells than feline cells (20).

Infection of cell cultures with FeLV or other oncornaviruses is not accompanied by any observable changes in the infected cells which continue to divide while the viruses multiply. This is in contrast to most other

animal viruses. Because of this, permanently infected FeLV cell lines have been established (70, 74). In order to detect the presence of viruses in cells, several of the following techniques have been used: electron microscopic visualization of the FeLV particles (79); detection of virus specific antigens by an agar-gel precipitin test (AGP) (35); complement fixation test (CF) (76, 77); direct fluorescent antibody (FA) test for detection of the production of FeLV antigen in cell cultures and its intracellular localization (95); labeling the viral RNA with ^{3}H-uridine and subsequently measuring the release of radioactive RNA which bands at a density of 1.15 g/cc (79); and either enhancement (helper effect) or interference in a focus assay using a modified murine sarcoma virus which grows in feline cells (68).

Vertical infection rather than horizontal infection is the usual mode of transmission for most RNA tumor viruses, either in the form of a virus or oncogene (38). However, there have been several reported observations that suggest classical horizontal transmission does occur (12, 16). In one case, previously healthy cats housed with leukemic cats became infected with leukemia (12, 16). Furthermore, FeLV has been found in salivary glands, lungs, and tonsils suggesting excretion in the saliva which could possibly lead to horizontal transmission (20).

Hardy (34) has recently illustrated horizontal transmission of leukemia in cats using Rickard's strain of virus.

Replication of FeLV

Very little is known about the actual mechanism of replication of RNA oncogenic viruses. Study of the kinetics and mechanism of viral replication was initially confined to avian and murine RNA tumor viruses but has recently been extended to other oncornaviruses including the FeLV with the development of improved tissue culture *in vitro* assay systems. Early studies with the Rous sarcoma virus (RSV) resulted in the observation that virus replication and cell multiplication coexist in infected cells, and replication of the virus requires early deoxyribonucleic acid (DNA) synthesis and is sensitive to actinomycin D (4, 22). It was found that the need for DNA is transient occurring only during the first 8 to 12 hours of infection (4, 90). These observations led to the proposed model that information was being transferred from viral RNA to DNA (a provirus DNA) (90). The DNA is then integrated into the cellular DNA and serves as a template for the production of progeny viral RNA (90). Evidence for the existence of the DNA provirus is still indirect, but there is now direct evidence that a unique enzyme exists within the RNA tumor viruses that can synthesize DNA from an RNA template (6, 91). Furthermore, other enzyme activities have been found in virions such as DNA dependent

DNA polymerase (89, 91); DNA endo-and exonuclease (53); and a polynucleotide ligase (53). These enzymes could act in the possible synthesis and integration of viral specific double-stranded DNA into the host cell genome, and they provide convincing support for the provirus hypothesis.

Although it appears that there is a DNA intermediate in the production of progeny viral RNA, a complete understanding about the molecular events in viral replication remains to be elucidated. Preliminary studies indicate that the site of viral protein formation appears to be in the cytoplasm while virus maturation occurs at the cytoplasmic membrane by a budding process (5). However, the mechanism by which the RNA tumor viruses enter the cells, the exact sites of replication of viral components, the rate of synthesis of these components, and the assembly of these components into complete virions need further study.

Strains of Feline Oncornaviruses

Feline oncornaviruses can be classified into two types depending on the types of neoplasms they produce: feline leukemia viruses (FeLV) which produce reticuloendothelial malignancies and feline sarcoma viruses (FSV) which produce solid tumors. Two strains of FeLV have been described and classified by their ability to cause lymphocytic leukemia in the cat--the Rickard's F-422 strain (70) and the Theilen F-74 strain (94). Until very recently,

little difference had been detected in these two strains with respect to biological and antigenic activity which was probably due to lack of extensive biochemical study. Various strains of feline sarcoma viruses such as Snyder-Theilen (ST-FSV) (88), Gardner (GA-FSV) (26), and Sarma's strain (SM-FSV) (80) have not been systematically classified and are named according to the individuals who first isolated them. Biological and antigenic characteristics appear to be generally very similar for all three strains except the type specific antigens. Sarma et al. (80) have preliminary evidence showing there are differences in type specific antigens as in the case with the murine and avian viruses. Furthermore, they have suggested that various strains of feline sarcoma viruses appear to be antigenic mixtures of FeLV and FSV with the FeLV being in a tenfold higher concentration than the FSV.

Recently, other RNA tumor viruses have been isolated from feline cells that appear to have different biological and antigenic characteristics from those mentioned above. The RD-114 virus isolated from a human tumor (rhabdomyosarcoma) after passage through feline cells (50, 57) was at first thought to be a human candidate RNA tumor virus (51) but recently has been shown to be a feline sarcoma virus (Livingston, personal communication). An RNA tumor virus, from Crandel cat kidney cells (CCC) (44) which were thought to be a normal cat cell line, has

been induced, isolated, and partially characterized (43). It also appears to have some differences in antigenic characteristics from the other feline RNA tumor viruses. It will be necessary to study the proteins of these viruses more thoroughly to determine their antigenic activities and relationships before a complete classification can be made of the viruses.

Antigenic Composition

Antigens of the oncornaviruses can be classified into two general types: (1) the type-specific (ts) antigens and the (2) group-specific (gs) antigens. The ts antigens are probably on the viral surface and constitute part of the envelope as has been shown with the murine and avian RNA tumor viruses. Several species of RNA tumor viruses, including feline oncornaviruses, can be separated into various subgroups on the basis of neutralization with type-specific antibody (80) and relative infectivity for generally susceptible or resistant cells (66). Early studies with RSV and murine leukemia virus (MuLV) by Duesberg (17, 18, 73) indicated that the glycoproteins of these viruses are part of or identical with the ts antigens. The gs antigens which do not react with neutralizing antibodies are considered to be internal components of the RNA tumor viruses (33, 43). They can be released from the virus particle by Tween ether treatment and appear in the water phase as soluble components (40).

Disagreements exist in the literature on the number of gs antigens in various RNA tumor viruses which may reflect differences in preparative and analytical procedures as well as the sera used to detect antigen activity. Schäfer et al. (83) have isolated two gs antigens from the FeLV and suggest that there may be more. Nowinski et al. (59), using a different method of isolation of gs antigens, suggest that there may be four.

Shared Antigens

Group-specific antigens are shared by avian leukemia-sarcoma viruses (8, 11), and different gs antigens are shared by each species of the mammalian leukemia-sarcoma viruses that has been studied extensively with the exception of the murine mammary tumor virus (27, 28, 30) and the bovine RNA tumor virus (21). Within the avian tumor viruses at least four polypeptides, bearing distinct determinants that are group-specific, have been isolated and described (10, 11, 24). These four gs antigens are shared only within the avian leukosis-sarcoma group and not with any of the different mammalian groups. Schäfer et al. (83) isolated and partially characterized two gs antigens from the FeLV which were designated gs-species (gs-spec.) and gs-interspecies (gs-interspec.) antigens (28). The gs-spec. antigen has thus far been found only in different serotypes of feline RNA tumor viruses and serves as an immunological basis for identifying

the feline group. The gs-interspec. antigen was shown to be present in RNA tumor viruses of other mammalian species, namely mouse, hamster, and rat. The gs-interspec. antigen (cross-reactive antigen) was designed as gs-3 by Geering et al. (28), and the major gs-spec. has been designated as gs-1 by Gregoriades et al. (33). The gs-interspec. antigen is of special interest since antiserum directed against it could be useful for detection and investigation of RNA tumor viruses in other species, including man (28).

It has been shown by Oroszlan et al. (64) that the major structural protein of the FeLV contains both the gs-1 as well as the gs-3 antigenic determinants as integral parts of its structure. This is a controversial issue which will be discussed further in the section on structural proteins.

Serological Studies

Various serological tests have been applied to the detection of antigens or antibodies specific for FeLV. These include the following: (1) The agar-gel precipitin test (AGP) (35, 90), a test system on which to base qualitative judgment of the number and kind of reactive antigen-antibody systems within a given mixture, and the only test in which cross-reacting components can be delineated. Using precipitating antisera against Tween 80-ether treated FeLV, three precipitin bands were detected in this study. (2) A complement-fixation test (CF) for the FeLV, devised

by Sarma (76, 77), is called the COCAL test. This test is more sensitive than the AGP test in detecting smaller amounts of antigens or antibodies, but a difficulty such as anticomplementariness is sometimes encountered. (3) Direct or indirect immunofluorescent techniques have been used effectively for detection of in vitro production of FeLV and/or antigens and their intracellular localization (72, 97). Using antisera prepared against Tween 80-ether disrupted FeLV and conjugated with fluroescein isothiocyanate, it was found that the gs antigens are located in the cytoplasm of infected cells (72). These techniques are also useful in studying virus-induced antigens on cell surfaces. (4) In the neutralization test, antibodies are produced against preparations of FeLV and are tested for their neutralizing activity in newborn kittens.

Structural Proteins

Information gained from study of several other types of RNA tumor viruses, especially the avian and the murine, has contributed greatly to the overall knowledge and understanding of the FeLV and its structural proteins. This section of the review will include some of the earlier work on analysis of proteins from these viruses. More specifically, it will concentrate on extraction and analyis of proteins from whole viruses with emphasis on the

internal gs antigens rather than the external envelope (type-specific) proteins.

Initially very little was known about the number, size, location, and function of RNA tumor proteins. Early studies with avian myeloblastosis (AMV) and Rous sarcoma virus (RSV) using SDS-PAGE and staining the protein bands with amido black revealed that four to eight components could be detected (1, 18). However, when RSV (18) and Friend leukemia virus (25) were labeled with radioactive amino acids, the radioactive peptides extracted from purified virus with phenol and SDS and then analyzed by SDS-PAGE, only three major electrophoretic peptide components were detected. Using a slight modification of the phenol-SDS extraction procedure and then analysis in SDS-PAGE, Bolognesi and Bauer (8) were able to detect four major peptide components from AMV after protein specific staining with amido black. Their molecular weights were estimated to be 13,000, 15,000, 23,000 and 28,000 daltons. In addition to these major components, a number of smaller bands of higher molecular weight were detected, the most distinct one having a molecular weight of 115,000 daltons. This component labeled highly with ^{14}C-glucosamine and stained well with the periodic acid Schiff stain suggesting that it was a glycoprotein. Unlike earlier work (18), Bolognesi and Bauer (8) attempted to correlate the electrophoretically separated components with specific antigens

such as the gs or ts antigens (7). He was able to recover most of the protein in an immunologically active form and illustrated their non-identity and immunological distinctness by complefixation and agar gel double diffusion using gs antisera. The largest of the major protein components (28,000 mol. wt.) was shown to be identical to the major gs antigen. A large portion of the ts antigenicity was lost, but some activity remained and was found in the region of the main glycoprotein component.

A later study by Hung et al. (39), using ^{14}C-amino acid labeled RSV which was dissociated with a neutral detergent (Brij 35), mercaptoethanol, and urea and analyzed by isoelectric focusing in urea, revealed seven radioactive peaks with pIs between 3.5 and 9.9. These peaks were further analyzed by SDS-PAGE to determine the number and molecular weights of the individual proteins in each peak. Eight separate proteins with the following molecular weights were identified: p1=96,000; p2=45,000; p3=37,000; p4=25,500; p5=21,000; p6=19,000; p7=16,500; and p8=14,000 daltons. The three largest proteins were glycoproteins and were identified as being surface or ts antigens by using neutralizing antibody. Proteins p4 and p8 reacted in complement fixation with hamster antiserum against gs antigen but showed non-identity in agar-gel double diffusion tests.

A number of investigators have attempted to extract glycoproteins from various RNA tumor viruses by a variety of procedures and to associate them with viral surface envelope antigens (9, 17, 71). Duesberg et al. (17) found that different strains of RSV and MuLV disrupted with tween-20 contained two glycoproteins with fucose and galactose in addition to glucosamine. They also found the major glycoprotein to have a molecular weight of about 100,000 and the minor one a molecular weight of about 37,000 daltons. Because both proteins inhibited virus neutralizing antibody activity, they were considered as part of or identical with the viral type- or sub-group-specific antigen. After treatment of AMV with the detergent NP-40, Bolognesi et al. (9) were able to isolate rosette-like structures which, after being analyzed by SDS-PAGE, revealed the presence of two glycoproteins having molecular weights of 37,000 (GI) and 115,000 (GII) daltons. In addition, two proteins were shown to carry the ts antigenicity by specifically absorbing neutralizing sera. The glycoproteins make up a large part of the structural proteins of the FeLV and are probably associated with the ts antigens.

Early studies made on the gs antigens from the avian RNA tumor viruses have provided techniques for the study and understanding of the gs antigens from the mammalian RNA tumor viruses. Allen (2) isolated two gs

antigens (gs-a and gs-b) from Tween ether treated AMV by Sephadex and carboxymethyl cellulose chromatography and extensively studied them. He was able to show that the two antigens were chemically and immunologically distinct by N- and C-terminal amino acid analysis, amino acid analysis, tryptic peptide fingerprinting, and N-terminal sequence determination.

Initially, most of the work with mammalian RNA tumor viruses involved analysis of gs antigens from tween-ether treated MuLV and comparison with gs antigens from the avian and other RNA tumor viruses. There have been several reports of multiple gs antigen components of MuLV (33, 81, 82), including one that is shared with the FeLV (13) and also with rat and hamster leukemia (28, 63). A number of procedures have been employed by several investigators to purify gs antigens from MuLV. Gregoraides and Olds (33) used isoelectric focusing to purify a gs antigen from the virus and found it to have a pI of 5.6 to 5.8. It also had a molecular weight of about 35,000 daltons and a sedimentation value $S_{20,w}$ of 2.7. In another study, Oroszlan et al. (62) purified the major gs antigens from MuLV by isoelectric focusing and found it to have a pI of about 6.7 and a molecular weight of about 26,000 daltons. The same workers purified the major gs antigen of the hamster tumor virus by isoelectric focusing and found it to have an isoelectric point of 6.9 and a molecular

weight of about 35,000 daltons. Differences between the two reported isoelectric points and molecular weights of the major gs antigen from the MuLV could be due to contamination with RNA (62, 63).

Radioactively labeled structural proteins of MuLV were separated by SDS-PAGE into five major peaks (63). The main protein peak had a molecular weight of 28,000 and was shown to be a gs antigen by reaction with a serum specific for gs-1. The two smaller proteins had molecular weights of 17,000 and 15,000 daltons. The 15,000 molecular weight protein was found to be argenine rich suggesting that it might be a basic protein associated with the RNA. A major and a minor glycoprotein were also found with molecular weights of 80,000 and 42,000 daltons, respectively.

In a similar study using radioactively labeled hamster and mouse viruses, three major polypeptides were separated by SDS-PAGE and ranged in molecular weights from 13,500 to 35,000 daltons (62). The third fastest migrating component from each virus--31,000 molecular weight species from the mouse virus and the 35,000 molecular weight species from the hamster virus--was shown to be the major gs antigen. Glycoproteins were not detected in this study, but there were several slow migrating minor components that could represent envelope proteins.

In still another study, Schäfer et al. (84) found three major protein components from the MuLV having molecular weights of about 14,000, 17,500, and 31,000

daltons; a series of minor proteins having higher molecular weights; and two glycoproteins, a major (molecular weight of about 100,000 daltons) and a minor protein (molecular weight of about 40,000 daltons).

Even though the FeLV have been isolated more recently, their proteins have been studied and compared with proteins from other RNA tumor viruses. Analysis with SDS-PAGE revealed three major components and two minor components, which is a similar pattern to the other mammalian RNA tumor viruses (30, 88). Schäfer et al. (83) designated the three major components as EI, EII, and EIII in order of decreasing electrophoretic mobility and reported their molecular weights as 15,000, 18,000 and 33,000 daltons, respectively. EI was characterized serologically as a gs antigen found only in feline leukemia and feline sarcoma viruses and was termed "gs-spec. antigen." The EIII protein was found to be a gs antigen which occurs in leukemia viruses of other mammalian species as well and was designated "gs-interspec. antigen" (27).

Deinhardt (15) used Sephadex chromatography and isoelectric focusing techniques to isolate the major gs antigen from FeLV. He found that it had an isoelectric point of 4.6 and a sedimentation coefficient of 2.9. Oroszlan et al. (64) also isolated the major gs antigen by isoelectric focusing and found it to have an isoelectric point of 8.3. When analyzed by SDS-PAGE, it migrated as

a homogeneous peak in the molecular region of 25,000 daltons. They also reported that this protein contains both the gs-1 and gs-3 antigenic determinants which were integral parts of its structure. This is in contrast to other reports indicating that the major gs antigen of the FeLV contains only the gs-3 determinant (83).

Due to the difference in opinion concerning the location of the gs-1 (33) and gs-3 (28) antigenic determinants, Parks and Scolnick (65) used a sensitive radioimmune precipitation assay that is ten-thousandfold more sensitive than immunodiffusion to resolve this problem. By using antisera prepared against heterologous gs antigens, they were able to provide further evidence that both gs-1 and gs-3 activities from both murine and feline leukemia viruses were located on the 30,000 molecular weight polypeptide.

Fleissner and coworkers (24) have used the technique of gel filtration in the presence of 6 M guanidine hydrochloride (GuHCl) and a reducing agent (23) for the separation of polypeptides from avian and mammalian RNA tumor viruses. This method has several distinct advantages over SDS-PAGE for separating viral proteins: a better resolution is obtained with proteins in the low molecular weight range, allowing for the identification of a protein not previously detected by SDS-PAGE; and after removal of

GuHCl by dialysis, a good recovery of antigenic activity can be obtained for most proteins.

Application of this method to AMV resolved seven major protein species according to their molecular weights. Two of the proteins (m1 and m2) of molecular weights $\geq$ 100,000 and 70,000 daltons, respectively, were glycoproteins and were shown to be on the surface of the viral membrane. Four proteins (p1=27,000, p2=19,000, p3=15,000 and p4=12,000 daltons) were internal components of the AMV and were considered gs antigens. The fifth protein (p5) had a molecular weight of 10,000 daltons but did not have antigenic activity, and the location of this protein is undetermined. This protein was not reported previously because its mobility was identical to p3 in SDS-PAGE and it lacked antigenic activity. The molecular weights of the proteins determined in GuHCl agree well with SDS-PAGE data with the following exception: m1 ($\geq$ 100,000 daltons) behaves as an aggregate in GuHCl eluting with the void volume and dissociates to a 32,000 molecular weight molecule in SDS-PAGE.

Soon after this study on the FeLV was initiated, Nowinski et al. (58) reported the analysis of the proteins from the mouse, cat, and hamster viruses by gel filtration in GuHCl. They found six major proteins as compared to seven for the avian virus. Proteins from the different mammalian species had similar molecular weights which

closely resembled the molecular weights of the six equivalent avian proteins suggesting a basic similarity in protein composition. The two glycoproteins (m1≥100,000 and m2=70,000 daltons) were shown to be constituents of the viral membrane, and the other four proteins (p1=31,000, p2=15,000, p3=12,000, and p4=10,000 daltons) were identified as internal components of the viruses. Although earlier work suggested the mammalian viruses lacked a 19,000 molecular weight protein found in the avian viruses, this study will illustrate that a minor protein component of that size does exist in the FeLV. In SDS-PAGE analysis, the largest glycoprotein (m1) did not dissociate readily into subunits as was the case for m1 of the avian viruses. Further, the gel filtration technique detected an extra small mammalian protein not previously identified by SDS-PAGE because proteins p3 and p4 migrated together as the fastest migrating component. The authors also reported that antisera prepared against the four internal gs antigens did not inhibit the activity of the reverse transcriptase enzyme. This suggested that the enzyme is not antigenically related to these proteins.

In searching for human RNA tumor viruses, three candidate viruses have been isolated: (1) the C-type virus from the human cell line ESP-1 (67); (2) RD-114 virus from human rhabdomysarcoma cells (51); and (3) the EFMU virus from a human tumor of the urinary tract (19). These

viruses have been analyzed by immunological and biochemical techniques described above to differentiate them from other mammalian C-type viruses. The virus from ESP-1 cell line has been shown to contain murine gs antigen which suggests that it is probably a murine virus (32). The RD-114 virus, which was isolated after being passed through prenatal cats, has been shown to have the mammalian gs-3 antigenic determinant but was completely free of feline gs-1 activity (61). Recently, the major internal gs-1 protein has been purified by isoelectric focusing (pI=9.1) and has a molecular weight of 33,500 daltons in SDS-PAGE (61). This is in contrast to the gs-1 protein of the feline C-type virus which has an isoelectric point of 8.3 and a molecular weight of 25,000 daltons. Antisera prepared against this protein was highly specific and did not react with the gs-1 antigens of other RNA tumor viruses including those isolated from the Woolly monkey and the Gibbon ape (61). This data suggests that RD-114 is a human virus activated by passage through fetal cats. However, very recent data by Livingston (45) has shown that this virus is actually an unique feline C-type virus with some biochemical and immunological properties distinct from the previously identified feline C-type virus. The EFMU virus has been isolated very recently and preliminary studies suggest that it is distinct from other mammalian RNA tumor viruses.

MATERIALS AND METHODS

Source of Virus

The chronically infected feline thymus tumor cell suspension, F-422, which was derived from a neoplastic thymus of a leukemic kitten, was used as the source of virus throughout all the experiments (70). This strain of FeLV, known as the Rickard's strain, was obtained from Dr. C. G. Rickard at Cornell University, Ithaca, New York.

Propagation and Maintenance of the F-422 Continuous Cell Culture

The thymus tumor cells were grown in spinner flasks (Bellco, Vineland, N.J.) in a cultural medium consisting of 40% McCoy's 5A and 60% Leibovitz' L-15 plus 15% heat inactivated fetal calf serum (GIBCO, Grand Island Biological Co., Grand Island, N.Y.) as reported by Rickard (70). Spinner cultures (50-1000 ml volumes) were set up with 1 to 2 x 10^5 cells/ml, incubated at 37°C either in a warm room or a fully humidified atmosphere of 8% CO_2 in air for three to four days until they reached a cell population of about 2 to 3 x 10^6 cells/ml. Part of the culture was then diluted to 1 to 2 x 10^5 cells/ml to repeat the growth cycle and the remaining culture was used for virus purification.

Storage of F-422 Cells

F-422 cells at a concentration of 2 x 10^6 cells per ml were stored in 1 ml ampules in 10% dimethyl sulfoxide (DMSO) and Leibovitz-McCoy's culture medium and placed in a -70°C freezer. At this point, they were transferred to a tank of liquid nitrogen and stored until used. At the time of use the cells were thawed out very rapidly in a 37°C water bath and the cells cultured in either spinner flasks or plastic tissue culture flasks (Falcon Plastics, Los Angeles, Calif.) with culture medium in the usual manner.

Storage of Culture Medium Containing FeLV

The culture medium was centrifuged at 1,000 rev/min for 5 minutes at 4°C in an International centrifuge (PR-6, International Equipment Co., Needham Hts., Mass.) to remove the F-422 cells which could be used for starting new spinner cultures or discarded. The supernatant fluid was then centrifuged at 10,000 rev/min for 10 minutes at 4°C in a Sorvall GSA rotor (RC 2-B, Sorvall, Inc., Newtown, Conn.) to remove cellular debris and then stored at -20°C until used for virus purification.

Preparation of Radioactive Labeled Virus

FeLV were labeled by including radioactive precursors into the medium of virus producing F-422 cells in spinner culture. Viruses labeled with ^{3}H-uridine were

prepared by addition of .5 to 2 uCi/ml of ^{3}H-uridine [specific activity of 26 curies/millimole (mM), New England Nuclear (NEN) Corp.] to a spinner culture of cells (approximately 2 x 10^{6} cells/ml) that were incubated in fresh culture medium one day previous to radioactive labeling at a concentration of approximately 1 x 10^{6} cells/ml. After 24 to 48 hours at 37°C, the culture was harvested and the virus purified as described in another section.

Viruses labeled with ^{14}C-amino acids were prepared by the addition of 0.5 uCi/ml of ^{14}C-amino acid mixture (NEC-445-NEN) to a spinner culture of cells at a concentration of 2 x 10^{6} cells/ml. The cells had been pelleted from normal culture medium and resuspended in a culture medium containing 90% amino acid free Leibovitz-McCoy's medium, 10% normal culture medium, and 10% heat inactivated fetal calf serum. The culture was incubated for 24 to 48 hours at 37°C after which time the cells were removed aseptically from the ^{14}C-amino acid culture medium and maintained in an equal volume of normal culture medium. The ^{14}C-amino acid culture medium was further clarified of cellular debris, and the labeled virus was pelleted as described previously, but this time under aseptic conditions so that the ^{14}C-amino acid culture medium could be reused. After the virus was pelleted, the ^{14}C-amino acid culture medium was added back to the cells that had been maintained in equal volume of normal culture medium and

the cell culture incubated again for 24 to 48 hours at 37°C. If healthy growing F-422 cells were available at this time, they were added to the mixture at a concentration of 1 x 10^6 cells/ml. After this incubation period, the labeled virus was pelleted from the culture medium, resuspended in a small amount of TNE [0.01 M Tris (hydroxymethyl) aminomethane, 0.1 M NaCl, 0.001 M ethylene diaminetetraacetate (EDTA), pH 7.4], mixed with the initial pellet, and purified as described in another section.

Glucosamine labeled virus was prepared by addition of 5 uCi/ml of ^{3}H-D-glucosamine (Spec. act. 3.6 C/mM, NEN) to a spinner culture containing 1 x 10^6 cells/ml in normal culture medium. Labeling was for 48 hours at 37°C after which time the virus was purified as described.

Purification of Virus

Viruses were purified from the cultural medium by a modification of the method of Robinson et al.(74) in which they were sedimented to equilibrium in multiple sucrose gradients (see Table 1). Polyethylene glycol (PEG; Powder Carbowax 6000, Union Carbide, New York, N.Y.; Stock Solution, 50% (w/v) in saline) was used instead of ammonium sulfate to precipitate and concentrate the virus. This permitted a hundredfold concentration instead of a tenfold and made it feasible to purify virus from large volumes of culture medium in one purification procedure. For example, 12 liters of culture medium that had accumulated

TABLE 1.--Purification of FeLV from Suspension Cell Culture.

1. Centrifuge the suspension cultures for 5 minutes at 1000 rev/min to remove the thymus tumor cells.

2. Centrifuge the resulting supernatant for 10 minutes at 10,000 rev/min to remove cellular debris.

3. Precipitate the virus from the culture medium with polyethylene glycol (5% final concentration) for at least 90 minutes (usually overnight) at 4°C.

4. Pellet the resulting precipitate by centrifuging for 30 minutes at 10,000 rev/min.

5. Resuspend the precipitate in a small volume of TNE buffer (approximately 1/100 original volume) containing 5% sucrose and layer this material on a discontinuous gradient of 15-50% sucrose.

6. Centrifuge the gradient for 3 hours at 25,000 rev/min at 4°C in the SW 27 rotor (Beckman) after which time the virus will band on the 50% sucrose cushion.

7. After puncturing the bottom of the gradient tube, collect the viral band from between the 15-50% sucrose layers.

8. Layer the virus on linear gradients of 20-50% (w/v) sucrose (dilute virus with TNE so that it will be less dense than the 20% sucrose) and centrifuge for 4 hours at 25,000 rev/min at 4°C.

9. Repeat the linear sucrose density banding once.

10. Collect the virus from the last gradient by puncturing the bottom of the gradient tube and collect the opaque viral band (density of 1.15-1.17 g/cc).

11. Dialyze the virus against cold TNE at 4°C using at least 5 changes of buffer.

12. Store the purified virus in 5 ml aliquots at -70°C or pellet it by centrifuging for 1 hour at 50,000 rev/min in the SW 50.1 rotor (Beckman) after which time the pellet is stored at -70°C.

over a period of time and stored at -20°C were thawed, concentrated to 120 ml, and used for virus purification.

Viruses labeled with ^{14}C-amino acids, ^{3}H-uridine, or ^{3}H-glucosamine were purified in a somewhat different manner. Instead of precipitating the virus from the culture medium with PEG, it was pelleted in a SW 27 rotor at 25,000 rev/min for 75 minutes at 4°C. The viral pellet was then resuspended in a small amount of TNE buffer, loaded onto a linear gradient of 20-50% (w/v) sucrose, and then centrifuged for 4 hours at 25,000 rev/min at 4°C. The remaining purification procedure was followed as outlined in Table 1.

If small amounts (100-250 ml of culture medium) of radioactive labeled viruses were being purified, they were first pelleted as described above and then loaded onto a 5 ml linear sucrose gradient (20-50% w/v) and centrifuged at 50,000 rev/min for 2 1/2 hours in the SW 50.1 rotor (Beckman Instruments Inc., Fullerton, Calif.). This was repeated twice, after which time the viral band at a density of 1.15-1.17 g/cc was collected, dialyzed in TNE buffer, and stored at -70°C as previously described.

Determination of Virus Density

Tritiated labeled virus was purified as described, but the collection of the virus differed. Instead of collecting only the viral band after puncturing the bottom of the gradient tube, 0.25 ml fractions were collected in

test tubes. The refractive index of each fraction was determined using the precision refractometer (Bausch and Lomb, Inc., Rochester, N.Y.) on a volume of 0.1 ml, and out of the remaining 0.15 ml fractions, 0.1 ml was removed, spotted on filter discs, and counted as described in the section on radioactive assay.

Determination of Protein Content

Protein concentrations were determined according to the procedure of Lowry et al. (46) using crystalline bovine serum albumin as the standard. The viral yield after purification was given as the amount of viral protein/ml of material.

Iodination of Proteins

The technique of Helmkamp et al. (37) using iodine monochloride (ICl) was employed for iodination of the following protein preparations; (1) individual purified proteins from the FeLV obtained by agarose chromatography (described in another section), and (2) marker proteins used for molecular weight determinations, transferrin, ovalbumin, chymotrypsinogen, hemoglobin, and cytochrome c. Four to five milligrams (mg) of each marker protein were dialyzed against borate buffer, pH 8.0, overnight at 4°C, diluted to 1 mg protein/ml with borate buffer, and then placed in an ice bath. Four molar equivalents of ICl (calculated for each protein) were added to 20 uCi of

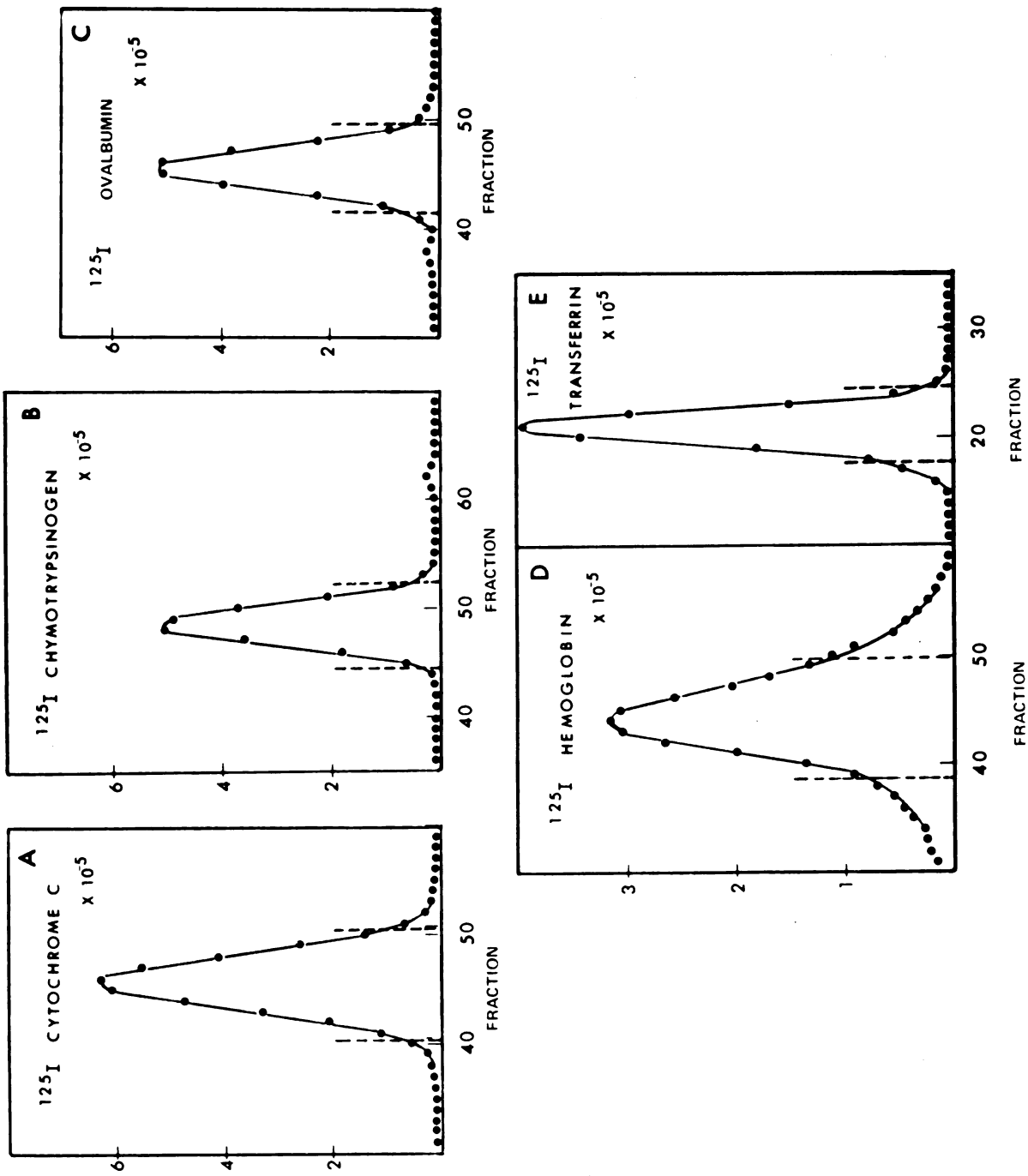
^{125}I (2mCi/.1 ml, NEN) which had been previously adjusted to a pH of 8.0 by adding 1 ml of 2x borate buffer (pH 8.0) or if necessary a few drops of 1 N hydrochloric acid (HCL). The ^{125}I-ICl mixture was immediately jetted into the respective protein solutions. After 5 minutes, the protein solutions were subjected to dialysis against several changes of TNE buffer followed by concentration from about 6 ml to 1 ml by negative pressure dialysis against TNE. The protein solutions were then chromatographed on G-200 Sephadex to remove remaining free iodine and separate single protein molecules from aggregates. TNE buffer was used as the eluent. Two ml fractions were collected and counted directly in a gamma counter. The results can be seen in Figure 1. The peak fractions (as indicated) were pooled, concentrated by negative pressure dialysis in TNE to 2 ml, and stored at -20°C.

Iodination of purified viral proteins from agarose chromatography was essentially the same as described for the marker proteins except smaller amounts of proteins were used because of the difficulty in obtaining large amounts. Also, iodinated viral proteins were not passed through Sephadex columns but were dialyzed extensively with several changes of TNE buffer to remove free iodine.

Radioactive Assay

Counting of all ^{3}H and ^{14}C labeled samples was carried out in a Packard Tricarb liquid scintillation

Figure 1.--Separation of iodinated marker proteins from free ^{125}I and aggregated material by Sephadex chromatography. The proteins were extensively dialyzed in TNE buffer, concentrated by negative pressure dialysis to 1 ml, and chromatographed on a G-200 Sephadex column (100 cm x 1.2 cm) using the same buffer. Two ml fractions were collected and counted directly in a gamma counter. Shown are chromatography profiles of (A) ^{125}I cytochrome c, (B) ^{125}I chymotrypsinogen, (C) ^{125}I ovalbumin, (D) ^{125}I hemoglobin, and (E) ^{125}I transferrin. Dashed lines indicate the peak fractions that were pooled for each iodinated protein and concentrated as described in Materials and Methods.


A
^{125}I CYTOCHROME C
x 10^{-5}
B
^{125}I CHYMOTRYPSINOGEN
x 10^{-5}
C
^{125}I OVALBUMIN
x 10^{-5}
D
^{125}I HEMOGLOBIN
x 10^{-5}
E
^{125}I TRANSFERRIN
x 10^{-5}
FRACTION

counter (Packard Instrument Co., Downers Grove, Ill.) for a sufficient time to obtain a minimum accuracy of 2%. Aliquots of 0.1 or 0.01 ml from fractions obtained from agarose columns, sucrose gradients, or other labeled preparations of proteins were spotted on 2.3 cm diameter Whatman 3MM filter discs and dried thoroughly at 60°C. The discs were then fixed with cold 5% trichloracetic acid (TCA) at 4°C for 20 minutes, washed with cold 5% TCA for 1 minute, and followed by extraction with acetone for 1 minute and dried at 60°C. The dried filter discs were placed in vials containing 5 ml of phosphor scintillation fluid and counted.

Phosphor Scintillation Fluid

PPO	22.7 gms
POPOP	1.9 gms
Toluene	8 pints

PPO - 2,5-Diphenyloxazole
POPOP - 1,4 bis-[2-(4-Methyl-5-Phenyloxazolyl)]-Benzene

When polyacrylamide gels were assayed for radioactivity, the vials with minced sections of gels (see another section) containing either ^{14}C or a mixture of ^{14}C and ^{125}I were mixed with 5 ml of Aquasol (NEF-934, NEN) and counted either in a liquid scintillation counter or in both a liquid scintillation counter and gamma counter. The recovery of counts using Aquasol was about 60%.

Counting of all ^{125}I labeled samples was carried out in a Packard autogamma spectrometer (Packard Instrument Co., Downers Grove, Ill.). Fractions from Sephadex and agarose chromatography columns (1 to 3 ml volume), isoelectric focusing columns (0.2 to 2 ml), and some sucrose gradients were collected in disposable test tubes and put directly into carrier tubes for counting. When sucrose gradient fractions were collected on filter discs (as in the case with determining sedimentation coefficients where there was a mixed label of ^{14}C and ^{125}I), the discs were dried, placed in scintillation vials, and counted. After samples had counted in the gamma counter for a sufficient time (± 2% error), 5 ml of phosphor scintillation fluid was added to the vials, and they were placed in a Tri-Carb scintillation counter and counted as described above. Ninety to 95% of the ^{125}I radioactivity was also recorded in the liquid scintillation counter at the setting used to count ^{14}C labeled protein. Therefore, it was necessary to count a ^{125}I standard during each experiment to make the necessary correction to obtain an accurate count of ^{14}C radioactivity. The amount of ^{14}C radioactivity recorded in the gamma counter was negligible.

Disruption of FeLV

A suspension of FeLV was treated with 0.2% Tween 80 and 4 to 5 volumes of anaesthetic grade ether. The suspension was shaken vigorously at room temperature for 5 minutes

after which time it was centrifuged at 1500 rev/min for 5 minutes to separate the aqueous and ether phases. Nitrogen gas was used to bubble off the ether phase. The above steps were repeated 3 times. After the final extraction, the aqueous phase was clarified by centrifuging at 50,000 rev/min in a SW 50.1 rotor at 4°C for 1 hour to completely remove undisrupted virus. After a protein determination was carried out, the material was stored at -20°C for use in immunizing rabbits, in immunodiffusion assays, or in isoelectric focusing experiments.

Preparation of Antisera

New Zealand whites or Dutch belted rabbits were inoculated in each of their four foot pads with Tween 80-ether treated FeLV preparation (2 ml total containing 1 mg of protein) and purified proteins (2 ml total containing 50 µg of protein p3, p4 , or p5 or 200 µg of protein p1 or p2) emulsified in complete Freund's adjuvant. The emulsion was prepared by the dropwise addition of the protein sample to the adjuvant with constant mixing by aspiration with a 2 ml syringe and a 20 gauge needle. Subsequent injections of equal amounts of protein emulsified with incomplete Freund's adjuvant were given subcutaneously at four sites 20 days after the initial immunization. The rabbits were ear bled 10 days after the second injection, and the sera checked by AGP test for precipitating antibody. Rabbits exhibiting precipitating antibody were bled by cardiac

puncture at 48 hour intervals obtaining approximately 30 ml of sera at each bleeding. Rabbits that did not exhibit precipitating antibody after the second injection were given subsequent injections every 10 days with the respective protein emulsified with incomplete Freund's adjuvant as described. An antibody response was not elicited against proteins p4 and p5 even after 3 and 4 subsequent injections, respectively.

Agar Gel Precipitation (AGP) Test

Agar gel diffusion (Ouchterlony) tests were preformed on slides in 2% agar (Special Noble agar, Difco Laboratories, Detroit, Mich., purified by washing repeatedly with distilled water, once with 70% ethanol, once with acetone, and dried) in phosphate buffered saline (PBS, 0.01 M phosphate, 0.85% NaCl, pH 7.2) and merthiolate (1/10,000) as a preservative for the following purposes: detection and quantitation of antibodies in rabbit sera; detection of antigens in isoelectric focusing fractions; and for the illustration of identity and nonidentity of antigens. The plates were stored at room temperature in a moist chamber and were evaluated after optimal precipitation had occurred (usually by 24 hours). Photographs of the precipitation reactions were taken with a polaroid camera (Polaroid Corp., Cambridge, Mass.).

Polyacrylamide Disc Gel Electrophoresis

FeLV labeled with ^{14}C-amino acids and separated viral proteins radioactively labeled with either ^{14}C or ^{125}I were analyzed by SDS-PAGE similar to the method of Maizel (47). Ten per cent resolving gels were prepared by mixing 3.33 ml of acrylamide-bis-acrylamide (30:0.8) [30 gm of acrylamide (electrophoresis grade, BIO-RAD Co., Richmond, Calif.) and 0.8 gm of bis-acrylamide(N-N'-bis methylene acrylamide; Eastman Kodak Co., Rochester, N.Y.) dissolved and diluted to 100 ml in water], 1.25 ml of gel buffer [48 ml of N-hydrochloric acid and 36.3 gm of Tris (Tris (hydroxy-methyl)-aminomethane) diluted to 100 ml in water, pH 8.9], 0.1 ml of 10% SDS [10 gm SDS (recrystalized from ethanol; Matheson, Coleman, and Bell, East Rutherford, N.Y.) dissolved and diluted in 100 ml water], 0.005 ml of TEMED (N, N, N', N',-Tetramethylethylene diamine; Matheson, Coleman, and Bell, Rutherford, N.Y.) and 5.75 ml of glass distilled water. The gels were polymerized by using 0.05 ml of 10% ammonium persulfate [1.0 gm ammonium persulfate (Phillipsburg, N.J.) dissolved in 9 ml of distilled water], in glass tubes 15 cm long with 0.6 cm inner diameters. After the 10% gel was poured into the gel tube to make a 10 cm long resolving gel, distilled water was carefully layered on and polymerization continued to completion in 30 minutes at 25°C. A 3% spacer gel was prepared by mixing 1.0 ml of acrylamide-bis-acrylamide

(30:0.8), 1.25 ml of spacer gel buffer (25.6 ml 1 M phosphoric acid and 5.7 gm Tris diluted to 100 ml with distilled water, pH 6.7), 0.1 ml of 10% SDS, 0.005 ml of TEMED, 7.54 ml of H_2O and 0.1 ml of 10% ammonium persulfate. A 1 cm long spacer gel was poured on top of the polymerized resolving gel and allowed to polymerize.

After the samples were dialyzed against spacer gel buffer (diluted 1:8) overnight, they were dissolved and dissociated by adding 1% SDS, spacer gel buffer diluted 1:8, 10% sucrose, and 0.1% 2-mercaptoethanol. A 0.2% phenol red was routinely used as a marker since it migrated more rapidly than any of the protein components. They were then heated at 100°C for 1 minute and then loaded on top of the gels. About 2×10^4 cpm of radioactive virus and at least 5×10^3 cpm of radioactive separated, individual proteins were used when the gels were to be sliced and counted. When the gels were to be stained, samples containing between 25 and 100 ug of protein (determined by Lowry method) were applied to the gels. In both cases, volumes ranging from 25 to 100 ul were used.

Electrophoresis was carried out at constant voltage of 100 volts at room temperature in fresh electrophoresis buffer (containing 0.1% SDS, 0.025 M Tris, and 0.2 M glycine, pH 9.0) until the dye marker reached the bottom of the gel tube (usually 5 hours). Electrophoresis was performed with a Polyanalyst (Buchler Instruments, Fort

Lee, N.J.) and a constant voltage, constant amperage power supply (Instrumentation Specialties Co., Lincoln, Neb.).

Gel Staining and Fractionation

Gels to be stained were recovered from the glass tubes by forcing a 0.1% SDS solution between the gel and wall of the tube. Fixing and staining with 0.25% Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, Mo.) in 50% methanol and 7% acetic acid, was carried out for one hour. The gels were then electrophoretically destained for about 15 minutes in 7% acetic acid containing 5% methanol. Lastly, the gels were photographed or scanned with a Gilford Spectrophotometer at 570 nm and then stored in 7% acetic acid.

If the gels were to be assayed for radioactivity, they were fractionated on a Gilson gel fractionator (Gilson Medical Electronics Inc., Middleton, Wis.) into 1 mm to 2 mm fractions which were minced and flushed directly into scintillation vials. Five ml of Aquasol were added to each vial, shaken for 10 minutes, and then counted as previously described.

Isoelectric Focusing

Isoelectric focusing was carried out in a 110 ml capacity glass column (model 1801, LKB Instruments Inc., Rockville, Md.) as described in the manufacturer's instructions with the cathode on the bottom except 5%

sucrose was used in the light solution. In a typical experiment, purified ^{14}C-amino acid labeled and unlabeled FeLV preparations were mixed (total counts approximately 1 x 10^5 cpm, total protein approximately 2 mg), treated with Tween 80-ether (as described in another section), and were digested with solid RNase at 0.2 mg/ml (Enzite-RNase, insolubilized enzyme, Miles Laboratory, Inc., Elkhart, Ind.) (64) and 20 µg/ml of bovine pancreatic DNase (Worthington, Freehold, N.J.) at 37°C for 20 minutes. Aggregates of subviral components, undisrupted virus (if any), and RNase were removed by centrifugation at 50,000 rev/min at 4°C in the SW 50.1 rotor for one hour before isoelectric focusing. The viral protein preparation was mixed with enough light solution (5% sucrose) containing 0.5 ml of carrier ampholine (ampholine carrier ampholytes, pH range 3-10, LKB Instruments, Inc., Rockville, Md.) to make a total volume of 55 ml. Dense sucrose solution (40%) containing 2 ml of carrier ampholine was adjusted to a total volume of 55 ml. Twenty ml of the dense cathode solution (60% sucrose containing 1.5% ethylenediamine) was placed in the bottom of the column. After a linear gradient was made from the dense and light solutions, (LKB 1821 gradient mixer-110 ml) the light anode solution (10 ml of 1% phosphoric acid) was layered on top of the gradient. Constant voltage at 5°C (temperature maintained constant with Lauda K-2/R, Brinkman Instrument Inc.,

Westbury, N.J.) was applied at a level not to exceed 1.5 watts. The voltage was increased at 100 volt increments as the amperage allowed, to a maximum of 700 V and held until the amperage remained constant for at least 12 hours.

Following the focusing procedure (usually 48 to 60 hours), 1.5 to 2.0 ml fractions (60 to 80) were collected from the bottom of the column and assayed for pH (Radiometer, London Co., Westlake, Ohio), radioactivity (by spotting 0.2 ml samples on filter discs, processed, and counted as described), and immunological activity (spotted sample aliquots, 20 µl, in immunodiffusion plates and reacted with antisera prepared against Tween 80-ether treated virus or purified proteins as described in another section). Fractions with positive immunodiffusion reaction and fractions making up radioactive peaks were stored at -20°C and used in other experiments.

Isoelectric points were determined for the individual proteins (total counts approximately 5×10^3; total protein approximately 200 µg) with the method described above using 2% carrier ampholyte solution in a narrower pH range of 3 to 6, 5 to 8, or 7 to 10. Experiments were conducted using narrow pH ranges for 60 to 72 hours with a final potential of 600 V. Fractions were collected and assayed as described above except antiserum prepared against the individual protein was used instead of antiserum prepared against Tween 80-ether disrupted virus.

Because of the small amounts of isolated viral proteins p3 and p4, a small scale isoelectric focusing technique (48) was used for determining their isoelectric points. The focusing column consisted of a glass tube 14.5 cm long, 0.8 cm in diameter, which had an enlargement 1 cm from the bottom of the tube. This enlargement prevented the acrylamide plug from being forced out of the tube during the isoelectric focusing procedure. A 5% acrylamide plug [0.84 ml of acrylamide-bis-acrylamide (30:0.8), 0.5 ml Tris buffer pH 8.8, 0.0025 ml TEMED, 3.6 ml water, and 0.05 ml of 10% ammonium persulfate to polymerize the gel] 2 cm long was poured, polymerized in the bottom of the tube and soaked in 3% sulfuric acid overnight. After a linear gradient was made from the dense solution [0.15 ml carrier ampholine, (pH range 3-6, 5-8, or 7-10) 1.0 gm sucrose, 1.85 ml water, and 0.1 ml protein (25 to 100 ug of protein, with 2×10^3 to 5×10^3 cpm)] and the light solution (0.05 ml carrier ampholine, 0.3 gm sucrose, 2.57 ml water, and 0.1 ml protein), the cathode solution (3% ethylene diamine) was layered on top of the gradient. The tube was inserted into the gel electrophoresis apparatus (Polyanalyst) having as the anode, 3% sulfuric acid and the cathode 3% ethylene diamine. A constant voltage of 200 volts was applied at 4°C for 9-12 hours. Iodinated hemoglobin, which has an electric point of 7.02, was focused separately

with each isoelectric focusing experiment as a control. Following the focusing procedure, 0.2 ml fractions were collected and assayed for pH, radioactivity, and immunological activity as described.

Preparation of Agarose Columns

Two types of agarose columns (6% and 8%) were prepared and operated in a similar manner as that described by Fish et al. (23) throughout this study. The 6% columns were used for separating viral proteins from dissociated FeLV where as the 8% columns were used for rechromatographing proteins with molecular weights between 10 to 50,000 daltons. To a thick slurry of Bio-Gel A-5M or A-1.5M (6 or 8% respectively, 200-400 mesh, Biorad Inc., Richmond, Calif.) was added sufficient solid guanidine hydrochloride (GuHCl, Heico Inc., ultra-pure, Delaware Water Gap, Pa.) to make the final concentration 6 M. Sodium phosphate buffer was included to make the final preparation 0.02 M and enough concentrated NaOH to make the final pH 6.5. The gel preparation was mixed well and allowed to stand overnight at room temperature. Prior to pouring the columns, the gel preparation was degassed for at least two hours. The glass columns (1.2 cm x 100 cm, Chromaflex, Kontes Glass Co., Vineland, N.J.) were filled about one-third full with eluting buffer (6 M GuHCl containing a 0.02 M sodium phosphate, pH 6.5) and the rest of the column including a 50 cm or 100 cm extender

(1.2 cm x 50 cm, Chromaflex, Kontes) was filled with a gel mixture. A 250 ml Marriot flask was attached to the column by tygon tubing. After a small amount of gel had settled, the effluent tube was opened, and the column packed under a pressure differential of 15 to 25 cm which was achieved by raising or lowering the Marriot flask. As the column packed, the clear buffer zone at the top was pipetted off and more gel was added until sufficient gel had been added to give a packed column of 85 to 95 cm long.

A newly poured agarose column was checked for proper packing by chromatographing a solution of 0.2% blue dextran (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) in eluting buffer with 10% sucrose. If the gel bed was properly packed (blue zone of dextran moves as a tight band down the column), the column was pre-equilibrated with eluting buffer containing 0.01 M dithiothreitol DTT prior to application of protein sample.

Preparation of Samples for Agarose Chromatography

To a viral pellet (containing 10 to 20 mg of viral protein) was added sufficient ^{14}C-amino acid labeled virus (usually 1 x 10^5 cpm) which was used to indicate the positions of the viral protein peaks in the effluent volume. If the unlabeled virus preparation was in suspension, the labeled virus was added and the virus mixture pelleted at 50,000 rev/min for 1 hour in the SW 50.1 rotor (Beckman).

The virus pellet was solubilized in 0.5 ml of 8 M GuHCl containing 2% mercaptoethanol or 0.05 M DTT, 0.01 M EDTA, and 0.05 M Tris-hydrochloride (pH 8.5) at 56°C for 45 minutes. At this time, 0.1 ml of eluting buffer containing 0.6% blue dextran and 50% sucrose was added to the viral protein solution and the protein solution loaded onto the 6% agarose column and chromatographed.

Sample Application on Agarose Columns

Prior to loading the protein sample onto the column, the top of the column (top 2 inches) was carefully stirred in a concentric fashion and allowed to repack and re-equilibrate with eluting buffer for a minimum of 4 hours. At this time, the column flow was momentarily stopped, the protein sample (0.5 to 0.8 ml) carefully layered under the solvent onto the top of the gel, and the column started again. The columns were run at room temperature at a flow rate of approximately 1 ml/hour with 1 ml fractions being collected.

Processing of Separated Viral Proteins

After the viral protein solution had been chromatographed and the effluent fractions assayed for radioactivity (as described in another section), the fractions from individual peaks were pooled and concentrated by negative pressure dialysis against 8 M GuHCl solution containing 0.01 M DTT to a volume of 0.5 to 1 ml.

Solid sucrose was then added to 10% and the material was rechromatographed on either a 6% or 8% agarose column similar to the procedure described.

After the proteins were rechromatographed two or three times, fractions from individual protein peaks were pooled, fresh DTT added (.77 mg/ml), and dialyzed against a low ionic strength buffer (2mM Tris-HCL pH 7.4) to remove GuHCl. Dialysis was performed at 4°C with large volumes of at least five changes of dialysis buffer. The protein samples were then stored in 2 ml aliquots at -70°C until further used for chemical and immunological analysis.

Estimation of Molecular Weights of Viral Proteins

Estimation of molecular weights of the viral proteins was carried out by electrophoresis in 7.5% and 10% SDS-polyacrylamide gels according to the method of Shapiro (87), and in GuHCl by the method of Fish et al. (23) using the following iodinated protein standards: cytochrome c (MW=12,400), hemoglobin (MW=16,600), chymotrypsinogen (MW=25,700), ovalbumin (MW=43,000), and transferrin (MW=76,600). These markers were obtained from Sigma (Sigma Chemical Co., St. Louis, Mo.) and iodinated as described previously. Iodinated marker proteins were mixed with the ^{14}C-amino acid labeled viral proteins and coelectrophoresed or cochromatographed. Standard curves were constructed by plotting the distance each marker

protein migrated in SDS gels or the elution position from GuHCl columns versus the logarithm of the respective molecular weights. The molecular weights of the viral proteins were determined by extrapolating from the standard curves.

Sucrose Gradient Analysis of Isolated Proteins

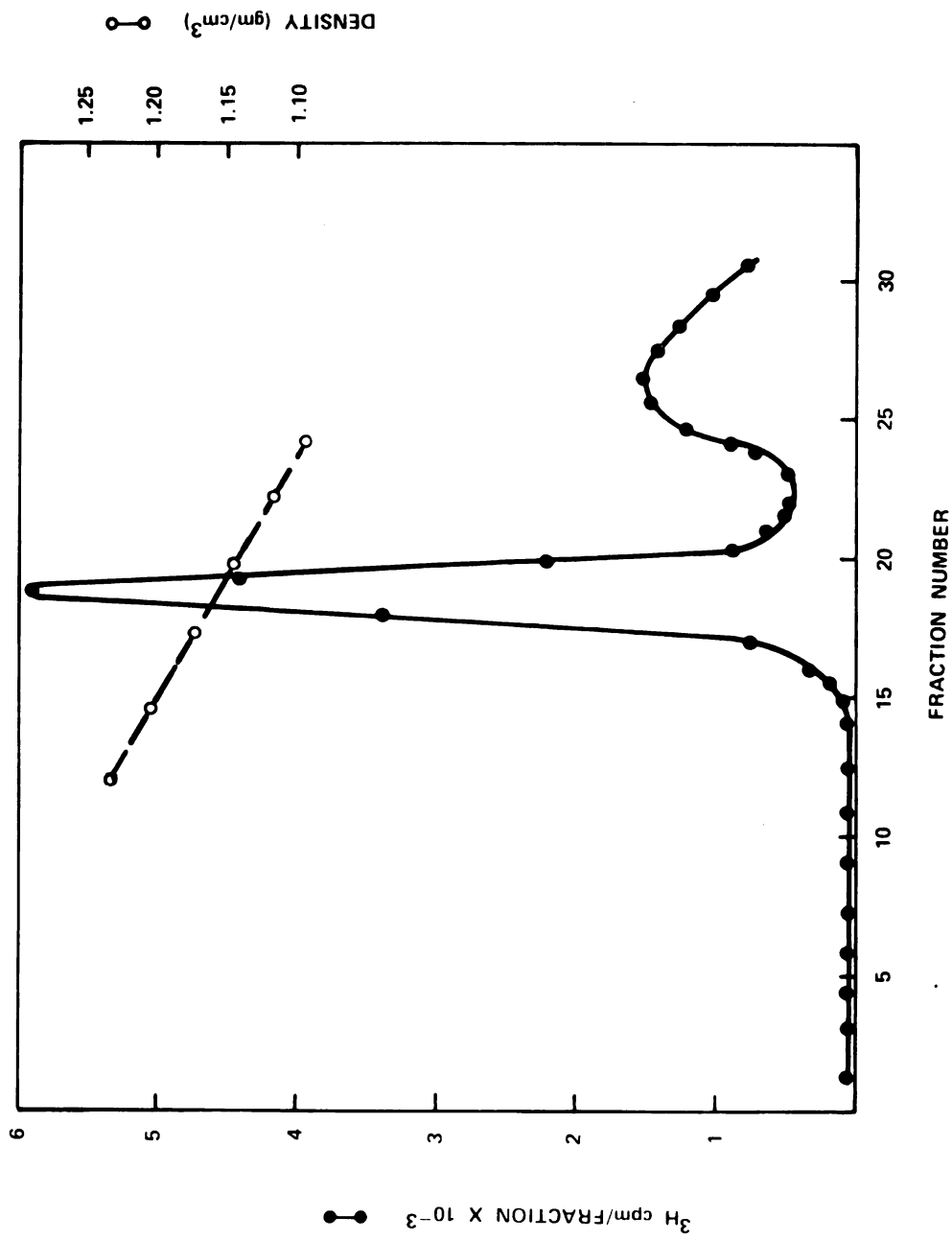
Isolated viral proteins were analyzed on 4.8 ml linear gradients of 5-20% (w/v) sucrose in TNE buffer at pH 7.4 containing 1 M GuHCl. Isolated proteins (3,000 to 9,000 cpm of ^{14}C) for analysis were prepared in final volumes of 0.5 ml containing iodinated bovine serum albumin (BSA) or iodinated chymotrypsinogen as markers (10,000 cpm per gradient). The gradients were centrifuged for 24 to 40 hours at 50,000 rev/min in the SW 50.1 rotor at 4°C. About 35 fractions (0.14 ml) were collected through the bottom of the tube onto filter discs and the discs processed and counted for ^{125}I and ^{14}C radioactivity as described. Approximate sedimentation coefficients were calculated by the method of Martin and Ames (50), assuming a value of 4.4S for BSA, and 2.54S for chymotrypsinogen (50).

RESULTS

Virus Purification and Yield

The chronically infected feline thymus tumor suspension cell culture (F-422) was chosen because the cells produce relatively large amounts of viruses and sufficient amounts of viral protein could be obtained economically. Under optimal conditions of growth, the F-422 cultures produce approximately 10 to 15 virions per hour per cell (Graves, unpublished results). The virus yield was determined by collecting the opaque band from the third sucrose gradient (as described in Materials and Methods), and a protein determination conducted using the method of Lowry (46). Approximately 3 to 5 mg of viral protein were obtained from each liter of culture medium. Consequently, a typical purification run starting with 12 liters of media produced 36 to 60 mg of viral protein. In order to demonstrate that C-type particles were actually being purified, small amounts of tritiated uridine labeled virus were analyzed on 5 ml sucrose gradients. When gradients were assayed for radioactivity and buoyant density, a sharp zone of acid-precipitable material was seen at a density of 1.15 g/cc (Figure 2). Electron microscopy of this material revealed a high concentration of C-type viral particles as has been found by other workers.

Figure 2.--Equilibrium density gradient analysis of FeLV. A suspension of ^{3}H-uridine labeled virus was layered onto a linear gradient of 20-50% (w/v) sucrose and centrifuged at 50,000 rev/min for 2 1/2 hours in an SW 50.1 rotor at 4°C. Sedimentation is from right to left. Density in g/cc and trichloracetic acid-insoluble radioactivity were determined for the fractions from the gradient as described in Materials and Methods.


DENSITY (gm/cm³)
1.25
1.20
1.15
1.10
6
5
4
3
2
1
³H cpm/FRACTION X 10⁻³
5
10
15
20
25
30
FRACTION NUMBER

Radioactive Labeling of Virus

A number of preliminary experiments were conducted to determine the best method for obtaining relatively large amounts of labeled virus with high specific activity. Virus with high specific activities of labeled protein was needed as a marker to simplify the monitoring of fractions from GuHCl columns and avoid the more complicated determination of optical density of each fraction. Individual labeled amino acids or standard labeled mixtures of amino acids were used to label the viral proteins. It was found that the standard mixture of ^{14}C-amino acids in amino acid free media was best for obtaining the largest amount of labeled virus. Up to 1 x 10^6 cpm were obtained using 0.5 uCi/ml of ^{14}C-amino acid mixture in 250 ml spinners with a cell concentration of 2 x 10^6/ml. Using the same cell concentration in 250 ml of normal culture medium with 5 uCi/ml of ^{3}H-D-glucosamine and 2 uCi/ml of ^{3}H-uridine, activities of 1 to 2 x 10^5 cpm and 5 x 10^5 cpm were obtained, respectively, for use in other experiments. Iodination of the viral proteins after disruption of the virus was attempted using the method of Helmkamp (37). High actitivies of up to 3 x 10^6 cpm/mg protein were obtained indicating these iodinated viral proteins would be useful in locating the protein peaks after gel chromatography. However, it was found that after storage for 2 to 3 weeks, the iodinated proteins gave variable results

upon analysis in gel columns. This could have been due to the breakdown of the proteins caused by the radioactive iodine.

Separation of Viral Proteins by Gel Filtration in Guanidine Hydrochloride

Purified unlabeled FeLV containing 10 mg protein was mixed with FeLV labeled with a mixture of ^{14}C-amino acid labeled virus, disrupted in 8 M GuHCl and 2% mercaptoethanol or 0.05 M DTT, and subjected to chromatography on 6% agarose gel in the presence of 6 M GuHCl and 0.01 M DTT. The elution profile of the ^{14}C-amino acid labeled proteins in Figure 3 indicates that six major proteins were resolved according to their molecular weights. In experiments where large amounts of viral protein (20-40 mg) were loaded onto the column, the separation of the viral peaks was not as good, whereas small amounts of viral protein (2-5 mg) resulted in separation of protein peaks that was as good or slightly better than when using 10 mg.

Gel filtration analysis of virus labeled with a mixture of ^{14}C-amino acids and ^{3}H-glucosamine revealed that the first two proteins eluting from the column contain carbohydrate and were considered glycoproteins, whereas the remaining four smaller proteins appeared to be free of significant carbohydrate as determined under the conditions of the experiment and were considered as non-glycoproteins (Figure 4). The two largest proteins

Figure 3.--Gel filtration analysis of FeLV proteins labeled with ^{14}C-amino acids. Purified FeLV (1×10^5 - 2×10^5 cpm; total protein 10 mg) were dissociated in 8 M GuHCl, loaded onto a 6% agarose column and eluted with 6 M GuHCl in the presence of 0.01 M DTT at a rate of 1 ml/hour. Eluted fractions (1 ml amounts) were assayed for radioactivity, and the peak fractions from each protein component were pooled and concentrated by negative pressure dialysis as described in Materials and Methods.

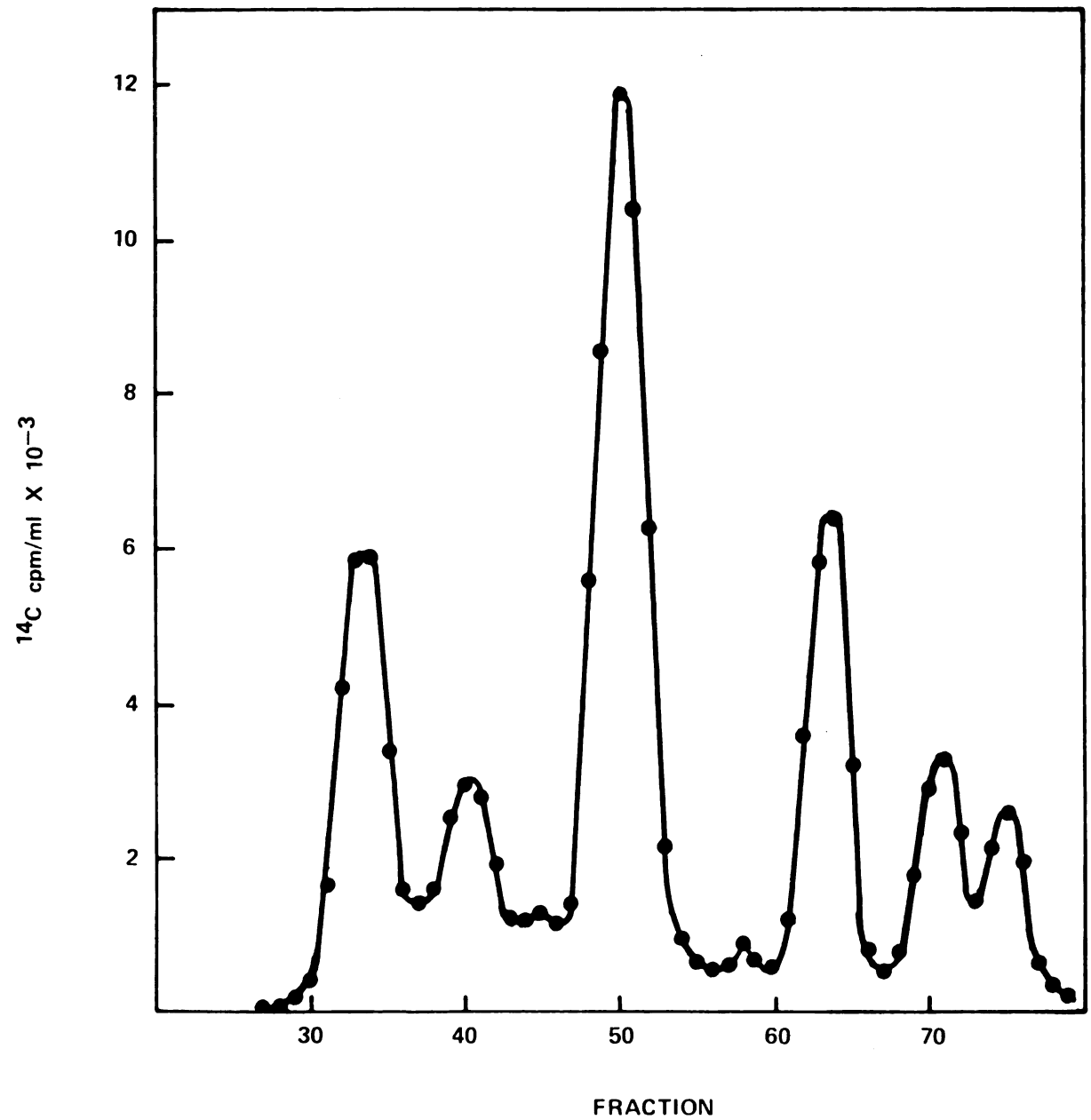
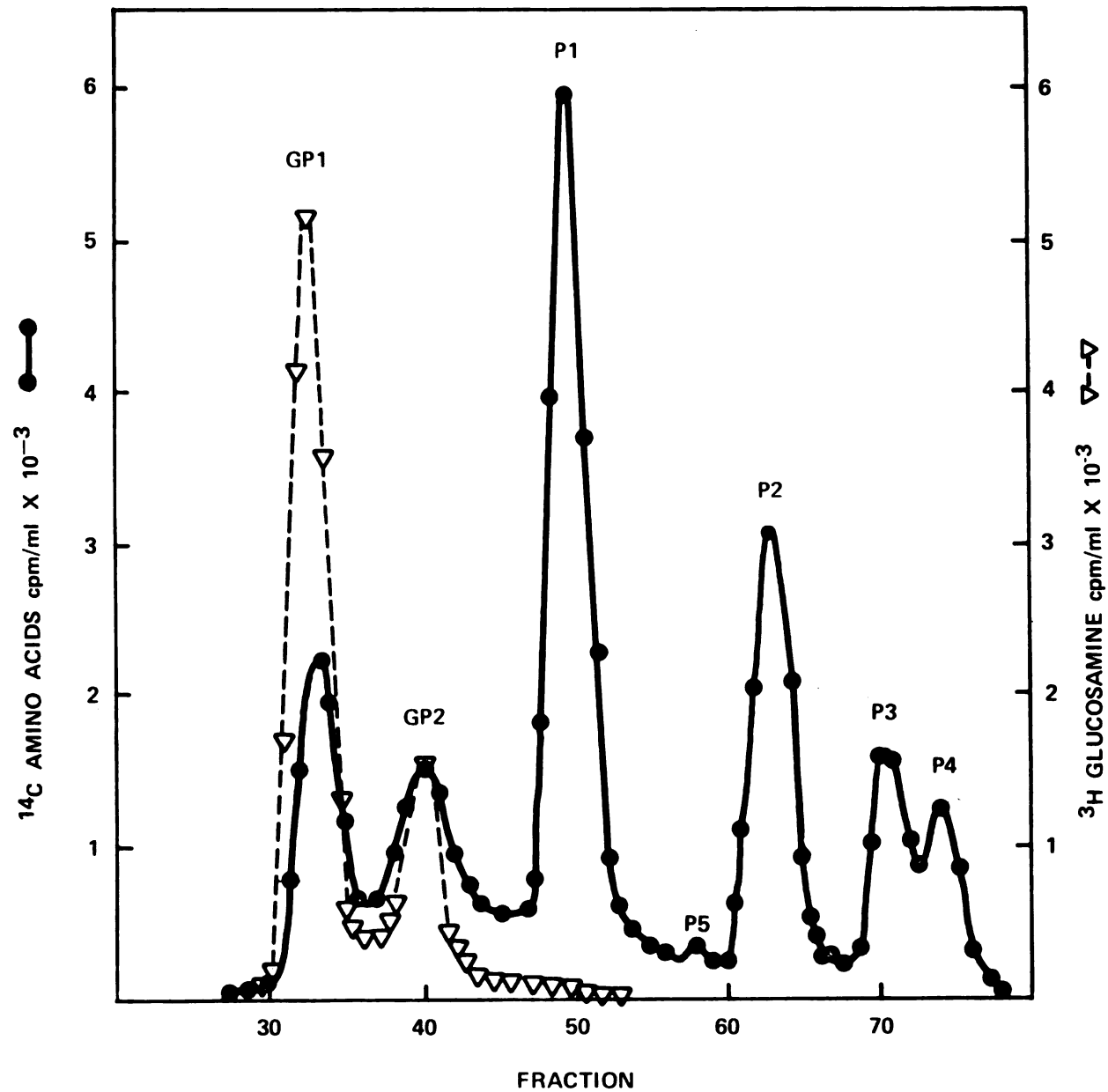

12
10
8
6
4
2
^{14}C cpm/ml X 10^{-3}
30
40
50
60
70
FRACTION

Figure 4.--Gel filtration analysis of FeLV proteins from a mixture of virus labeled ^{14}C-amino acids and ^{3}H-glucosamine. The two labeled virus preparations (1×10^5 cpm of ^{3}H-glucosamine and 1×10^5 cpm of ^{14}C-amino acids; total protein 10 mg) were mixed and analyzed as in Figure 3.


P1
GP1
P2
GP2
P3
P4
P5
6
5
4
3
2
1
30
40
50
60
70
FRACTION
^{14}C AMINO ACIDS cpm/ml X 10^{-3}
^{3}H GLUCOSAMINE cpm/ml X 10^{-3}

were designated as gp1 and gp2 and the remaining proteins as p1 to p4 in the order of decreasing molecular weight. Initially only six major proteins were analyzed, but upon repeated analysis of FeLV on 6% agarose, a minor component eluting between p1 and p2 was consistently observed. This minor component was of interest because it may be equivalent to a seventh major protein component in the avian system which is reported to be missing in mammalian RNA tumor viruses. This minor component, designated as p5, was iodinated to facilitate further analysis.

Since proteins p3 and p4 were usually poorly separated on 6% agarose (Figures 3 and 4), preliminary experiments were run to separate them more effectively. It was thought that if one of the two proteins was considerably more basic than the other, it would be feasible to try to separate them on the basis of their isoelectric points. Earlier work by Fleissner (24) demonstrated that some of smaller proteins of the avian RNA tumor viruses were more basic than others. With this in mind, virus labeled with ^{3}H-arginine was purified and chromatographed on 6% agarose and found p4 to contain more arginine than p3, suggesting the former was more basic. Despite this difference, another technique was found more feasible, and this was not further exploited.

Further separation of proteins p3 and p4 was accomplished by using 8% agarose in the presence of

6 M GuHCl. Figure 5A illustrates the improved separation on 8% as compared to 6% agarose (Figure 3). In this particular experiment, peak fractions of p3 and p4 were pooled after chromatography on 6% agarose and then chromatographed on an 8% agarose column (Figure 5A). Further purification of p3 and p4 on the same 8% agarose resulted in each protein forming symmetrical peaks well separated from one another (Figure 5B and C). When p3 and p4 were well separated on 6% agarose; peak fractions of each protein were pooled separately, concentrated, and rechromatographed on 8% agarose twice as described above.

In preliminary experiments, p1 and p2 obtained from an initial 6% agarose column were analyzed by SDS-PAGE and immunodiffusion and found to be contaminated with adjacent proteins. It was therefore necessary to rechromatograph them also for further separation. P1 was first rechromatographed on 6% agarose (Figure 6A) and then on 8% agarose (Figure 6B) whereas p2 was rechromatographed twice on 8% agarose (Figure 6C and D). After being rechromatographed twice, they appeared as homogeneous peaks (Figure 6B and 6D) free of their nearest neighbors, an observation further supported by analysis with SDS-PAGE and immunodiffusion. Occasionally p1 and p2 were rechromatographed a third time for further purification as a precaution.

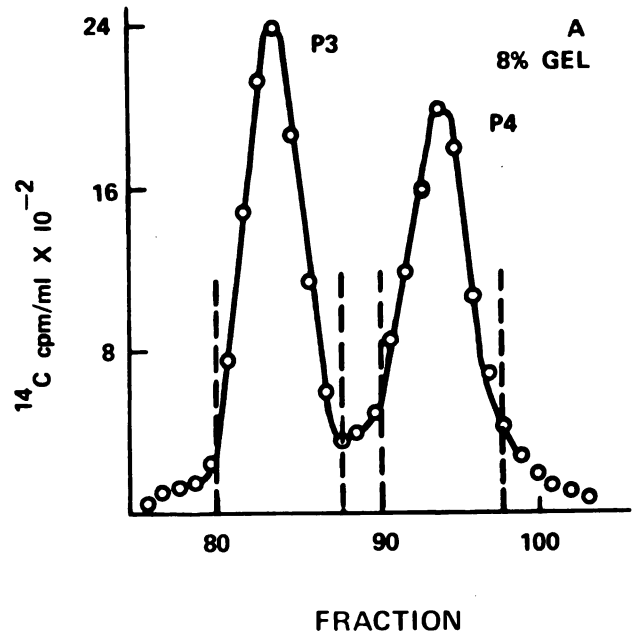
P5 was available in very limited amounts and had to be iodinated before it could be rechromatographed.

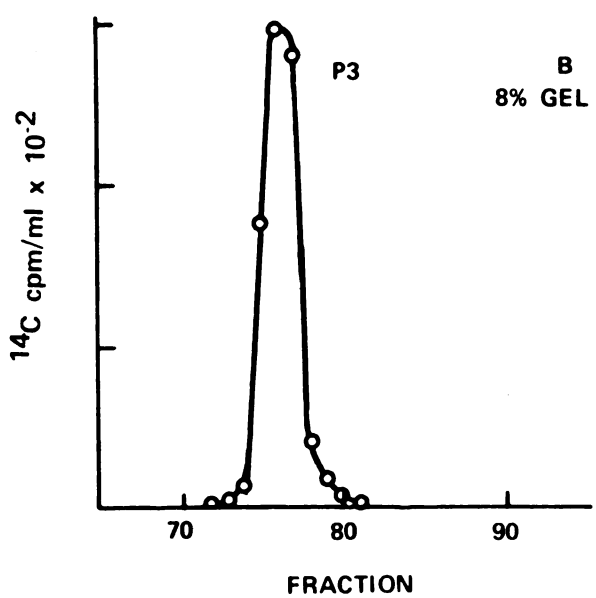
Figure 5.--Gel filtration analysis of p3 and p4. ^{14}C labeled p3 and p4 obtained from 6% agarose were concentrated, chromatographed on 8% agarose, and assayed as in Figure 3.

A. Elution profile of rechromatographed p3 and p4.

B. Elution profile of p3 after being rechromatographed twice.

C. Elution profile of p4 after being rechromatographed twice.


24
16
8
P3
P4
A
8% GEL
^{14}C cpm/ml X 10^{-2}
80
90
100
FRACTION


P3
B
8% GEL
^{14}C cpm/ml x 10^{-2}
70
80
90
FRACTION

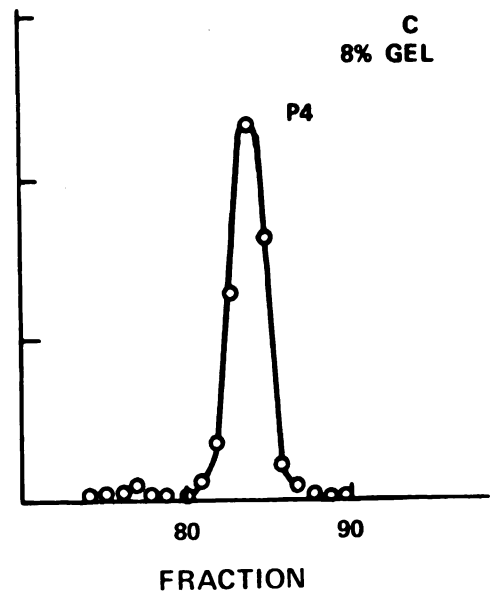

C
8% GEL
P4
80
90
FRACTION

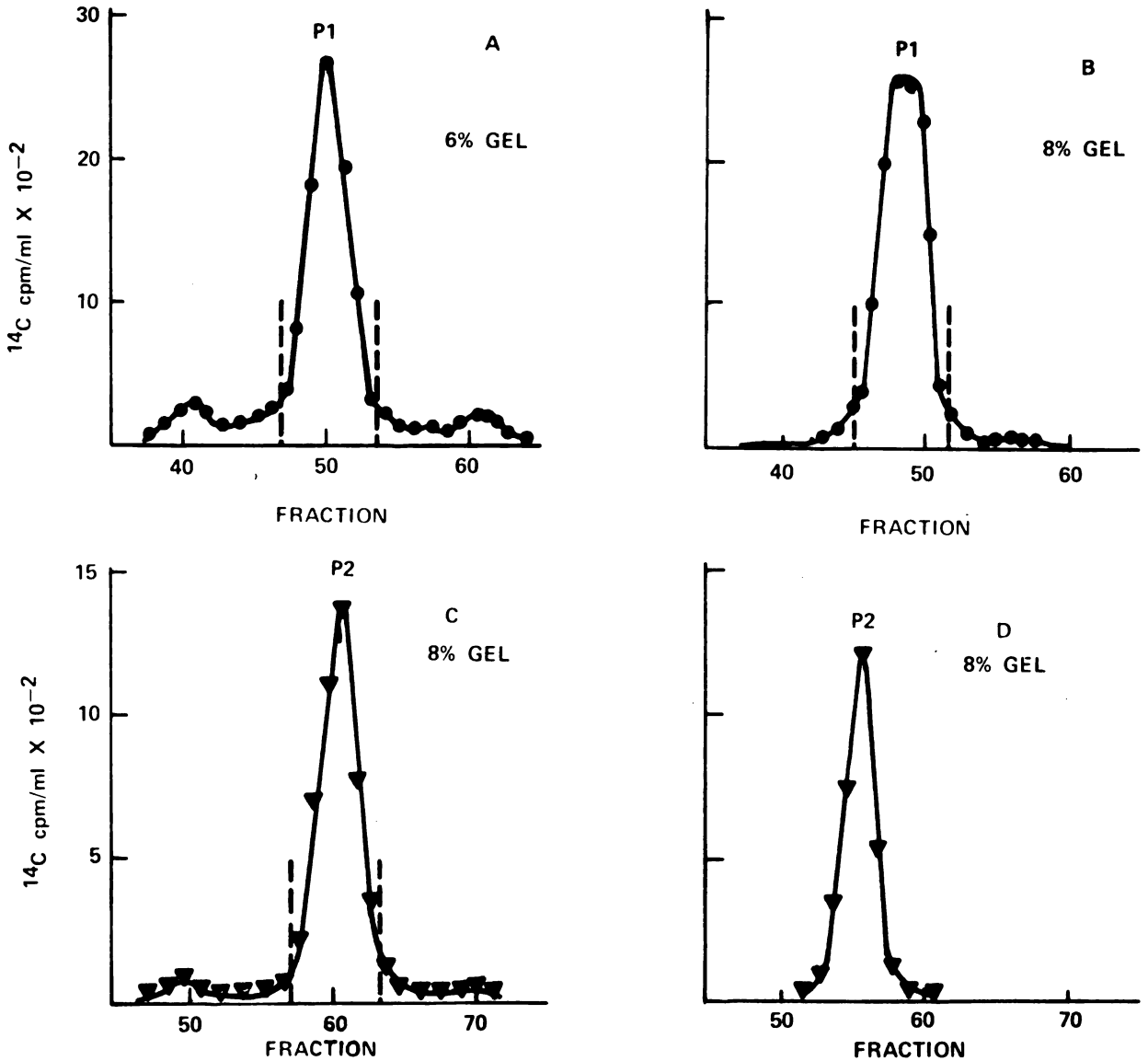
Figure 6.--Gel filtration analysis of p1 and p2. ^{14}C labeled p1 and p2 were chromatographed on 6% and 8% agarose and analyzed as in Figure 3.

A. Elution profile of p1 rechromatographed on 6% agarose.

B. Elution profile of p1 rechromatographed on 8% agarose.

C. Elution profile of p2 rechromatographed on 8% agarose.

D. Elution profile of p2 rechromatographed twice on 8% agarose.


30
20
10
P1
A
6% GEL
^{14}C cpm/ml X 10^{-2}
40
50
60
FRACTION
P1
B
8% GEL
40
50
60
FRACTION
15
10
5
P2
C
8% GEL
^{14}C cpm/ml X 10^{-2}
50
60
70
FRACTION
P2
D
8% GEL
50
60
70
FRACTION

Figures 7A and 7B are profiles of p5 after it was rechromatographed on 6% agarose and 8% agarose, respectively.

After rechromatographing proteins p1 to p5 two times on 6% and on 8% agarose, they were dialyzed against a low ionic strength buffer as described in Materials and Methods and then used for further immunological and chemical analysis.

Gp2 rechromatographed on 6% agarose as a single peak free from neighboring proteins gp1 and p1. Analysis of this single rechromatographed peak in 7.5% polyacrylamide gels in the presence of SDS revealed that it was heterogeneous and could be resolved into six polypeptide components (Figure 8). Six visible bands were detected only in the molecular weight range of 40,000 to 80,000 daltons and none coincided with the lower molecular weight proteins of the virus. Gp1 was not analyzed as extensively.

Relative Amount of Each Protein

In order to chemically or immunologically characterize the viral proteins, it was necessary to determine the relative amounts of each one within the virion and determine if it was economically feasible to work with them. An estimate of the relative amounts of each protein as a per cent of the total viral protein was determined from 6% agarose columns and is presented in Table 2. These data were calculated by dividing the radioactivity in each

Figure 7.--Gel filtration analysis of p5. ^{125}I labeled p5 was chromatographed on 6% and 8% agarose and analyzed as in Figure 3.

A. Elution profile of p5 on 6% agarose.

B. Elution profile of p5 rechromatographed on 8% agarose.

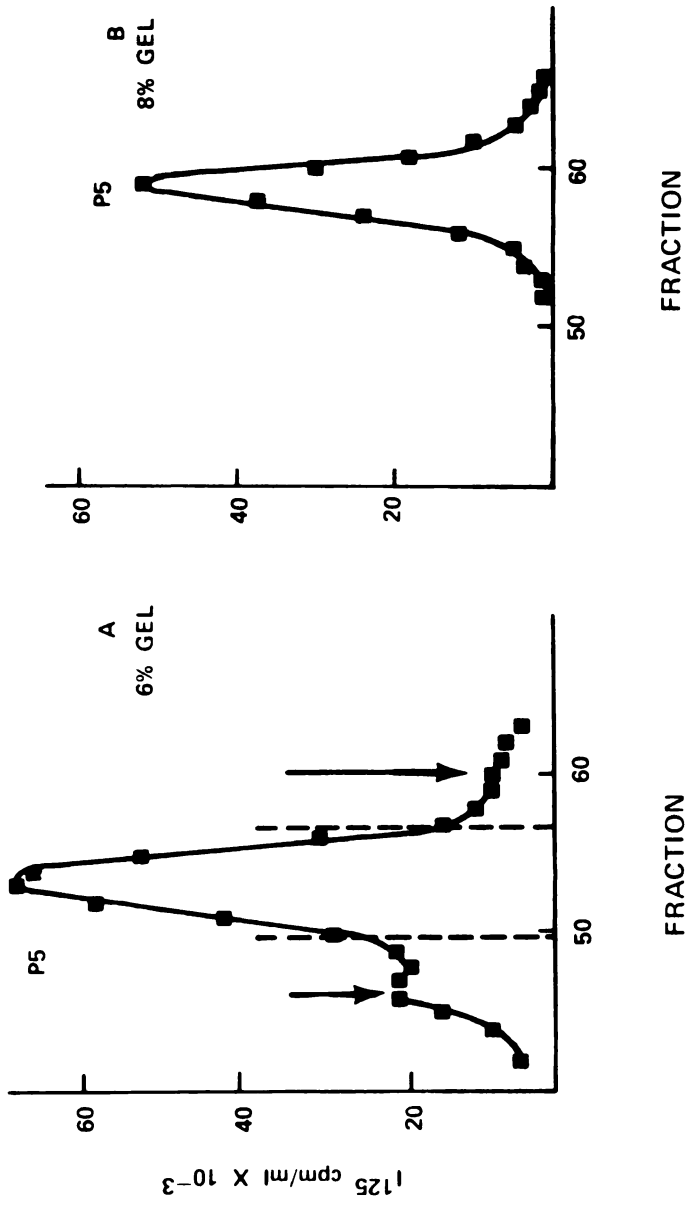

A
6% GEL
P5
B
8% GEL
P5
^{125} cpm/ml X 10^{-3}
60
40
20
50
60
FRACTION
FRACTION

Figure 8.--Electropherograms of rechromatographed gp2 and proteins from whole FeLV. Unlabeled FeLV and gp2 were subjected to electropherises in 7.5% gels containing 0.1% SDS for 4 hours at 100 volts after being solubilized in 1% SDS and 2% 2-mercaptoethanol. The protein bands in the gels were stained with Coomassie Blue as described in Materials and Methods. Absorbance profiles of the gels are shown.

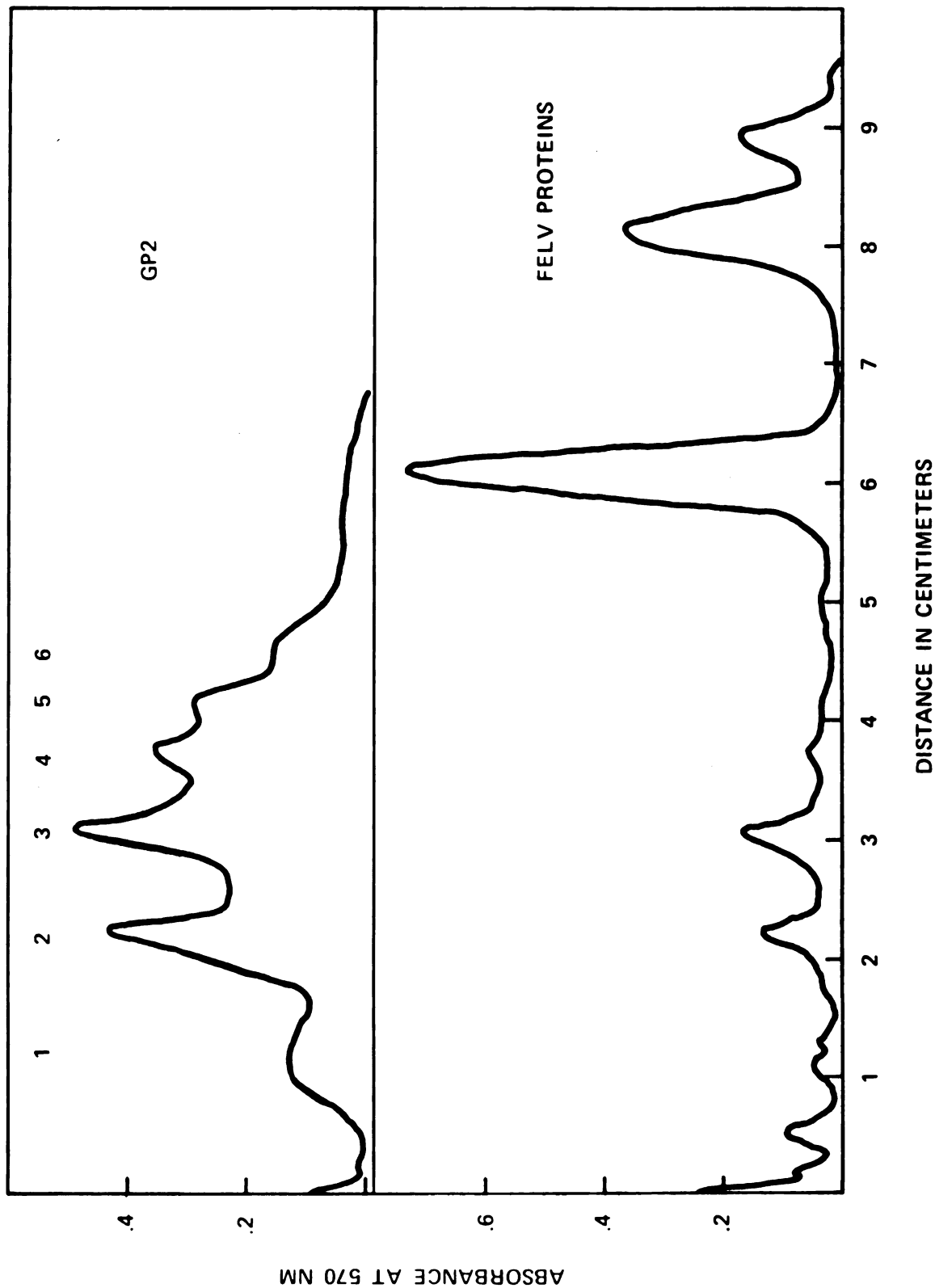

GP2
FELV PROTEINS
ABSORBANCE AT 570 NM
.2
.4
.6
1
2
3
4
5
6
7
8
9
DISTANCE IN CENTIMETERS

TABLE 2.--Per Cent of Individual Proteins per Total Viral Protein and their Molar Ratios.

Protein	Average[a] %	Molar Ratio[b]
gp1	17.0 ± .5	.12
gp2	10.7 ± 1.1	.15
p1	31.5 ± .7	1.05
p2	17.2 ± .8	1.10
p3	7.9 ± .5	.65
p4	5.3 ± .3	.53
p5	.53 ± .1	.03

[a]The per cent of each protein was determined from typical ^{14}C-amino acid radioactivity profiles as seen in Figures 3 and 4. The total radioactivity of each protein species was divided by the total radioactivity recovered from the column. The data are averages of three separate determinations.

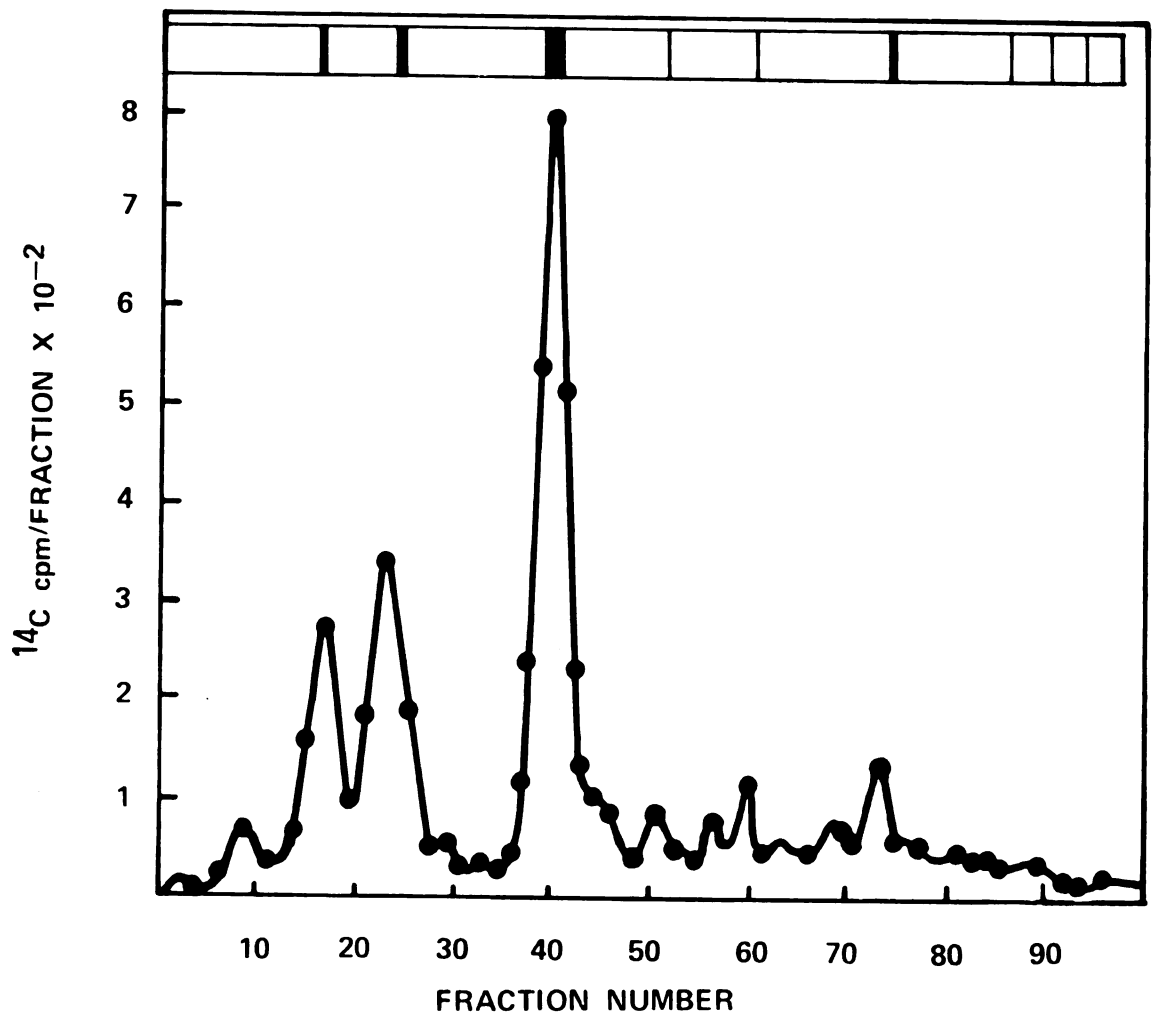
[b]Molar ratios were computed by dividing the per cents of total radioactivity for each protein species by its molecular weight as summarized in Table 4.

protein peak by the total radioactivity recovered from the agarose column. Since proteins p1 and p2 together make up nearly 50% of the total viral protein recovered (Table 2), extensive analysis could be conducted on them. Proteins p3, p4, and p5 together make up less than 15% of the total, and only limited amounts were available for analysis. Molar ratios of the proteins were determined by dividing the per cent of the total radioactivity in each protein species by the molecular weights (determined in GuHCl columns) of polypeptide chains (Table 2). P1 and p2 are present in almost equimolar amounts as are proteins p3 and p4. This is similar to the results obtained with proteins from the avian RNA tumor viruses (24).

SDS-Polyacrylamide Gel Electrophoresis of Viral Proteins

In order to compare the elution profile obtained from gel filtration (Figure 3) with that of an electropherogram profile of FeLV on SDS-PAGE, a ^{14}C-amino-acid labeled virus preparation was dissociated with SDS and 2-mercaptoethanol and electrophoresed in 10% acrylamide gels in the presence of SDS. The electropherogram pattern obtained was characteristic of other C-type viruses (56) (Figure 9). Three major and several minor peaks were distinguishable. In preliminary experiments with proteins obtained by gel filtration, it was shown that p1 was the larger and the third fastest migrating peak; p2 was the

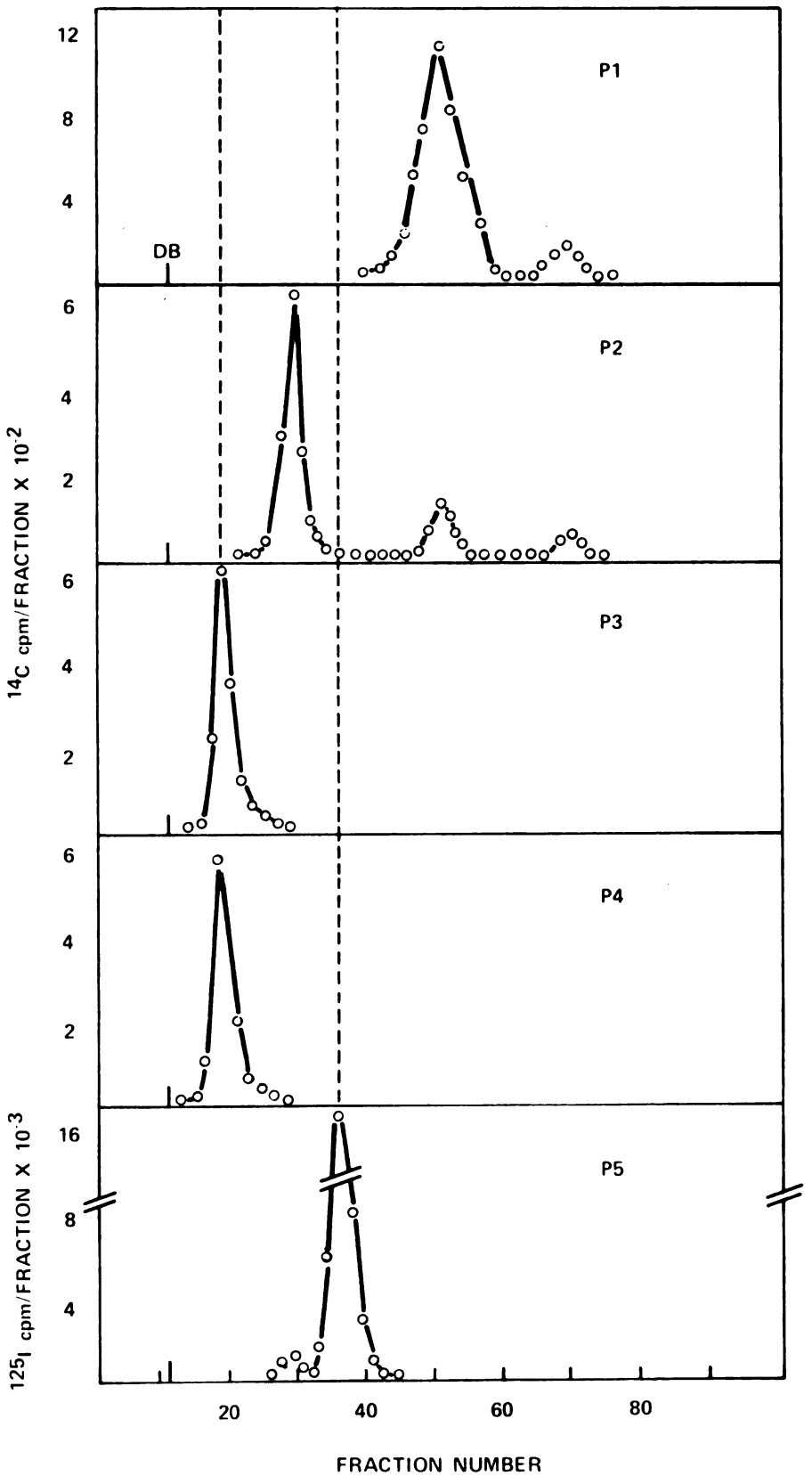
Figure 9.--Polyacrylamide gel electropherogram of ^{14}C-amino acid labeled FeLV proteins. Purified FeLV (2×10^4 cpm) was solubilized in 1% SDS and 0.1% 2-mercaptoethanol and then boiled for 1 minute. A 50 ul sample was applied to a resolving gel (1 cm 3% spacer gel, 10 cm, 10% resolving gel) and electrophoresed at 100 volts for 5 hours. Fractionation and assay of the fractions are described in Materials and Methods. The positions of Coomassie Blue stained protein bands as shown in the gel diagram were measured accurately from a duplicate gel and drawn to scale for comparison with labeled peaks. Migration is from right to left.


8
7
6
5
4
3
2
1
^{14}C cpm/FRACTION X 10^{-2}
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

intermediate and second fastest migrating peak; and p3 and p4 comigrated to make up the smallest and fastest migrating peak of the three major peaks. Occasionally a minor peak of activity was seen in the area of the dye marker ahead of the three major peaks suggesting that some radioactivity attached to the dye. This small peak was not seen in the absence of dye marker.

It has been suggested by Nowinski et al. (58) that protein p2 of the mammalian RNA tumor viruses migrated ahead of protein p3 and comigrated with p4 on SDS-PAGE. In order to determine the relative distance of migration of proteins p1-p5, rechromatographed individual proteins were analyzed by SDS-PAGE in 10% gels (Figure 10). Proteins p3 and p4, rather than p2 and p4, migrated in an identical region and appeared as one band when stained with Coomassie Blue. Attempts to separate proteins p3 and p4 on 12.5 and 15% gels in the presence of SDS were also unsuccessful. P5 migrated between p1 and p2 in a manner similar to its elution pattern off of agarose columns (Figures 10 and 3). Comparison of the data in Figures 3 and 9 shows that better separation of lower molecular weight proteins was obtained with agarose chromatography than with SDS-PAGE, especially in the case of p3 and p4.

Figure 10.--Polyacrylamide gel electropherograms of individual rechromatographed FeLV proteins. ^{14}C-amino acid labeled proteins, (A) p1, (B) p2, (C) p3, (D) p4 and ^{125}I labeled protein (E) p5 obtained from agarose columns, were analyzed on 10% gels as described in Figure 8. Fractionation and analysis of gels are described in Materials and Methods. Migration is from right to left. DB represents the position of the dye band.


P1
P2
P3
P4
P5
DB
12
8
4
6
4
2
6
4
2
6
4
2
16
8
4
20
40
60
80
^{14}C cpm/FRACTION X 10^{-2}
^{125}I cpm/FRACTION X 10^{-3}
FRACTION NUMBER

Molecular Weight Estimations of Polypeptide Chains from FeLV in SDS-Polyacrylamide Gels

SDS-PAGE analysis of rechromatographed ^{14}C-amino acid labeled viral proteins in 10% gels with iodinated marker proteins permitted an estimation of their molecular weights. A standard curve was constructed by plotting the distance each marker migrated versus the logarithm of the molecular weights. The molecular weights of the viral proteins were determined by extrapolating from the standard cruves (Table 3). P1 had a molecular weight of 27,000 daltons which was similar to that obtained by others (30, 40). Molecular weights of p2 and p5 were 15,000 and 18,000 daltons, respectively. As illustrated in Figure 10, p3 and p4 migrated in an identical region of about 12,000 molecular weight. In experiment 1 (Table 3), gp1 was shown to have a molecular weight of 110,000 daltons with minor bands in the higher molecular weight region; gp2 gave six bands in the molecular weight region of 40-80,000 daltons (Figure 8, and Table 4). These data suggest that gp1 and gp2 are actually heterogeneous consisting of several polypeptides.

Molecular Weight Estimations of Polypeptide Chains from FeLV by Gel Filtration in 6 M Guanidine Hydrochloride

Molecular weight determinations of the FeLV proteins were conducted as described by Fish *et al.* (23) using 6% and 8% agarose columns. Iodinated marker proteins were

TABLE 3.--Molecular Weight Estimations of FeLV Proteins in SDS-Polyacrylamide Gels.

Protein[b]	Molecular Weight x 10^3 (daltons)[a]			
	Expt. 1	Expt. 2	Expt. 3	Average[d]
gp1	110[c]	ND	ND	110
gp2	40-80[c]	ND	ND	40-80
p1	27.5	28.5	27	27
p2	13.5	16.5	14.5	15
p3	12.4	12.0	11.8	12
p4	12.4	12.0	11.8	12
p5	17.0	18.5	17.5	18

[a]Molecular weight determinations were made using the following iodinated protein markers: transferrin, ovalbumin, chymotrypsinogen, hemoglobin, and cytochrome c. Calibration curves were constructed after electrophoresis in 7.5% (gp1 and gp2) or 10% (p1-p5) gels and the molecular weights of the proteins extrapolated from standard curves as described in Materials and Methods.

[b]Rechromatographed proteins from agarose gels were used for molecular weight determinations.

[c]Protein markers, gp1, and gp2 were run on parallel gels and stained with Coomassie Blue. Calibration curves were constructed by measuring the distance the marker had migrated and the molecular weights of gp1 and gp2 extrapolated as described.

[d]The average molecular weight for each protein was determined from 3 experiments and rounded off to the nearest tnousand.

TABLE 4.--Molecular Weight Estimations of FeLV Proteins on Agarose Columns.

Protein	Molecular Weight x 10^3 (daltons)[a]			
	Expt. 1[b]	Expt. 2[c]	Expt . 3[c]	Average[d]
gp1	≥110	ND	ND	110
gp2	70	ND	ND	70
p1	31.0	30.0	28.5	30
p2	16.0	14.5	15.0	15
p3	12.0	11.2	11.0	11
p4	10.5	10.5	10.0	10
p5	21.5	21.0	21.0	21

[a]Molecular weight determinations were made using the iodinated protein markers described in Table 3 except with experiments 2 and 3 where transferrin was not used. Calibration curves were constructed from the elution profiles of the marker proteins and the molecular weight of the viral proteins extrapolated as described in Materials and Methods.

[b]Experiment 1: 6% agarose column used.

[c]Experiments 2 and 3: 8% agarose columns used and molecular weights of gp1 and gp2 were not determined (ND).

[d]Average molecular weights for each protein were determined from the three experiments and rounded off to the nearest thousand.

used to construct a calibration curve from which molecular weights of viral proteins could be estimated. In experiment 1 (Table 4), a 6% agarose column was used and an estimation of molecular weights for all seven proteins was possible. Gp1 eluted with void volume (Table 4) suggesting that it had a molecular weight equal to or greater than 100,000 daltons; gp2 had a molecular weight of 70,000 daltons. In experiments 2 and 3, 8% agarose columns were used and data were obtained only for the small proteins (Table 4). The average molecular weights for p1-p5 from the three experiments were as follows: p1=30,000; p2= 15,000; p3=11,000; p4=10,000; and p5=21,000.

A comparison of the molecular weights of p1-p5 obtained in GuHCl gel columns with that of SDS gels revealed the following (Tables 3 and 4): p1 and p5 had relatively higher molecular weights in GuHCl than they did in SDS; p2 had identical molecular weights in both systems; and p3 and p4 had slightly greater molecular weights in SDS gels.

Isoelectric Focusing of the FeLV Proteins

To further analyze the FeLV proteins, purified ^{14}C-amino acid labeled virus was disrupted in Tween 80-ether, digested with RNase and DNase, clarified at 50,000 rev/min for 1 hour, and then focused in a pH gradient of 3 to 10. Two distinct major peaks were repeatedly observed, one at a pH of 8.7 and one at a pH of 5.6

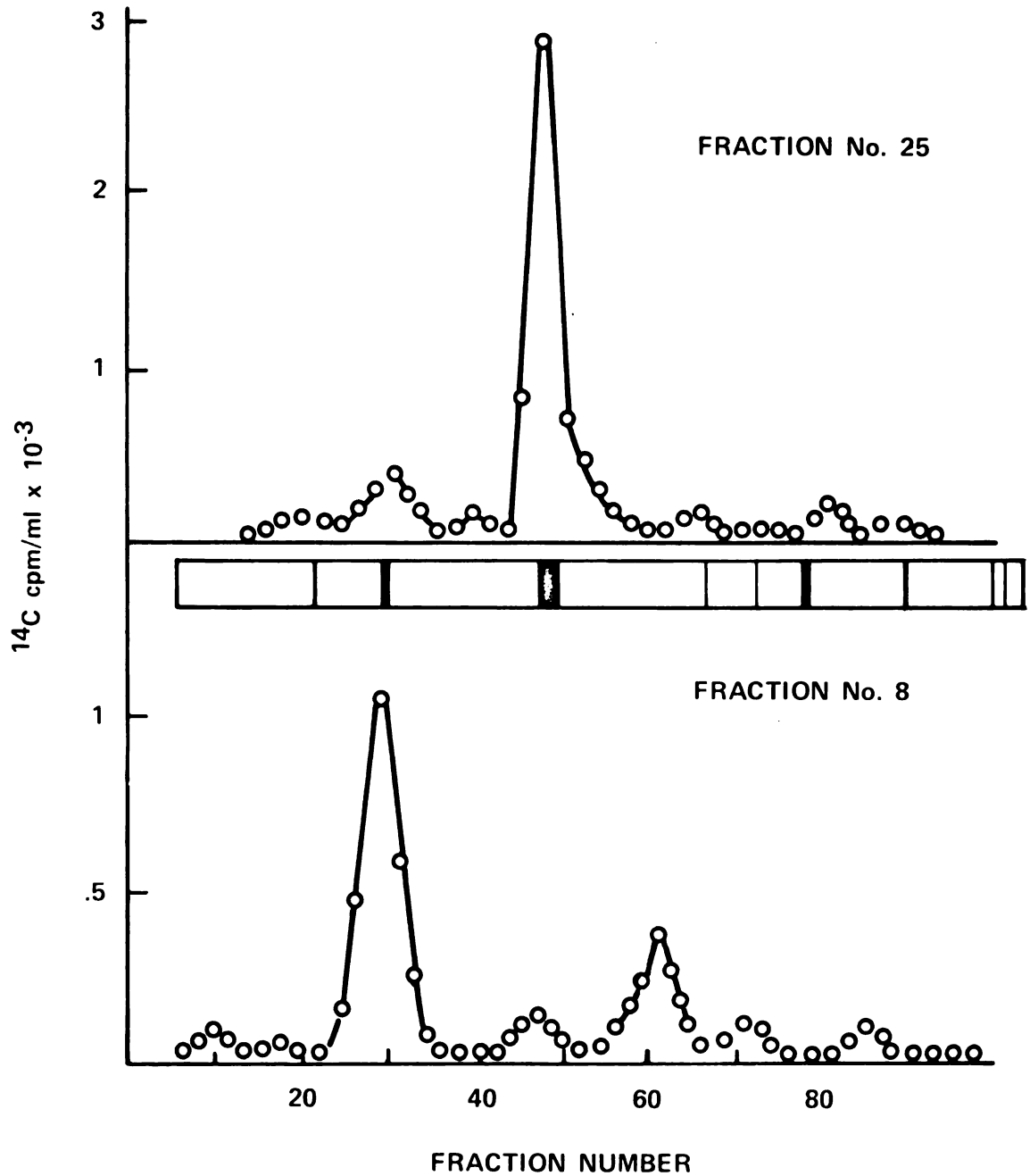
(Figure 11). In experiments where 0 to 6 M urea gradients were used, similar results were obtained. To determine which proteins these peaks represented, fractions 8 and 25 from the center of each peak were analyzed by SDS-PAGE and immunodiffusion. Protein from fraction 8 migrated in a region identical with p2 in SDS gels (Figure 12). In immunodiffusion, this same material reacted with only antiserum against p2 (Figure 11). These data indicate that the protein in fraction 8 which focused at a pH of 8.7 was p2. Protein from fraction 25 migrated in a region identical with p1 in SDS gels (Figure 12). In immunodiffusion analysis, this material reacted only with antiserum against p1 (Figure 11). These date indicate that the protein in fraction 25 which focused at a pH of 5.6 was p1.

Because only small amounts of p3 and p4 were available for analysis, a small scale isoelectric focusing column was used to determine their isoelectric points. Hemoglobin labeled with ^{125}I was included with each isoelectric focusing experiment for a reference. In a typical experiment, ^{14}C-amino acid labeled p3 or p4 (1 to 2 x 10^3 cpm) and ^{125}I labeled hemoglobin (5 x 10^3 cpm) were incorporated into separate linear gradients containing 3.3% ampholine (pH range of 3-6) and focused as described in Materials and Methods. Isoelectric points of p3 and p4 were 4.2 and 3.8, respectively

Figure 11.--Isoelectric focusing anysis of Tween 80-ether disrupted FeLV proteins. A sample of disrupted ^{14}C-amino acid labeled virus (1×10^5 cpm; 2 mg viral protein) was extensively dialyzed against 0.001 M Tris pH 7.4 buffer, digested with RNase (0.2 mg/ml) and DNase (20 ug/ml) at 37°C for 20 minutes, clarified by centrifugation at 50,000 rev/min, and then focused in a linear gradient of sucrose containing 2% ampholine with a pH range of 3-10. Isoelectric focusing, collection of fractions, and assay of fractions for radioactivity, pH, and antigens by immunodiffusion (see insert) were performed as described in Materials and Methods. Numbers 7-12 and 19-26 are isoelectric focusing fractions that were reacted with antisera prepared against p2 (a) and p1 (b), respectively.

pH (o-o)
14C cpm/ FRACTION x 10-2 (▼-▼)
FRACTION NUMBER (2ml/fr.)

Figure 12.--Polyacrylamide gel electropherograms of peak fractions from isoelectric focusing. Proteins from the center of each peak (fractions 8 and 25, Figure 11) were dialyzed and concentrated by negative pressure dialysis in spacer gel buffer (see Materials and Methods), dissociated and electrophoresed as in Figure 9. Fractionation of gels and assay of the fractions for radioactivity are described in Materials and Methods. The positions of the Coomassie Blue stained FeLV protein bands were measured accurately and drawn to scale from a gel run in parallel. Migration is from right to left.


3
2
1
FRACTION No. 25
1
.5
FRACTION No. 8
^{14}C cpm/ml x 10^{-3}
20
40
60
80
FRACTION NUMBER

(Table 5). When p1 and p2 were analyzed with this technique, they were found to have isoelectric points of 5.5 and 8.9, respectively. Hemoglobin focused repeatedly in a pH range of 6.8 to 7.1.

Immunological Analysis of Tween 80-Ether Treated Virus

Antiserum produced against Tween 80-ether disrupted FeLV in rabbits was employed in AGP tests. The antiserum appeared highly specific for the ingernal gs antigens because it reacted only weakly with whole FeLV (obtained by pelleting purified virus) and did not react with fetal calf serum. This antiserum formed three precipitin lines with Tween 80-ether disrupted virus (Figure 13). In preliminary experiments, the heavy precipitin line (1) was shown to contain p1, the major protein component of the virus; the middle precipitin line (2), p2; and the minor precipitin band (3), could not be identified. In some experiments, precipitin line 3 was very faint and occasionally not detectable.

Immunological Analysis of Viral Proteins Renatured from Guanidine Hydrochloride

Gel filtration in GuHCl was not only suitable for preparative fractionation of viral proteins but allowed subsequent recovery of native antigenic activity of several of the proteins after dialysis. The isolated viral proteins were dialyzed free of GuHCl and the reducing agent

TABLE 5.--Isoelectric Points of Proteins Isolated from FeLV.

Protein	Isoelectric Point[a]
p1	5.5
p2	8.9
p3	4.2
p4	3.8
p5	ND[b]

[a]Isoelectric points were determined for p1-p4 after they were rechromatographed on agarose gel columns as in Figures 5 and 6. The proteins were dialyzed extensively against 0.001 M Tris pH 7.4 buffer, treated with RNase (0.2 mg/ml) at 37°C for 20 minutes, clarified by centrifugation at 10,000 rev/min for 10 minutes, and then focused in a linear gradient of sucrose with 3.3% ampholine of the following pH ranges: p1, 5-8; p2, 7-10; p3, 3-6; and p4, 3-6. Isoelectric focusing, collection of fractions, assay for radioactivity, and pH determinations were performed as described in Materials and Methods. Isoelectric points are averages from three experiments.

[b]Isoelectric point for p5 was not determined (ND).

Figure 13.--Agar gel precipitin analysis of FeLV antigens.

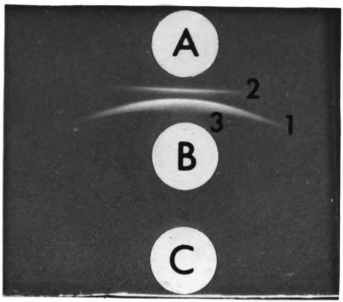
Well A: Tween 80-ether disrupted FeLV.

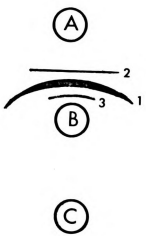
Well B: Antiserum produced against Tween 80-ether treated virus (see Materials and Methods).

Well C: Fetal calf serum.

Numbers 1, 2, and 3 represent precipitin bands.

A schematic drawing of the precipitin lines is shown in the lower half of the figure.


A
2
3
1
B
C


A
2
3
1
B
C

and tested in immunodiffusion with antisera produced against either Tween 80-ether disrupted virus or against the rechromatographed renatured individual proteins (Figure 14). Antiserum against Tween 80-ether disrupted virus formed precipitin lines only with p1, p2, and p3 (Figure 14A). The single lines obtained with each protein cross one another indicating non-identity between these three proteins (non-identity reaction between p2 and p3 not shown in this figure). Antisera produced against rechromatographed proteins p1, p2, and p3 reacted only with their homologous proteins (Figure 14B, C, and D). Single precipitin lines were obtained suggesting homogeneity. Antibodies were not detected against p4 and p5 with either antiserum against Tween 80-ether treated virus or antiserum against individual proteins (Figure 14A, E, and F).

FeLV and MuLV share at least one antigen as demonstrated by immunodiffusion analysis with selected gs antigens and antisera (27, 28), and the antigenic determinant appears to be associated with the major polypeptide of the virus (31). To determine if this cross-reactive determinant (interspecies or gs-3 antigen) can be detected after gel chromatography in the presence of GuHCl, antiserum prepared against gs-3 of the Rauscher MuLV (gift from Erwin Fleissner and William Hardy) was reacted with isolated p1, p2, p3 in immunodiffusion along with the specific antiserum

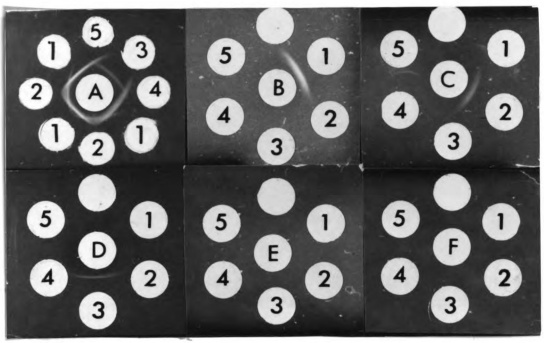
Figure 14.--Agar gel precipitin analysis of the FeLV proteins isolated by gel filtration in 6 M GuHCl.

Center well A: Antiserum against Tween 80-ether disrupted virus.

Center wells B, C, D, E, and F: Antisera prepared against rechromatographed proteins p1, p2, p3, p4, and p5, respectively (Figures 5, 6, and 7), after dialysis.

Outer wells 1, 2, 3, 4, and 5: Isolated proteins p1, p2, p3, p4, and p5, respectively, that have been rechromatographed 2 times (Figures 5, 6, and 7) and dialyzed to remove GuHCl (see Materials and Methods).

A schematic drawing of the precipitin lines that developed is shown in the lower half of the figure.


5
1
3
2
A
4
1
2
1
5
1
4
B
2
3
5
1
C
4
2
3
5
1
D
4
2
3
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1
E
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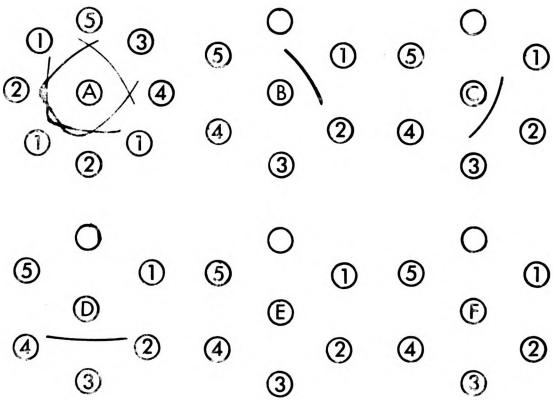
3
5
1
F
4
2
3


5
1
3
2
A
4
1
2
1
5
1
B
2
3
5
1
C
2
3
5
1
D
4
2
3
5
1
E
2
3
5
1
F
2
3

for each protein. Anti-gs-3 and anti-p1 exhibited a line of identity with p1 further illustrating that the gs-3 antigenic activity was associated with the major polypeptide of the FeLV (Figure 15). Furthermore, recovery of this antigenic determinant was still possible after the protein was rechromatographed two times on agarose columns with subsequent removal of the GuHCl.

Sucrose Gradient Sedimentation Analysis of Isolated Proteins

In order to obtain additional information about the relative size of isolated viral proteins, they were analyzed in linear gradients of 5-20% (w/v) sucrose. Proteins isolated from agarose columns tend to aggregate after removal of GuHCl and may exhibit false sedimentation values. To minimize this problem, the proteins were treated with 1 M GuHCl and then analyzed on sucrose gradient containing 1 M GuHCl. Protein p1 was centrigued in a sucrose gradient with the 4.4S iodinated BSA, and p2, p3, and p4 were centrifuged in separate gradients with 2.54S iodinated chymotrypsinogen so that direct estimations of sedimentation values could be made. P1 sedimented at a 2.5S (Figure 16A); p2, 1.4S (Figure 16B); p3, 1.17S (Figure 16C); and p4, 1.14S (Figure 16D). A second smaller peak with a higher sedimentation value was observed when p1 was analyzed (Figure 16A) and probably represented an aggregate of p1 that was not dissociated in 1 M GuHCl.

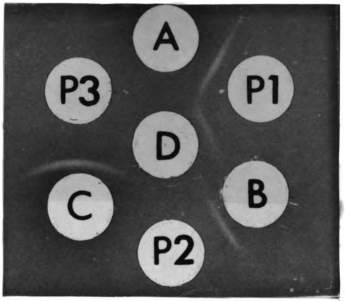
Figure 15.--Agar gel precipitin analysis of rechromatographed proteins p1, p2, and p3 with antiserum prepared against MuLV gs-3.

Wells A, B, C: Antisera prepared against rechromatographed proteins p1, p2, and p3, respectively (Figures 5 and 6).

Well D: Antiserum prepared against gs-3 antigen of MuLV (gift from Erwin Fleissner and William Hardy).

P1, p2, and p3: Isolated proteins that had been rechromatographed 2 times on agarose columns.

A schematic drawing of the precipitin lines that developed is shown on the lower half of the figure.


A
P3
P1
D
C
B
P2

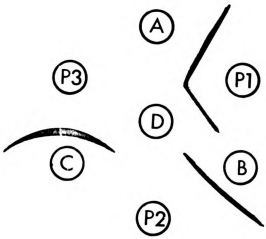

A
P3
P1
D
C
B
P2

Figure 16.--Sucrose gradient sedimentation analysis of isolated FeLV proteins. ^{14}C-amino acid labeled proteins were isolated and rechromatographed as described (Figures 3, 5, and 6). The proteins were treated with 1 M GuHCl prior to analysis.

A. Nine thousand cpm of p1 and 10,000 cpm of iodinated BSA were mixed and analyzed on linear 5-20% sucrose gradient in 1 M GuHCl at 50,000 rev/min for 24 hours as described in Materials and Methods.

B, C, D. Ten thousand cpm of iodinated chymotrypsinogen were added to p2 (4×10^3 cpm), p3 (6×10^3 cpm) and p4 (4×10^3 cpm) and each mixture analyzed as described in A, except the gradients were centrifuged for 40 hours instead of 24.

A
BSA
4.4 S
P1
2.5 S
B
2.54 S
P2
1.4 S
C
2.54 S
1.17 S
P3
D
2.54 S
P4
1.14 S
NORMALIZED FRACTIONS
^{14}C cpm/FR X 10^{-2}
^{125}I BSA cpm/FR X 10^{-2}
^{125}I CHYMOTRYPSINOGEN cpm/FR X 10^{-2}

Protein p2 formed very strong aggregates after the GuHCl was removed by dialysis making it a very difficult protein to study chemically and immunologically. Treatment of these aggregates with 2 M urea and 2% mercaptoethanol for 24 hours at 20°C, resulted in complete loss of immunological activity, whereas SDS, Brij 35, and DTT appeared to have little or no effect. These same dissociating and reducing agents had little effect on p1 and p3, the other two proteins that could be monitored for immunological activity. In addition, the immunological activity of p2 was destroyed after iodination, whereas p1 and p3 were unaffected, further indicating that p2 is a labile protein and slight changes in its configuration may result in loss of its antigenic activity.

Even though the viral proteins appear to be homogeneous after being rechromatographed twice on agarose, it is felt that another procedure based on charge or electrophoretic mobility such as preparative disc electrophoresis or isoelectric focusing, should be used in addition to assure purity. That way proteins would be separated not only on the basis of molecular weight but also on the basis of charge and possible contaminants of identical molecular weight can be eliminated.

DISCUSSION

The purpose of this study was to separate and purify the structural proteins from purified FeLV and to begin chemical and immunological analysis. The Rickard's strain of virus was a good model system to work with because: (1) a high yield of virus (approximately 3 to 5 mg of viral protein per liter of cultural media) could be obtained from the F-422 suspension cell cultures; (2) the FeLV proteins had not been studied extensively; and (3) it could be used for comparison with viruses of other species, especially the human candidate RNA tumor viruses.

There was concern in this study that purified virus may be contaminated with host cell protein which would complicate subsequent studies of virus specific proteins. With the intent of quantitating possible contamination, attempts were made to establish a normal thymus cell culture which could be labeled with ^{14}C-amino acids and then mixed with F-422 cells. After purifying the FeLV from the mixture, an estimate of contamination with host cell protein could have been made from the amount of radioactivity found in the viral band. However, neither a primary thymus cell culture nor a thymus cell line could

be established. Attempts were made to infect the "normal" Crandel cat kidney cells (45) with FeLV until it was discovered than an endogenous virus existed in the cells (Livingston, personal communication) rendering their usefulness questionable. A normal cell line is needed that can be infected and subsequently produce relatively large amounts of virus so mixing experiments can be conducted. At present the control experiments cannot be done and this limitation must be considered in the analysis of FeLV structural proteins. In a study by Nowinski et al. (58), virus preparation isolated by a similar method of density gradient centrifugation was examined in the electron microscope and found to predominately contain virions. Contaminating cell vesicles rarely exceed 10% of the banded material. It should be noted, however, that since the FeLV is a budding virus, host cell membrane proteins may be an integral part of the virion envelope; therefore, it may be impossible to avoid all host cell proteins in a virus preparation even if purification of the virion is accomplished.

Agarose gel filtration in 6 M GuHCl offers an effective, simple reproducible method for separation of proteins from RNA tumor viruses according to their molecular weights (24). This method may be employed for either analytical purposes since it can separate low molecular weight proteins better than SDS-PAGE (Figure 9), or as a

preparative step in a purification procedure where large amounts of dissociated virus (10 to 20 mg) can be applied to the column at one time with a 90-100% recovery of material. In addition, removal of the GuHCl permits a good recovery of the original antigenic activity of some of the proteins. However, this method does have a few disadvantages: It is expensive and time consuming because of the cost of GuHCl and the slow flow rate (1 fraction/ hour); many of the proteins tend to aggregate after the GuHCl is removed; and limited resolution is obtained with proteins in the molecular weight region of 60,000 daltons and greater.

Application of the above method in the present work permitted resolution of FeLV into six major and one minor protein components (Figure 3). Use of a mixture of virus labeled with ^{14}C-amino acids and ^{3}H-glucosamine revealed that the first two proteins eluting from the column were glycoproteins (gp1 and gp2) whereas the remaining five proteins (p1-p5) contained no glucosamine and were non-glycoproteins (Figure 4). In preliminary studies (Velicer, unpublished data), gp1 and gp2 were found to contain fucose and galactose in addition to glucosamine whereas p1-p5 appeared to be free of carbohydrates within the limits of sensitivity of our method. Since gp1 eluted with the void volume when chromatographed on 6% agarose, it was assumed to have a molecular weight $\geq$ 100,000 daltons.

A glycoprotein (m1) of the avian RNA tumor virus system was shown to have a similar molecular weight on agarose columns (24) but disaggregated into a 32,000 molecular weight polypeptide when analyzed by SDS-PAGE, indicating that it was an aggregate in GuHCl. This is in contrast to the preliminary observation made when gp1 was analyzed by SDS-PAGE. Several stained polypeptide bands were found in the molecular weight range of 110-115,000 daltons and none in low molecular weight regions. However, this must be re-analyzed with glucosamine labeled material because glycoproteins with a high carbohydrate content may have stained poorly with Coomassie Blue and were not detected. Nowinski et al. (58) also found that the major glycoprotein of the mammalian RNA tumor viruses, after isolation by gel filtration, only partially dissociated upon subsequent analysis by SDS-PAGE. The 70,000 molecular weight glycoprotein (m2) of the avian and mammalian tumor viruses, obtained from agarose gel chromatography by Nowinski et al. (58) and Fleissner (24), did not dissociate further when analyzed by SDS-PAGE. In this study, gp2 also had a molecular weight of 70,000 daltons on agarose columns (Table 4); but after being rechromatographed as a single peak on 6% agarose and analyzed by SDS-PAGE, six polypeptide components were detected in the molecular weight region of 40-80,000 daltons (Table 3, Figure 8) suggesting considerable heterogeneity. It is possible that the two

smaller polypeptides (40-50,000 daltons) are subunits of an aggregate in gp2 but the 4 larger components appear to be individual polypeptides in the 60-80,000 molecular weight range that are not resolved on agarose. Although it has been reported that 6% agarose permits useful molecular weight estimation between extreme limits of 80,000 and 1,000 daltons (23), our data indicate its practical usefulness for separating complex mixtures is limited to those proteins below 60,000 molecular weight. Therefore, it appears that separation of larger proteins is better obtained by SDS-PAGE.

Since p1, p2, and p5 were not completely separated and p3 and p4 poorly separated on 6% agarose columns (Figures 3 and 4), they were rechromatographed on 8% agarose which resulted in improved separation into symmetrical peaks (Figures 5-7). When these rechromatographed proteins were analyzed separately by SDS-PAGE, more than one protein band was occasionally observed in a higher molecular weight region (Figure 10A) implying the protein preparation was not pure. However, this is not consistently observed when the same preparations were re-analyzed suggesting these additional bands may be aggregates of the monomeric polypeptide. Since the proteins aggregate very rapidly after removal of the GuHCl, the conditions used to dissociate these proteins before analysis by SDS-PAGE may have been insufficient to achieve 100% dissociation. Upon

analysis of these same protein preparations of p1, p2, and p3 by the AGP test, single precipitin lines were obtained when each protein was reacted with its homologous antiserum produced against rechromatographed proteins (Figure 14), and no lines formed when reacted with heterologous serum. Even though these data give further proof of homogeneity, it is felt an additional, more sensitive, immunological technique, such as radioimmune assay (65), is needed to further prove homogeneity of each polypeptide.

The molecular weights of p1-p5 obtained by gel filtration in GuHCl (Table 4) generally agree quite well with values determined in SDS-PAGE (Table 3). Small variations exist which may be due to the differences in binding of SDS by viral proteins (69). Perhaps this is why p3 and p4 migrate in an identical region in SDS gels (Figure 10), giving the appearance of one less protein than was found by gel filtration. Therefore, separation of lower molecular weight proteins appears to be better accomplished in 6 and 8% agarose than with SDS-PAGE (69), with 8% agarose being the most superior technique.

Analysis of Rickard's strain of FeLV by SDS-PAGE revealed three major protein peaks (Figure 9): third fastest migrating peak, p1=27,000 daltons; intermediate peak, p2=15,000 daltons; and the fastest migrating peak, containing p3 and p4=12,000 daltons; and several minor peaks. Earlier, Schäfer (83) identified a similar

pattern, but there exist slight variations among the molecular weights of the three major components in the two studies. He found the following: third fastest migrating protein, EIII=33,000 daltons; the intermediate protein, EII=18,000 daltons; and the fastest migrating component, EI=15,000 daltons. No attempt was made by Schäfer to resolve EI into the two protein components detected by gel filtration (Figure 3). Furthermore, EII was not considered an antigenic component of the FeLV. This is in contrast to this study where p2 is definitely an antigenic component (Figure 14).

Protein p2 did not migrate ahead of p3 in SDS gels under the conditions of this study which is in contrast to what Nowinski et al. (58) suggested for two corresponding proteins in their study. This could be attributed to the per cent of acrylamide gels used; 7.5% in Nowinski et al. (58) experiments, and 10% gels in this experiment.

Protein p5 may represent a component that has been reported missing in the mammalian RNA tumor viruses (Figure 3). It migrated between p1 and p2 with a molecular weight of 21,000 daltons in GuHCl similar to that of a seventh protein from the avian RNA tumor viruses. If this protein is equivalent to the 19,000 molecular weight component of the avian virus (24), this may suggest that all C-type RNA tumor viruses have the same number of protein components, but the amount of each component in different species

may vary. This observation is supported by Nowinski et al. (58) who found that amounts of each of the four main low molecular weight polypeptides of several mammalian RNA tumor viruses varied from one species to another. However, it is possible that p5 is a cellular contaminant and further analysis is needed to rule this out.

The isoelectric point for the major protein of the Theilen strain of FeLV was shown to be 8.3 (67). These data obtained in this study indicate that the major protein (p1) of Richard strain has an isoelectric point of 5.6 (Table 5, Figure 11). It is not known why there is such a deviation between the two values since identical techniques were employed. It is possible that these major proteins of the two strains have very different content of several amino acids but this would be surprising since they share immunological activity and presumably have the same function or structural role. Protein p2 was shown to have an isoelectric point of 8.7; p3, 4.2; and p4, 3.8 (Table 5). Fleissner (24), Nowinski et al. (58), and Bolognesi (personal communication) have shown that proteins equivalent to p3 and p4 in other RNA tumor viruses are basic. However, either p3 and p4 are not basic as originally expected, or they are actually basic proteins but appear as acidic proteins which may be due to attachment of small pieces of RNA that are difficult to remove by treatment with RNase (63).

Immunization of rabbits with proteins p1-p5 resulted in antisera being produced against p1, p2, and p3 but not p4 and p5 (Figure 14). These antisera appear to be monospecific as illustrated by immunodiffusion analysis (Figures 14 and 15), suggesting that the proteins are purified to homogeneity. It is not known for sure why antibodies were not produced and detected against p4 and p5. It could have been due to inefficient renaturation of the polypeptides from GuHCl, the small amounts of protein available to immunize the rabbits, or as in the case of p4, the small size of the protein may be a factor. Similar results were obtained for a 10,000 molecular weight protein of the avian (24) and mammalian RNA tumor viruses (58). Further attempts should be made treating p4 as a hapten by attaching it to a larger molecule such as sepharose and immunizing rabbits. With p5, the small amount of this protein available may limit antibody production against it.

Evidence has been presented demonstrating the gs-3 antigen (interspecies or cross-reactive antigen) is on the major polypeptide (30,000 daltons) of the Theilen strain of FeLV. Antisera prepared against hamster and mouse gs-3 antigens (31) were used in complement-fixation, immunodiffusion, and adsorption experiments to clearly show that they cross react with the major protein of the FeLV. More recently, the major polypeptide has been iodinated (65) and used in a very sensitive radioimmunoassay test showing

its reactivity with antisera against other mammalian RNA tumor virus antigens. In this study antiserum prepared against Rauscher MuLV gs-3 antigen and rechromatographed p1 of the Rickard strain of FeLV exhibited a line of identity with p1 (Figure 15) further supporting the contention that the gs-3 antigenic activity was associated with the major polypeptide. These data also illustrated that recovery of the antigenic determinant was still possible after p1 was rechromatographed two times on agarose with subsequent removal of GuHCl. In contrast, Nowinski et al. (58) could not show the gs-3 antigenic activity on the major polypeptide from the MuLV after chromatography in GuHCl. This may indicate the activity was not renatured from GuHCl or that the amount of material used in their study was insufficient to be detected in the immunodiffusion assay.

Two other interspecies antigens have now been illustrated, the RNA-dependent DNA polymerase (96) (molecular weight 69,000) and a new interspecies antigen designated as interspec II (3) (molecular weight 71,000). Although it was not the purpose of this study to characterize these antigens, it is interesting to speculate that the six proteins obtained by SDS-PAGE from gp2 (Figure 8) with molecular weights between 40-80,000 daltons, may represent one or more of these interspecies antigens.

Further studies using specific antisera will be necessary for more positive identification.

The minimum estimate of total molecular weight of the virus specific proteins is about 500-600,000 daltons assuming that p1-p5 and polypeptides gp1 and gp2 illustrated by SDS-PAGE represent distinct individual virus coded proteins. Since the total mass of viral RNA is about 1.2×10^7 daltons, at least twice as many proteins could be coded for if the genome is nonrepetitive. However, if the genome is redundant containing 3 to 4 repeating 35S subunits of 3.0 to 3.5×10^6 daltons, then these proteins appear to exceed the apparent limit of what might be coded for by such a subunit (3).

Information and reagents obtained from this study can be used for further analysis of the FeLV and comparative studies with other RNA tumor viruses including a human virus if one is isolated. Assuming that p1-p5 are purified proteins and are in sufficient amounts, more extensive physical and chemical characterization can be conducted. Amino acid composition, peptide mapping, C- and N-terminal amino acid determinations and possibly determination of the primary structure can be carried out on those individual rechromatographed proteins available in sufficient quantity. If the proteins are found not be be completely homogeneous based upon the fact that they have been separated only on the basis of molecular weights, then

further purification steps such as isoelectric focusing or preparative disc electrophoresis will be necessary. A technique of radioactive labeling of proteins in vitro with ^{14}C or ^{3}H-formaldehyde has been established in our laboratory that does not destroy the immunological activity of the proteins. This procedure may make possible physical, chemical, and immunological analysis of much smaller amounts of protein.

Now that the major proteins are separated from the virus and partially characterized, they can be used as standards for comparing and identifying viral proteins synthesized in vitro with cell free extract in experiments now being planned. In addition, viral polypeptides obtained by in vitro synthesis can be tested for specific immunological reactivity by using antiserum produced against each rechromatographed protein.

The antisera produced against p1, p2, and p3 can be used to locate these proteins in the virion and also in the infected cells by using the immunoferritin labeling technique and immunofluorescence, respectively. In addition, specific antisera can be used in kinetic studies for detecting newly synthesized polypeptides providing they are immunologically reactive. They may also be used to study relationships between antigens of other RNA tumor viruses since there appears to be several cross-reactive

antigens. Also, they may be used to locate antigens in apparently normal cells suggesting the presence of either the virus or viral genome.

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