ANALYSIS OF RIBONUCLEIC ACID FROM FELINE LEUKEMIA VIRUS

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ABSTRACT

ANALYSIS OF RIBONUCLEIC ACID FROM
FELINE LEUKEMIA VIRUS

By

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The Rickard strain of feline leukemia virus (FeLV-R) is continuously produced by the permanently infected feline thymus tumor cell line F-422. Attempts to synchronize F-422 cells using the methods of thymidine excess and serum-free medium were unsuccessful.

FeLV-R (density of 1.14 g/cc) concentrated by salt precipitation, polyethylene glycol precipitation, or pelleting and purified by isopycnic centrifugation appears to contain particulate (possibly vesicular) material as a contaminant derived from the host cell. This is suggested by electronmicroscopy of purified virus, velocity sedimentation studies and by RNA analysis of particles resolved by velocity sedimentation. FeLV-R can be purified from the contaminant by rate zonal centrifugation or by rapid pelleting through a 20% w/w sucrose barrier. Purified FeLV-R can infect the Crandell-CCC feline kidney cell line and produce progeny virus with a density of 1.14 g/cc.

By labeling F-422 cells with a continuous source of ^{3}Huridine and quantitating the radioactivity in purified virus, the minimum interval between the synthesis of viral RNA and its release into virus

was measured to be 30 min. or less. By labeling F-422 cells with a 15 min. pulse, the average interval between the synthesis of viral RNA and its release into virus was measured to be between 4 and 5 h.

RNA from FeLV-R was resolved into three size classes, 50 to 60S (comprising 50 to 73% of the total), 8S (comprising 3 to 7% of the total), and 4 to 5S (comprising 7 to 21% of the total) when analyzed by electrophoresis on 2.0% polyacrylamide -0.5% agarose gels. The 50 to 60S RNA from virus harvested after 4 h. of labeling electrophoretically migrated faster and sedimented more slowly than the same RNA harvested after 20 h. of labeling. This argues for an intravirion modification of the high molecular weight RNA. The high molecular weight subunits from dissociated 50-60S RNA cosedimented with 28S ribosomal RNA when analyzed by velocity sedimentation through 99% dimethylsulfoxide (DMSO) giving them an estimated molecular weight of 1.8×10^6 daltons. Aggregates intermediate between 50 to 60S ($s_{25,DMSO} = 7.09$) and 28S ($s_{25,DMSO} = 4.22$) suggest that conditions other than 99% DMSO alone are needed for complete dissociation of the RNA. The 8S RNA in FeLV has not previously been reported.

ANALYSIS OF RIBONUCLEIC ACID FROM FELINE LEUKEMIA VIRUS

By

David A. Brian

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To Donna, Matt, and Molly

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AMV	avian myeloblastosis virus
BSA	bovine serum albumin
CCC	Crandell feline kidney cells
DMSO	dimethylsulfoxide
F-422	feline thymus tumor cells, Rickard's number
FCS	fetal calf serum
FeLV-R	feline leukemia virus, Rickard's strain
HBSS	Hank's balanced salt solution
HnRNA	heterogeneous nuclear RNA
MAK	methylated albumin kieselguhr
MLV	murine leukemia virus
MMTV	mouse mammary tumor virus
mRNA	messenger RNA
MSV-SD	murine sarcoma virus, Soehner-Dmochowski strain
NDV	Newcastle disease virus
oligo(dT)	oligodeoxythymidilic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
poly(A)	polyadenylic acid

POPOP	1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene
RAV	Rous associated virus
RD-114	feline oncornavirus, originally thought to be of human origin
rRNA	ribosomal RNA
s	sedimentation coefficient
SR-RSV	Rous sarcoma virus, Schmidt-Ruppin strain
TCA	trichloroacetic acid
TNE	tris-sodium chloride-EDTA buffer
tRNA	transfer RNA
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate

INTRODUCTION

There are several reasons why at least one approach to oncology should be the study of RNA tumor (oncorna) viruses.

1. Oncornaviruses are known to cause neoplasia in animals: leukemia (37) and sarcomas (96) in chickens, leukemia (56), sarcomas (59) and mammary adenocarcinomas (14) in mice, and leukemias (66) and sarcomas (104) in cats.
2. Oncornaviruses transform cells in vitro (75, 98) and therefore allow biochemical analysis of the transformation process.
3. Mutants, both conditional (76) and non-conditional (77), for transformation defectiveness exist enhancing the ability to mechanistically understand transformation. While the number of genes is probably no more than 50 (54) the problem of identifying the transformation gene or its product does not seem insurmountable.
4. The genetic material in the virion is single-stranded RNA with probably the same polarity (+) as intracellular messenger RNA coding for viral structural components (9). A reasonable approach to the study of controls functioning during virus replication (and possibly transformation) would be to study

control mechanisms functioning during in vitro translation of virion RNA (49, 116).

5. Oncornavirus infection (and therefore disease) can be transmitted horizontally (i.e., associate to associate) (58). To what extent oncogenesis could be controled as an infection process should be thoroughly investigated.
6. Oncornavirus genetic material can reside in the genetic material of the host (94) giving rise to vertical (parent to offspring) transmission of the disease. Oncogenesis is therefore more than just an infectious process and requires study of the entire cellular biochemistry as well as the virus for understanding the oncogenic process.
7. Oncornaviruses cross species barriers. While the murine leukemia and sarcoma virsus have been adapted to grow in rat (51) and human cells (1), feline leukemia and sarcoma viruses can infect and cause disease in dogs (89), rabbits, marmosets and monkeys (111) without adaptation. FeLV and FeSV also grow very well on human cells (64) transforming them in the latter case (98) without adaptation. The feline leukemia and sarcoma viruses are therefore important to study both epidemiologically and biochemically since their role in human neoplasia is an open question (114).

The feline thymus tumor cell line F-422 (89) used in this study has the advantages of growing rapidly and producing large quantities of virus (3-4 mg viral protein/liter/24 h.) (52). It therefore is an excellent system for studying viral replication and for producing

virus for structural studies. A disadvantage is that no uninfected thymocyte line is available as a control cell.

The purpose of this study was three-fold: (1) to study the effects of the cell cycle on virus replication, (2) to measure the rate of viral RNA synthesis and incorporation into intravirion RNA, and (3) to characterize the intravirion RNA with respect to molecular weight and percentage composition of molecules present and to establish preparative procedures for further analysis of the RNA.

One objective of this laboratory is to study the intracellular origin and behavior of FeLV RNA as well as the in vitro translation of FeLV RNA. In view of this it was imperative that a careful characterization of the intravirion RNA be made. At the time these studies began one paper existed on the characteristics of FeLV RNA (65).

LITERATURE REVIEW

There are many recent reviews on the oncornaviruses (53, 57, 109, 110, 114, 120). The purpose of the following review is to focus only on that literature which guided my experimentation and to bring the results which I have obtained into perspective.

1. Chemical Composition of RNA Tumor Viruses

AMV is composed of 35% lipids, 63% protein, and around 2% RNA (16). Based on similarities in structure, all oncornaviruses are assumed to have this general composition.

Lipids.--Lipids are present in the form of phospholipids and are evidently all derived from host cell origin since no unique phospholipids are found in viruses which are not found in the membranes of uninfected host cells (88).

Proteins.--The structural proteins of FeLV-R have been characterized (52). In summary these include two glycoproteins of 200,000 and 80,000 daltons mol wt, and five other proteins of 27,000, 18,000, 15,000, 12,000, and 12,000 daltons mol wt.

These totaled would translate to a single-stranded RNA gene equivalent to approximately 1.1×10^6 daltons mol wt in size.

The extent to which the carbohydrates found in the glycoproteins is coded for by the virus is unknown.

Nucleic acids.--The nucleic acid in oncornaviruses is RNA. Very minor amounts of DNA reported are very likely of cellular origin (117). All RNA is single-stranded and is susceptible to digestion by pancreatic ribonuclease.

2. RNA of RNA Tumor Viruses

Table 1 summarizes the analyses of native (undernatured) RNA species extracted from several oncornaviruses. Methods of analysis include both velocity sedimentation in neutral sucrose gradients and gel electrophoresis.

Table 2 summarizes the analyses of denatured high molecular weight (genomic) RNA from several oncornaviruses. Methods of analysis include velocity sedimentaion in neutral sucrose gradients and gel electrophoresis.

The native RNA from all oncornaviruses studied to date is resolved for the most part into two major size classes: a 50-75S class and a 4S class. In some cases minor amounts of RNA are seen between these two extremes. The high molecular weight 50-75S RNA is the putative genome, and itself is an aggregate of 3-5 smaller 28-38S molecules and some 4S molecules. What follows is a detailed review of viral RNA. Native RNA refers to undernatured RNA as it is found in the virion and 70S-associated in the titles refers to that RNA which becomes apparent after denaturing 50-70S RNA; 70S in this case is used as a general term for the high molecular weight genomic RNA which actually ranges from 50 to 75S depending upon the report.

Table 1.--Native RNA from RNA tumor viruses.

Year	Virus	Species of RNA present (% of total)						Reference
		50-75S	35S	28S	18S	7-10S	4S	
1965	Bryan RSV	64S(70-80%)	. .	. .	. .	. .	4S(20-30%)	(91)
1965	AMV	71S(30-60%)	. .	. .	. .	4-10S(40-70%)	. .	(90)
1969	AMV	65S(70%)[a]	36S(9%)[a]	. .	. .	. .	4S(21%)[a]	(42)
1969	Rauscher MLV	$1.2x10^7$ daltons(95%)	. .	. .	. .	. .	. .	(7)
1970	Bryan RSV with RAV_1	75-80S(65-70%)	. .	28S(6%)	18S(4%)	7S(3%)	4S(20-25%)	(12)
1970	SR-RSV	70S(68%)	. .	28S(6%)	18S(4%)	7S(3%)	4S(19%)	(12)
1971	FeLV-Strain 5	75S(35%)[a]	35S(14%)[a]	. .	. .	4-10S(51%)[a]	. .	(65)
1971	MLV	70S(67-72%)	. .	. .	. .	4-12S(33-28%)	. .	(122)
1972	RSV Prague 5 min	68S(44%)[a]	36S(16%)[a]	28S(10%)[a]	18S(15%)[a]	. .	4S(16%)[a]	(22)
1972	RSV Prague 24 h.	68S(69%)[a]	36S(14%)[a]	. .	. .	. .	4S(17%)[a]	(22)
1973	MSV-MLV	69S(80%)[a]	. .	. .	. .	. .	4S(20%)[a]	(79)
1973	MSV-SD 4h.	50S(88%)[a]	. .	. .	. .	4-10S(12%)[a]	. .	(33)
1973	MSV-SD 24 h.	58S(88%)[a]	. .	. .	. .	4-10S(12%)[a]	. .	(33)
1973	FeLV-R 4 h.	50S(60%)[a]	. .	. .	. .	4-10S(30%)[a]	. .	(34)
1973	FeLV-R 24 h.	58S(60%)[a]	. .	. .	. .	4-10S(30%)[a]	. .	(34)
1973	FeSV-R 4 h.	50S(62%)[a]	. .	. .	. .	4-10S(30%)[a]	. .	(34)
1973	FeSV-R 24 h.	58S(62%)[a]	. .	. .	. .	4-10S(30%)[a]	. .	(34)
1973	Moloney MSV	70S(62%)[a]	. .	28S(17%)[a]	18S(8%)[a]	8S(5%)[a]	4S(8%)[a]	(68)

[a]Calculated by author from published data.

Table 2.--Subunits of 50-75S RNA from RNA tumor viruses.

Year	Virus	Species of RNA present (% of total)					Reference
		35S	28S	18S	7-10S	4S	
1969	MLV	35-44S(90%)	. .	. .	4-10S(10%)		(7)
1971	AMV	35S	. .	. .		4S(1-2%)	(43)
1971	MLV	38S(75%)[a] ($4x10^6$ daltons)	28S(10%)[a]	18S(8%)[a]	. .	4S(7%)[a]	(122)
1972	RSV-prague 5 min	36S(33%)[a]	28S(19%)[a]	18S(17%)[a]	. .	4S(32%)[a]	(22)
	RSV-prague 24 h.	36S(19%)[a]	10-36S(60%)[a]			4S(15%)[a]	
1973	MSV-MLV	37S(50%)[a]	. .	18S(20%)[a]		4S(30%)[a]	(79)
1973	MSV-SD	28S(78%)		. .	. .	4S(22%)	(33)

[a]Calculated by author from published data.

Native 4S RNA.--By the criteria of elution from methylated albumin kieselguhr columns, mobility during polyacrylamide gel electrophoresis and degree of methylation, but not by the criterion of nucleotide composition, the 4S RNA in RSV was identical to cellular 4S RNA (12). Bishop, et al., suggested that viral 4S RNA is cellular 4S RNA somehow carried along into the virion, and that differences in nucleotide composition between the two reflect population differences perhaps due to a selection process operating during incorporation into the virion (12).

The suggestion that 4S RNA is a breakdown product from 70S RNA (7) is negated by base compositional differences between 4S RNA and 70S RNA, and the degree of methylation (12).

A number of laboratories have demonstrated that at least some of the 4S RNA from AMV can undergo aminoacylation and is therefore transfer RNA (38, 40, 93). When testing for amino acid accepting activity of 4S RNA from AMV a non-random distribution of viral tRNA was found by comparison to the tRNA population of normal liver or from AMV infected leukemic myeloblasts (47). This suggested a selective incorporation. There appeared to be no selectivity for isoaccepting tRNA species, however, when the isoaccepting species were analyzed by reversed-phase 5 chromatography.

Seventy S-associated 4S RNA.--4S RNA molecules were reported to be associated with the 60-70S RNA molecules of SR-RSV, AMV, and MSV-MLV which dissociate under denaturing conditions of heat, DMSO or formamide (39, 40, 45). It represents 2.5 to 4% of the 70S RNA, is

present at the rate of 4 to 5 molecules per 35S RNA molecule and is structurally similar to tRNA by the criterion of base composition.

Rosenthal and Zamecnik established that in AMV, the 70S-associated 4S RNA as well as free 4S RNA contained tRNA on the basis of ability to esterify amino acids and their minor base composition (92, 93). Since the percentage of minor base composition was lower in the 70S-associated species than in cellular 4S RNA, they inferred that an additional 4S molecule was also present in association with 70S RNA.

It was suggested early that 70S-associated 4S RNA possibly functions as a linker molecule holding 35S subunits together (39) or as a primer molecule for DNA synthesis by the RNA-directed DNA polymerase (20, 107, 118). Recent studies favor the hypothesis that some, but probably not all, of the associated 4S RNA functions as a primer (23). Canaani and Duesberg found that the T_m of 60-70S RNA in RSV dissociating into subunits of 35S and 4S was 55°C, whereas the T_m for loss of reverse transcriptase activity (due to loss of primer) was 70°C (40). They concluded from this that most of the associated 4S RNA does not have primer activity, but that the primer is a small molecule less than 10S in size. Dahlberg, Faras, and others have identified the primer molecule for DNA transcription from 70S RSV RNA to be a 4S molecule approximately 75 nucleotides long, having pG at the 5' terminus and $C_pC_pA_{OH}$ at the 3' terminus (23, 45). All primer molecules had a common identity with respect to base composition, and as a class differed in base composition from other tRNAs.

Native 5S RNA.--Free 5S RNA was reported in MSV (38, 68) but no function was proposed for it.

Seventy S-Associated 5S RNA.--A 5S RNA is associated with the 70S RNA of RSV. It is 120 nucleotides in length, constitutes 1% of the 70S RNA and is present at the rate of 3 to 4 molecules per 70S RNA molecule. It has a nucleotide composition and structure identical to the 5S RNA associated with the large ribosomal subunit from uninfected chicken cells. Since it melts from the 70S complex at the same temperature required to dissociate 70S RNA into 35S subunits it is presumed to play a role in linking the 35S subunits together (45).

Native 7S (8S) RNA

Bishop, et al., first reported a 7S RNA species in RSV (13) which was resolved using MAK chromatography. It is single-stranded with a molecular weight of 80,000 daltons, constitutes 3-5% of the total viral RNA, and is present at the rate of 5-10 molecules per virion. It is not methylated and does not hybridize to 60-70S RSV genomic RNA. Its function is unknown. A species like it was not found in cells. Erikson reported that oligonucleotides produced by RNase T_1 digestion of 7S RNA from AMV and from MSV yielded identical fingerprints and suggested that there must be a conservation of sequences among the viruses that replicate in different hosts (41). The same molecule was found in infected host cells, and its function in either case is not known. Two species of 8S RNA were resolved by polyacrylamide-agarose gel electrophoresis from Maloney strain of MSV (68). Its function is unknown.

Seventy S-Associated 7S (8S) RNA

Emanoil-Ravicovitch, et al., report a 70S-associated 8S RNA in MSV (38). It has exactly the same electrophoretic mobility as free 8S RNA from MSV and has a very similar base composition. It was presumed to play a linker role among the subunits.

Native 18S and 28S RNA.--Bishop, et al., report the presence of small amounts of 18S (approximately 4%) and 28S (approximately 6%) in Bryan RSV + RAV_1, and SR-RSV (12). They were resolved by gel electrophoresis and were shown by mixing experiments to result from cellular debris adventitiously purifying with virus.

The 18 and 28S RNA species in immature RSV Prague on the other hand were concluded to be molecules destined for aggregation into the 68S molecule (22).

Seventy S-Associated 18S and 28S RNA.--These are discussed below with 70S RNA and its subunits.

Native 35S RNA.--In three instances, free 30-40S subunits of the genomic RNA was reported (22, 42, 65). They were viewed as pre-assembled genomic RNA subunits.

Seventy S-Associated 35S RNA.--This is discussed below with 70S RNA and its subunits.

Seventy S RNA and Its Subunits.--The 50-75S RNA is believed to be the genetic material of the virus but there is no direct evidence for this since it has not been conclusively shown to be infectious. It is predicted to be the same polarity (+) as the mRNA which codes for viral proteins within the infected cell (9).

It can be seen from table 1 that a wide range in sizes exist among the different virus species from 50S for FeLV-R, FeSV-R, and MSV-SD (33, 34) to 75S for Bryan RSV and RAV_1 (12) for the high molecular weight genomic RNA. This corresponds to a range of molecular weights from 5.7×10^6 daltons to 13.4×10^6 daltons using Spirin's equation (105):

$$M = 1550 \times s^{2.1}$$

Even within one virus group, such as the avian tumor viruses, a maximum sedimentation coefficient difference corresponding to a molecular weight difference of 1.3×10^6 daltons is found (15). The significance of such a wide range in sizes is not yet known. The observed differences in the avian viruses did not correlate with any biological function such as transformation (15).

The 50 to 75S RNA from all oncornaviruses, when collected from a neutral sucrose or glycerol gradient and heated or subjected to agents known to destroy hydrogen bonding such as urea, formamide, formaldehyde, or DMSO, is converted into two size classes, namely 4S and 28-35S. Occasionally intermediate sizes are found. Data from many sources are summarized in table 2. The 70S-associated 4S, 5S, and 7S RNA species are discussed above. McCain, et al., reported that an 18S subunit was a consistent finding in MLV-MSV (79). Its base composition was unlike 18S rRNA. An 18S peak appeared in addition to the 36S RNA in MLV as reported by Watson (122), but no significance was made of it. In most cases, RNA between 4S and 36S in size was heterogeneous and its presence was attributed to degradation of higher molecular weight RNA (7, 28) by nucleases present in the virion (62).

Bader and Steck demonstrated that the heterogeneous RNA increases with the increasing time a virus spends at 37C and this correlates with a decrease in virus infectivity (7).

In virions that are collected over short periods (1-4 h.) and not allowed to age at 37°C, a majority of the 50-75S RNA dissociates into 28-35S subunits. East, et al (32, 33), reported that the subunits from MSV-SD, FeLV-R, and FeSV-R are 28S as determined by sucrose gradient centrifugation, whereas, Bader and Steck (7), report the subunits in MLV to be 35-44S based on sedimentation data. These are the two extreme estimates for sedimentation coefficients reported. In general, it is estimated that 3 or 4 28-35S subunits each with a molecular weight of approximately 2 or 3 x 10^6 daltons are linked together to form a 50-75S molecule which exists at the rate of one per virion. The 28-35S subunits are thought to be held together by double-stranded regions of hydrogen bonding (28, 82). The possibilities that protein or DNA bind the subunits together is ruled out by their resistance to separation upon treatment with pronase or deoxyribonuclease (7).

One notable feature is that the 50-75S molecule denatures to 28-35S molecules in a step-wise fashion. This was demonstrated under controlled conditions by Travnicek and Riman (115). 60-70S AMV RNA was converted to a 50-54S molecule and then to a 30-40S molecule when using increasing formamide concentrations and detected by sedimentation in glycerol gradients. This phenomenon is noted in other published data although not extensively discussed (7, 20, 33, 42).

Cheung, et al. (22), noted that an intermediate molecule of 55-60S (intermediate between 36S and 60-70S) was present in RSV

labeled for 5 minutes and East, et al. (33, 34), noted that an intermediate molecule of 50S (intermediate between 28S and 58S) was present in virions labeled for only 4 h. The common hypothesis is that the molecule of intermediate size is an aggregate with fewer than the maximum number of subunits (i.e., three rather than four or two rather than three). The intermediate then can either be a product of aggregation or disaggregation. Travnicek and Riman concluded that different double stranded regions have different thermal stabilities (115). Canaani and Duesberg found 30-40S subunits present in immature (freshly released) RSV collected at 3 min intervals which assemble to 60-70S molecules upon incubation, but no aggregates of intermediate size were seen (21).

Duesberg and Vogt (30) report the interesting finding that the 30-40S subunits in avian RNA tumor viruses can be grouped into *a* and *b* subunit classes based on electrophoretic mobility in polyacrylamide gels. The *a* subunits are electrophoretically slower than *b* subunits and are found in viruses which can transform cells <u>in vitro</u>, whereas *b* subunits are found in viruses which are unable to transform cells. From oligonucleotide digest studies in which all oligonucleotides from *b* can be found in *a*, but not vice versa, Duesberg hypothesises that *b* subunits arise from *a* by deletion of a piece of genetic material (which includes the gene for transformation) (31, 33, 85). That is, $a = b + x$ where x is the deleted genetic material. On denaturing formamide-polyacrylamide gels, *a* subunits have a measured molecular weight of $2.4\text{-}3.4 \times 10^6$ daltons, and *b* subunits have a measured molecular weight of $2.2\text{-}2.9 \times 10^6$ daltons (31). The *a* subunits are demonstrated to be the cause and not the consequence of transformation by Martin and

Duesberg using mutants which are temperature sensitive for transformation but not temperature sensitive for virus production (77). In these experiments, the *a* subunit was present in progeny virus produced at the temperature restricting transformation. The above results hold true for cloned stocks of virus (32). Sheele and Hanafusa were not able to distinguish clearly between *a* and *b* subunits from transforming and non-transforming avian oncornaviruses using polyacrylamide gel electrophoresis (100). Distinct subunit classes have not been distinguished in the mammalian RNA tumor viruses (33, 34, 123).

One fundamental question concerning the 28-35S subunits remains to be answered. That is whether or not each of the 3 or 4 subunits possess identical genetic information (i.e., a haploid, segmented 70S genome) or possesses unique genetic information (i.e., a polyploid genome) (121). At present, the polyploid model is favored by Duesberg and Vogt (32) simply because it is easier to envision a single deletion giving rise to *b* subunits from *a* than it is to envision a multiple (3 or 4) deletion.

The 3' terminus of AMV is almost exclusively an unphosphorylated uridine residue as determined by periodate oxidation and reduction with tritiated borohydride and analysis of the tritiated base residues following hydrolysis (43). The 3' terminus of MLV (AKR), FeLV, hamster leukemia virus, and viper leukemia virus 35S RNA also contain uridine as shown by the same method (78).

The 5' terminus of RSV 35S RNA is 5'-OH adenosine as determined by radioactive phosphorylation with polynucleotide kinase and analysis of the degraded nucleosides (103).

Poly (A).--At least some of the 35S subunits of the high molecular weight RNA contain stretches of polyadenylic acid (poly (A)) as part of their primary structure (50, 55, 67, 95). This is shown to be true for all of the oncornaviruses looked at to date, i.e., the RSV, AMV, FeLV, Mason-Pfizer agent, MMTV, MSV, and Rauscher MLV. The tract of poly (A) can be selectively isolated and studied because of its resistance to enzymatic degradation by pancreatic and T_1 ribonucleases in high (0.3M) salt (11). It is estimated to be 100 to 300 nucleotides in length from its sedimentation coefficient and electrophoretic behavior, with a corresponding molecular weight of 30,000-60,000 daltons. It comprises roughly 1-3% of the total 70S RNA (50, 55), and is composed of 91-97% adenosine, 6% cytidine, and trace amounts of guanosine and uridine (55). It is estimated to be present in the amount of 1-8 stretches per 70S genome (55). It may not be a part of all 35S subunits, however (55, 67). In one case (67) it was reported to be present in the 4-12S RNA portion of RSV RNA but this was thought to be derived from degraded 35S RNA.

Certain chemical properties of the poly (A) can be utilized to purify it and the additional RNA molecule which is covalently attached to it. (1) It is partitioned into the aqueous phase during phenol extraction in pH 9.0 Tris buffer, and into the phenol phase in pH 7.5 Tris buffer (17). A low ionic strength is necessary for this phenomenon. (2) It binds to Millipore (nitrocellulose membrane) filters in 0.5 M KCl and is eluted in low (no KCl) buffer (70). (3) It binds by hydrogen bonding to oligo(dT)-cellulose in high salt (0.5 M KCl) buffer, and is eluted in low salt (no KCl) buffer (3).

(4) It binds by hydrogen bonding to poly(U) glass filters in high salt buffer and is eluted in low salt (no salt) buffer (102). Each of these properties can be utilized during the isolation and purification of 28-35S subunit RNA free of RNA which does not contain poly(A) such as ribosomal RNA.

Remarkable similarities exist between the covalently attached poly(A) found in the oncornavirus 28-35S subunits and those found in eukaryotic messenger RNA, high molecular weight heterogeneons nuclear RNA, and adenovirus-specific mRNA, the synthesis of which are all in the nucleus of the cell. The poly(A) in these cases is 100-200 mucleotides long (25, 35, 70), resides on the 3'-OH terminus (81), is added to heteogeneous nuclear RNA after transcription is completed (2, 35, 87) and is transported from the nucleus to the cytoplasm. This information alone raises the question of whether the oncornavirus RNA is synthesized in the nucleus in the same fashion. In this respect it would be interesting to know if 3' deoxyadenosine (cordycepin) would inhibit the synthesis or transport of oncornavirus RNA, as it does in the case of adenovirus specific mRNA. Such an effect would doubtlessly manifest itself by affecting virus production and should be seen at this level. It is interesting to note that cordycepin inhibits transformation of mouse embryo fibroblasts by MSV (73).

The full significance of poly (A) is not yet known.

Methylation.--Until recently only ribsomal 28S and 18S RNA and transfer RNA were shown to be methylated (18, 24). Perry and Kelly (86) have shown that mRNA from mouse cells is methylated in

both base and ribose moieties with approximately 2.2 methyl groups per 1000 nucleotides, one-sixth the level of ribosomal methylation. This finding is confirmed and extended by Desrosiers, et al. (26). This raises the question of whether oncornavirus RNA is likewise methylated in view of other similarities existing between viral RNA and mRNA, namely, poly(A).

Erikson (42) labeled avian myeloblasts with (methyl-^{3}H)-methionine and found a high level of methyl labeling in 4S RNA with 19% of the total counts in 65S RNA. Too few counts were available for analyzing the quality of the methylation. Also, the 65S RNA had not been treated so as to remove the associated tRNA-like 4S RNA. McCain, et al. (79), found 15% of the methylated counts in 69S MSV-MLV RNA. After heating all methyl counts sedimented with 4-10S RNA. Base-specific methylase activity, namely N^2-guanine RNA methyltransferase, is associated with AMV, RSV, and RAV_1, and N^2-adenine RNA methyl-transferase is associated with RSV (48). This along with circum-stantial evidence implicating a relationship between nucleic acid hypermethylation and the malignant conversion of cells (106) suggest that a close look should be taken at the methylation of oncornavirus RNA.

Summary of Questions on the High Molecular Weight Subunits.--In summuary, questions remaining to be answered about the 28-35S subunits are:

1. Exactly how many subunits exist for each 50-75S molecule?
2. How are the subunits held together in the 50-75S molecule?
3. Does each subunit have poly(A)? Where is the poly(A) within the molecule? What is the function of poly(A)?

4. Are the molecules methylated? If so, what kind of methylation (base or sugar)? On what nucleotides and where in the molecule does it exist? What is its function?
5. Is each subunit in the 50-75S molecule identical (polyploidy) or unique (haploid-segmented)?
6. What is the primary structure of the subunits?
7. Exactly what genetic information is present?
8. What are the secondary and tertiary structures of these molecules?

3. Replication

Determining what biochemical events take place during oncornavirus replication from the time of infection to the time of progeny virus release proves to be an arduous task since it requires detection and quantitation of minute intracellular quantities of viral components (DNA, RNA, and protein) (97). There is no shut-off or even diminution of cellular synthesis during oncornavirus infection (110).

Two approaches have been taken as a start toward elucidating replication events: (1) to study the kinetics of virus replication in permanently infected cells, and (2) to study the effects of the cell cycle on virus replication in synchronized cells.

Kinetics of Labeling.--The interval between the addition of radioactive RNA precursors (uridine) to infected cells and the appearance of radioactive RNA in progeny virus has been measured for RSV-RAV, MLV systems (6), MLV and AMV systems (10), and the MSV-SD system (33).

Interpreting these results involves knowing the behavior of uridine uptake and distribution by cells. Uridine is actively accumulated in mammalian cells, becomes phosphorylated immediately to UMP, UDP, and UTP and cannot escape from the cells (8). At a level of 20μM radioactive uridine for 10^6 HeLa cells/ml, 80% of the radioactive uridine is taken up by the cells from the medium (8). Intracellular uridine pools are sufficiently large that they cannot be diluted by added uridine within a chase period of 12 minutes (8). A chase period of 4 hr. can effectively dilute the intracellular acid soluble pool to 30% (6). Trichloracetic acid precipitable radioactive cellular RNA is detected as early as 3-5 minutes after addition of label (6, 10).

When infected cells were labeled with a continuous source of radioactive uridine, radioactive RNA from progeny virus appeared as early as 2 h. for RSV-RAV_1 (6), 80 minutes for MLV (6), 30 minutes for MSV-SD (33), and 1.5 hr. for AMV (10).

When infected cells were pulse-labeled 15 minutes with radioactive uridine, radioactive RNA from progeny virus is released at a maximum rate at 5 hr. for RSV-RAV, and MLV (6) and between 3 and 6 hr. for AMV (10).

The above results are interpreted to mean that the minimum interval between labeling of viral RNA intracellularly and its release in progeny virus ranges from 30 min. to 2 hr., and the interval of 5 hr. is an average interval which viral RNA spends in an intracellular pool before being incorporated into a virion (10).

Synchronization.--Early key experiments suggested that oncornavirus replication was under the control of cellular events. RSV had a specific requirement for DNA synthesis during the infections cycle (5, 108) and DNA-dependent RNA synthesis was a requirement for replication at all times after infection (4). Another experiment (60) demonstrated that in synchronized cells, RSV production was seen at early G_1, only after cells had gone through mitosis, and regardless of when cells were infected in the cell cycle. Non-oncogenic RNA viruses show none of these metabolic dependencies for infection and replication. The synthesis and release of RSV was further studied in infected chick embryo fibroblasts synchronized in serum-free medium (71). The synthesis of virus-specific RNA, quantitated by hybridization to *in vitro* synthesized DNA, and of virus-specific protein, quantitated after its appearance into the virion, occurred in early S phase. Virus release occurred in early G_1 phase. Leong postulated that a controling factor was produced during early G_1 which allowed subsequent virus release, and a second factor was produced at the onset of DNA synthesis which was important for RNA and protein synthesis. Kirsten MLV and MSV-MLV production is synchronized in chronically infected cells synchronized by a double thymidine block. Maximal virus release coincided with the time of mitosis in both cell systems and is in agreement with avian sarcoma-leukemia systems as reported by Temin (110), Hobom-Schnegg, et al. (60), and Leong, et al. (71, 85).

The synthesis of cell products have been studied in synchronized cells and appear to be under the control of cell cycle events. I_gG and I_gM production was greatest during G_1 and S phase and smallest

during mitosis in human lymphoid cells synchronized by thymidine and colcemid, indicating that transcription of immunoglobulin genes occurs during a limited portion of interphase (19).

The above methods for cell synchronization utilize chemically modified environments (excess thymidine, isoleucine-free medium, serum-free medium) to effect synchronization. One method which completely avoids chemical modifications is that of Shall and McClellan (101) in which suspended cells from a heterogeneous population are grouped on the basis of size (diameter) by velocity sedimentation differences. The synthesis of cell products by cells synchronized in this fashion was not studied.

MATERIALS AND METHODS

PART I

Source of Cells and Virus

The permanently infected feline thymus tumor cell line, F-422, which produces the Richard strain of feline leukemia virus (FeLV-R) was obtained from Dr. C. G. Rickard (Cornell University). It is propagated in medium which is a mixture of 60% Leibovitz and 40% McCoys and 15% fetal calf serum (Grand Island Biological Co. (GIBCO)). Crandell (CCC) feline kidney cells were obtained from Dr. K. M. Lee (Cornell University) and propagated in McCoys 5A medium and 15% FCS. MOPC-21F cells were obtained from Dr. R. J. Patterson (Michigan State University).

Determining Viable Cell Numbers

Viable cells exclude trypan blue dye and remain colorless. Cells were counted using a hemocytometer in a 0.08% final concentration of trypan blue dye obtained as a 0.4% solution (GIBCO).

Electronmicroscopy of F-422 Cells and FeLV-R

Embedding.--The fixation, agar infiltration and embedding procedures of Howatson (61) were used. Twenty ml of cells (10^6/ml) were fixed directly in situ with an equal volume of 6% glutaraldehyde. Gleutaraldehyde was added slowly while cells were mixing. After fixing for 24 h. at 4°C, cells were pelleted at 1000 r.p.m., 5 min.

in an International PR-6 centrifuge, washed 2X with PBS and pelleted. The cell pellet was mixed with an equal volume of 2% agar at 50°C for 10 min., then cooled. The pellet was thinly sliced and fixed 1.5 h. at 4°C in 1% osmium tetroxide made up in PBS. Slices were dehydrated in sequential baths of 50%, 75%, 95%, and 100% ethanol (3X in 100%), followed by 3 washes in propylene oxide. Slices were embedded in Dow Epoxy Resin (DER)-812 (74), sectioned, and mounted on unfilmed copper grids. Sections were stained with uranyl acetate and lead nitrate before electronmicroscopic examination in a Hitachi-11 electron-microscope.

Negative Staining.--Virus was purified by pelleting followed by isopycnic centrifugation (see below). The viral band was collected then dialyzed 20 h. against 1% ammonium acetate at 4°C. Five µl of the virus preparation was dried onto carbon-coated, collodian-filmed copper grid, then stained 2 min. in 1% aqueous solution of uranyl acetate before electronmicroscopic examination.

Synchronization of F-22 Cells

Thymidine Excess.--The thymidine excess method as described by Mueller and Kajiwara (83) was used. Cells at 0.8 x 10^6/ml in a 25 ml volume were treated with 2mM thymidine for 8 to 20 h. after which cells were washed 1X with HBSS and resuspended in fresh medium containing .01 mM levels of deoxyadenosine, deoxycytidine and deoxy-guanosine. At times 0.1mM, 0.5mM, and 1.0mM thymidine were used in place of 2.0mM thymidine.

Serum-Free Medium.--The serum-free medium method as described by Leong, et al. (71), was used. Cells at 0.5-0.8 x 10^6/ml in a 25 ml volume were grown in medium without serum for 24 h. Cells were then resuspended into fresh medium containing 15% FCS or serum was added to the already existing cultures to make 15% final concentration. Duplicate cell counts were made at hourly intervals.

BSA Gradients.--Sterile continuous gradients of bovine serum albumen were made by a modified method of Miller and Cudkowicz (80). A 35% W/W stock solution of BSA (fraction V power, nutritional Biochemical Corp.) was made using T.N.E. 7.5 buffer (0.01 M Tris, pH 7.5, 0.1 M NaCl, 0.001 M EDTA), and filtered through a millipore (0.22 μM) filter and stored at 4°C. Four ml 35-17% W/W gradients were made in 14 cm x 6.5 cm i.d. home-made test tubes using a gradient maker prewashed with 70% ethanol and dried with 90% ethanol in a laminar flow hood. One to 10 million cells were suspended in 10% BSA solution and centrifuged 2000 r.p.m. for 30 min. at 10°C in an International PR-G centrifuge. Cell bands were collected using a sterile pasteur pipette. This density gradient ranges from 1.0525 to 1.1004 g/cc.

Isoleucine deficient medium.--The method of Tobey and Ley (112) was used. A mixture of Leibovitz-McCoys medium (60:40 respectively) in powder form, without amino acids and without bactopeptone, was obtained from GIBCO. Amino acids and bactopeptone in powder form were obtained separately from the same source. The composition of this medium is described in table 3. Bactopeptone was omitted from the reconstituted medium with no ill consequences for cell viability.

Table 3.--Procedure for reconstituting amino acid-free and bactopeptone-free Leibovitz-McCoys medium (GIBCO medium number 72154) for isoleucine deficiency.

Constituent	Quantity	Solubilized in
Medium (GIBCO medium number 72154, control number 330150)[a]	powder for 5 liters	
$NaHCO_3$	4.7 grams	4.800 liters H_2O
Penicillin	500,000 units	
Streptomycin	500,000 µg	
Amino acids:		
L-α alamine	702.8 mg	
L-arginine	1899.2 mg	
L-asparagine	840.0 mg	
L-aspartic acid	39.9 mg	
L-cysteine	613.4 mg	
L-glutamic acid	44.2 mg	
L-glutamine	1338.4 mg	
glycine	615.0 mg	
L-histidine	1055.0 mg	
L-isoleucine[b]	453.7 mg	
L-leucine	453.7 mg	0.06 liters H_2O
L-lysine	354.4 mg	
L-methionine	254.8 mg	
L-phenylalamine	408.0 mg	
4-hydroxyproline	39.4 mg	
L-proline	34.6 mg	
L-serine	652.6 mg	
L-threonine	935.8 mg	
L-tryptophane	66.2 mg	
L-valine	335.2 mg	
L-tyrosine	936.2 mg	0.005 liters 5N HCl

[a]A mixture consisting of 60% L-15 (Leibovitz) medium without amino acids and 40% McCoy's 5a medium without amino acids and bactopeptone.

[b]Omitted when making isoleucine deficient medium.

Cells at 0.34-0.4 X 10^6/ml were grown 48 h. in medium deficient in isoleucine. Isoleucine was then added to make a final concentration of .608 mM, the concentration found in normal medium. Cells were counted periodically.

Isotopic Labeling of Virus

FeLV-R was isotopically labeled by incubating cells with ^3H uridine (>40 Ci/m mole) or ^{14}C uridine (57 mC/m mole) obtained from New England Nuclear. The exact labeling conditions are described for each experiment.

Purification of Feline Leukemia Virus, FeLV-R

FeLV-R was purified by any one of five methods, the exact method being identified in each experiment. For each method, cell supernatant was first clarified by pelleting cells at 1000 r.p.m. (300xg) for 5 min. in an International PR-6 centrifuge, then further clarified by pelleting cell debris at 10,000 r.p.m. (16,000xg) in a Sorval GSA rotor.

1. Salt Precipitation Followed by Isopycnic Centrifugation.-- Virus was precipitated by adding an equal volume of saturated ammonium sulfate (pH 7.5) at 4°C (93). Precipitate was pelleted by centrifuging 10,000 r.p.m. for 30 min. in a Sorvall GSA or SS-34 rotor, resuspended in 1/100 original volume in T.N.E. 7.5 buffer, and isopycnically centrifuged on a 40-15% w/w sucrose gradient for 4 h. at 25,000 r.p.m. (84,000xg) in a SW 27 rotor or for 2.5 h. at 45,000 r.p.m. (190,000xg) in a SW 50.1 rotor. FeLV-R was recovered from the fractionated gradient at the density of 1.14 g/cc.

2. Polyethylene Glycol Precipitation Followed by Isopycnic Centrifugation.--A 50% solution of polyetylene glycol in T.N.E. 7.5 buffer was added to clarified cell supernatant to make a final concentration of 5% PEG at 4°C. After 2 to 16 h., the precipitate was pelleted, and isopycnic centrifugation was carried out as described above.

3. Pelleting Followed by Isopycnic Centrifugation.--FeLV-R was pelleted through a barrier of 20% w/w sucrose made up in T.N.E. 7.5 by centrifuging 3 h. at 25,000 r.p.m. in a SW 27 rotor at 4°C or 1.5 h. at 45,000 r.p.m. in a SW 50.1 rotor at 4°C. Pellet was resuspended in 0.5 ml T.N.E. 7.5 and subjected to isopycnic centrifugation as described above.

4. Pelleting.--FeLV-R was pelleted 2X through a barrier of 20% w/w sucrose as described above. This method is similar to one used by Bader and Steck (7) and virus purified this way is called minimally purified virus.

5. Pelleting Followed by Rate Zonal Centrifugation Followed by Isopycnic Centrifugation.--FeLV-R was pelleted once as described above. The pellet was then suspended in 0.5 ml T.N.E. 7.5, by sonicating 15 sec. in a Branson ultrasonic cleaner, and rate zonally centrifuged in a 30-15% w/w sucrose gradient (22) for 10 min. at 45,000 r.p.m. in a SW 50.1 rotor at 4°C. Virus collected from the fractionated gradient was concentrated by pelleting as described above.

Radioactivity Assay

Virus radioactively labeled with RNA precursors (^{3}H uridine or ^{14}C uridine) was detected and quantitated by totaling the number of radioactive counts isopycnically banded at the density of FeLV-R (1.14 g/cc). To accomplish this, 5 ml gradients (from the SW 50.1 rotor) were fractionated into approximately 0.19 ml fractions from the bottom by dripping with a needle on to 2.3 cm Whatman paper filter pads. The pads were then dried, precipitated 20 minutes in 5% TCA at 4°C, washed 1X with acetone, dried and counted in toluene and POPOP (52).

Infecting Crandell (CCC) Cells

CCC cells were freshly grown to just less than a complete monolayer in 75 cm^2 Falcon plastic flasks. FeLV-R was prepared by growing F-422 cells with greater than 90% viability in 100 ml medium at 3-4x10^6 cells/ml. for 4 h. and purified under sterile conditions. Virus was pellet from clarified cell supernatant at 30,000 r.p.m. (75,000xg) for 1 h. in a type 30 rotor, using 33 ml screw capped polycarbonate tubes. Virus pellets were resuspended in 1 ml total volume, and cells were absorbed with the virus suspension for 1 h. at 37°C, then washed 3X with 10 ml. HBSS. Cells were either assayed immediately for virus production or passed with trypsin at 1:2 or 1:4 dilution and assayed at a later date.

A flask of cells was assayed for virus production by incubating with 5 ml medium containing 16μCi ^{3}H uridine/ml. Virus was purified by PEG precipitation and isopycnic centrifugation and TCA precipitable radioactivity was quantitated as described in the radioactivity assay.

Cordycepin Treatment

To determine the effect of cordycepin (3'-deoxyadenosine) on the growth and viability of F-422 cells, $5x10^7$ cells were incubated at $1x10^6$ cells/ml with 50µg/ml of cordycepin. Viable cell counts were periodically made.

To determine the effect of cordycepin on viral synthesis, $6x10^7$ cells in 1 ml were incubated with 50µg of cordycepin for 15 min., washed 2X with 4 ml medium, then pulsed for 15 min. with 50µCi ^3H uridine in 1 ml. Cells were then diluted to 2 x 10^6/ml with medium and incubated 6 h. Virus was purified by PEG precipitation and isopycnic centrifugation and then assayed by radioactivity content as described above. Control cells were handeled identically except for cordycepin treatment.

RESULTS

Electronmicrographs of F-422 Cells and FeLV-R

Electronmicrographs of plastic-embedded, thin-section F-422 cells are shown in figures 1 and 2. The completed extracellular virion and the budding virus are identified. They fit the description for the FeLV-R by Rickard, et al. (89); the virions being 100mμ in diameter and having a darkly stained, centrally located nucleoid, and the nucleoid material in the budding virus becoming apparent only as the bud is assembling. No intracellular particles with this appearance could be found. Figure 3 is a higher magnification of embedded virions which were grouped and external to cells. Note that the diameter of these particles is 100mμ. Crescent shaped forms have been reported (89). Figure 4 is a high magnification of virus purified by isopycnic centrifugation and negatively stained. Note the non-spherical shapes which is apparently an artifactual phenomenon resulting from the uranyl acetate staining procedure (89).

Synchronization of F-422 Cells

Thymidine Excess.--The doubling time for feline thymus tumor cells, F-422, is between 16 and 20 h. (Fig. 5) as measured by cell numbers. When synchrony of cell DNA occurs as a result of thymidine excess synchrony of cell division also occurs and cell numbers

Figure 1. Electronmicrograph of F-422 cell. F-422 cells were fixed in situ with glutaraldehyde, embedded in DER-812, sectioned and strained with uranyl acetate and lead nitrate. A budding virion is noted (B).

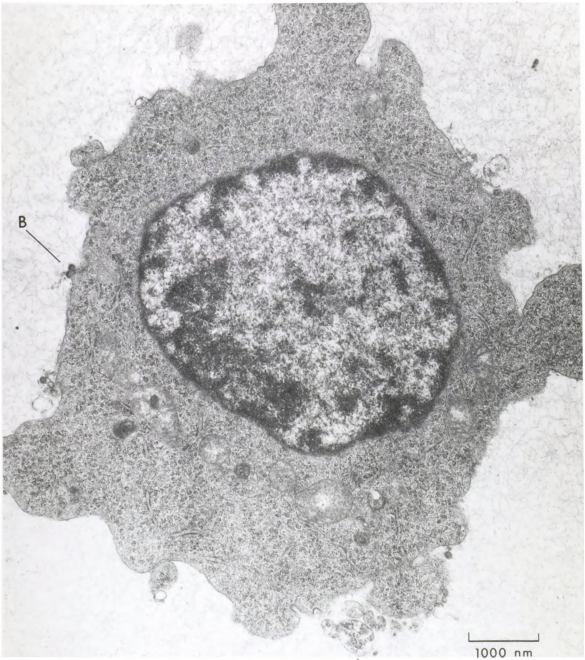

B
1000 nm

Figure 2. Electronmicropgraph of F-422 cells and FeLV-R. Same preparation as described in figure 1. Note virions (V) and budding virions (B).

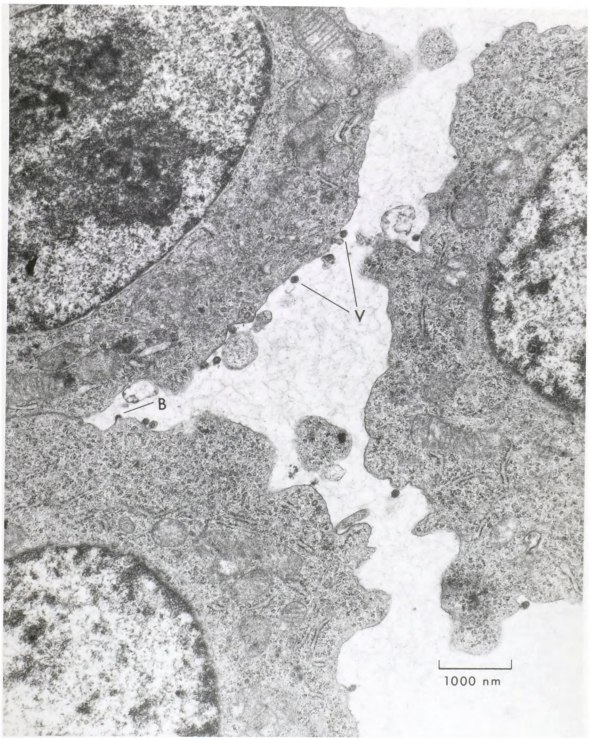

V
B
1000 nm

Figure 3. Electronmicrograph of FeLV-R. Same preparation as described in figure 1.

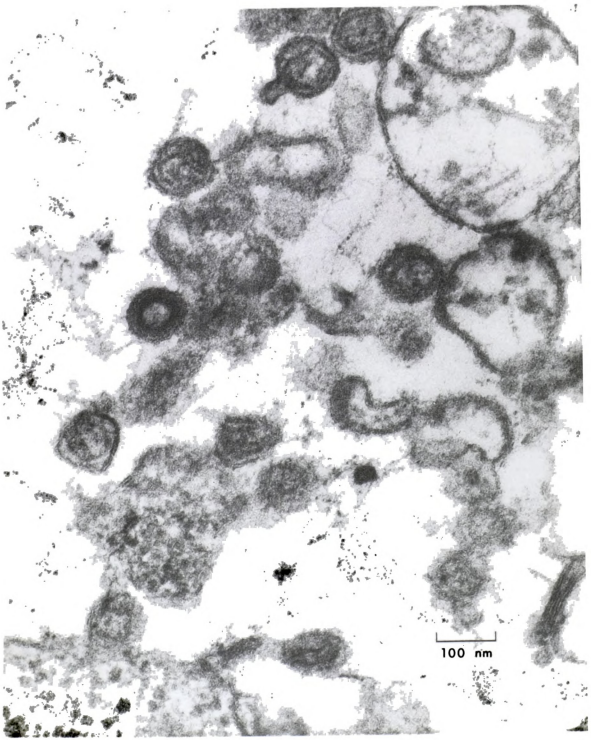

100 nm

Figure 4. Electronmicrograph of purified FeLV-R. FeLV-R purified by isopycnic centrifugation was dialyzed against ammonium acetate and negatively stained with uranyl acetate.

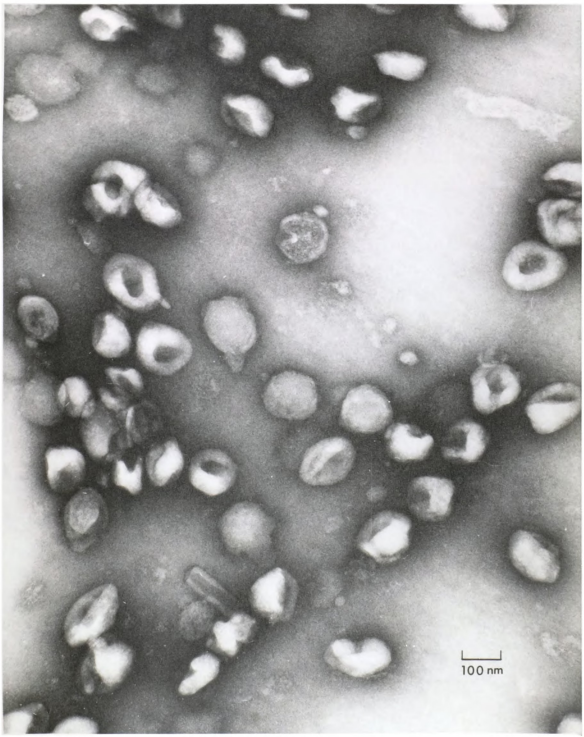
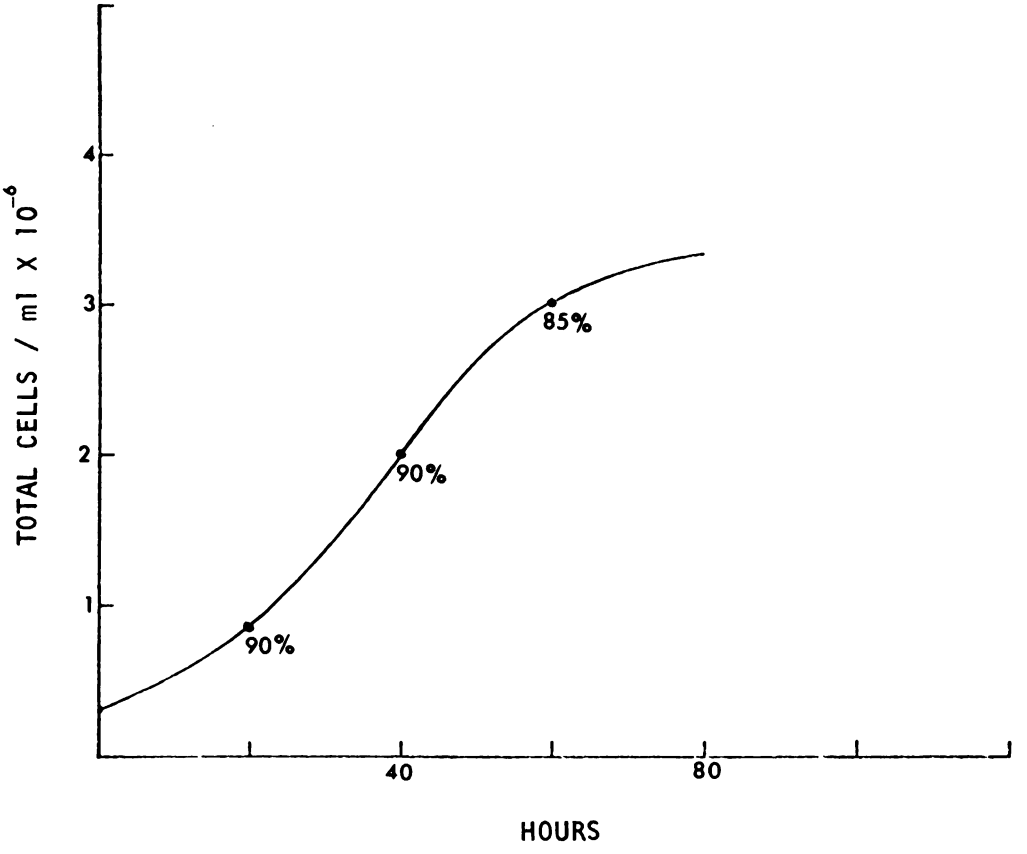

100 nm

Figure 5. Growth curve for F-422 cells. Cells were seeded in fresh medium at 0.3×10^6 cells/ml. Viable cell numbers were determined by using trypan blue dye. The percentage of viable cells is noted.


TOTAL CELLS / ml X 10^{-6}
4
3
2
1
85%
90%
90%
40
80
HOURS

double within a period of 8-10 h. (83). Synchrony was assayed by cell numbers only. As shown in figure 6 thymidine at concentrations of 2 mM had an inhibitory effect on cell replication both while the thymidine was with the cells (expected) and after it was removed (not expected) for a period of 30 h. or greater. There was also a toxic effect since cell viability decreased from 90% to 50% during the experiment. The growth inhibition and toxicity were also seen (1) when concentrations of 1 mM, 0.5 mM, and 0.1 mM were used rather than 2 mM thymidine, (2) when cells were washed free of thymidine and placed in fresh medium or washed free of thymidine and placed in medium supplemented with 1 mM each of deoxycytidine, deoxyadenosine, and dexyguanosine (83), or (3) when cells were treated for periods of 14, 12, 10, and 8 h. rather than for 16 h. prior to washing and resuspending. In each case, cells would not increase in number until after a period of 20 h. and then at a rate of only 25% of controls.

To determine whether F-422 cells do incorporate thymidine an experiment as described in figure 7 was performed. F-422 cells take up thymidine at a rate equal to or faster than MOPC-21F cells.

Serum-Free Medium.--After either (1) resuspending cells into fresh medium with 15% FCS, or (2) adding FCS to a 15% final concentration little cell growth was noted for a period of 10 h. After a 10 h. lag period, cell growth continued asynchronously with a curve paralleling the curve of control cells (data not shown). Cells therefore did not appear to be synchronized with serum-free medium.

Figure 6. Growth curves for F-422 cells during and after excess thymidine treatment. Cells were incubated for 20 h. in 0.4 mM, 0.2 mM, and 0.1 mM thymidine then (at arrow) resuspended in fresh medium containing 0.01 mM each of deoxyadenosine, deoxycytidine, and deoxyguanosine. Control cell (handeled in parallel) growth curves are shown. a. 0.4 mM b. 0.2 mM c. 0.1 mM.

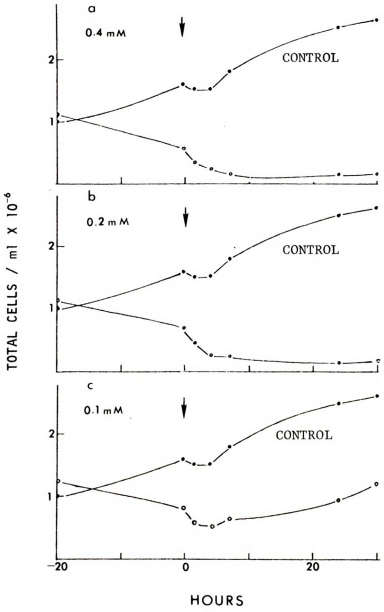
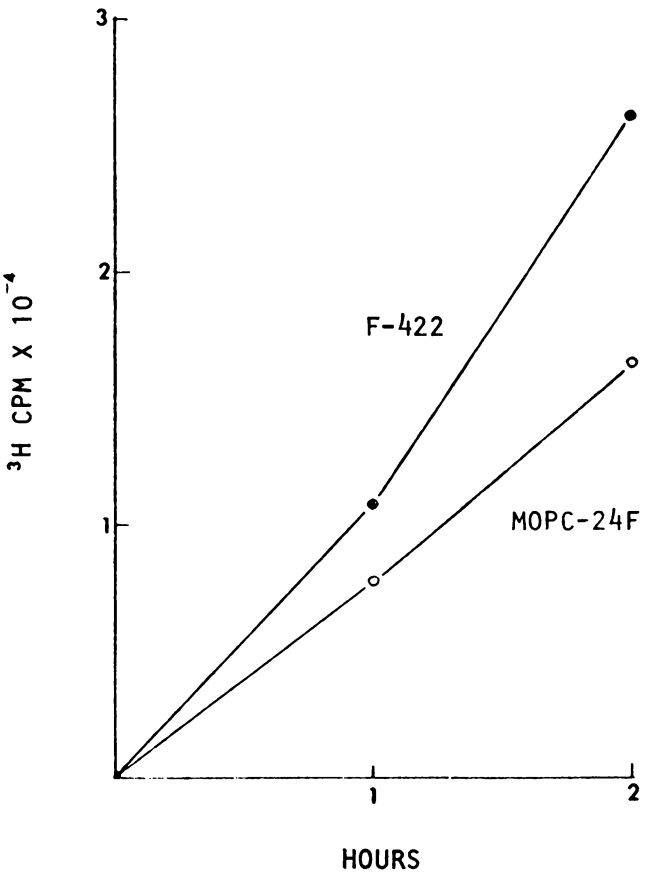

a
0.4 mM
CONTROL
b
0.2 mM
CONTROL
c
0.1 mM
CONTROL
2
1
2
1
2
1
TOTAL CELLS / ml X 10⁻⁵
-20
0
20
HOURS

Figure 7. Incorporation of ^{3}H thymidine into F-422 cells and MOPCI-24F cells. F-422 and MOPCI-24F cells, 1.40×10^{5} of each, were incubated separately in .58 ml medium containing 8μCi ^{3}H thymidine (20 Ci/m mole). At hourly intervals triplicate 0.8 ml samples were taken and precipitated with 1/10 vol. of 50% TCA at 0°C. Precipitate was washed 2X with 5% TCA. The washed precipitate was resuspended by sonication in a Branson ultrasonic cleaner, solubilized in NCS tissue solubilizer, and counted in 5 ml Aquasol.


3
2
1
^{3}H CPM X 10^{-4}
F-422
MOPC-24F
1
2
HOURS

Isoleucine-Free Medium.--All experiments were done with special medium formulated by GIBCO, MED-72154, control number 230628, which, when completely reconstituted with all amino acids, failed to support the normal growth of F-422 cells. It was later discovered, after completion of these experiments, that the medium was improperly formulated by GIBCO, and has since been replaced with MED-72154, control number 330150 which does support the normal growth of cells. Synchronization of F-422 cells was not attempted with MED-72154, control number 330150.

BSA Gradients.--Populations of synchronized cells have been obtained by fractionating an asychronous population of cells based on sedimentation rate differences within the asynchronous population (101).

Attempts were made to likewise fractionate a F-422 cell population on density gradients of BSA (80). Conditions of these experiments were such, however, that isopycnic centrifugation resulted rather than rate-zonal centrifugation. Centrifugation times for longer than 30 min. gave the same results.

During centrifugation two cell bands resulted; a broad band containing greater than 90% of the cells positioned near the middle of the gradient, and a very narrow band resting on top of the 17% BSA. Cells under the light microscope from the large, dense band, appeared as cells from a heterogeneous population. From the upper band all cells appeared to have distinctly visible nuclei. Cells from either band when suspended in fresh medium grew with no detectable synchrony (data not shown) but had a normal growth curve. Cells in the upper

band were presumed to be daughter cells or premitotic cells based on the distinct appearance of the nuclei, but their asynchronous growth did not support this presumption. F-422 cells were not synchronized using this procedure.

Purification of FeLV-R

Because electronmicrographs of F-422 cells and of isopycnically purified FeLV-R hinted at the existence of cell vesicular contamination and because a trailing shoulder (less dense that the virus) was frequently observed during isopycnic centrifugation of the virus, the question was raised whether isopycnic centrifugation alone was enough to purify FeLV-R from cell vesicular contamination, assuming cell vesicles do exist and do have a density near 1.14 g/cc.

The experiment described in figure 8 was performed for the purpose of determining if FeLV-R truely sedimented to its density (1.15 g/cc) in 2.5 h. (an established protocol for isopycnically centrifuging virus in our laboratory). Three observations were made. (1) Yes, the virus is at 1.14 g/cc in 2.5 hr. (2) What appears as a trailing shoulder at 2.5 hr. is a resolved peak at 45 min. (3) No trailing shoulder appears at 15 h., but rather a shoulder appears to be leading toward the bottom of the tube.

Attention was focused on the resolved trailing peak at 45 min. It was hypothesized to be the vesicular contaminant and a sedimentation velocity experiment was performed as described in figure 9. The purpose was to resolve more clearly the two peaks seen in figure 8 a. Two observations were made. (1) Three peaks were resolved rather than two. (2) The most slowly sedimenting peak (the presumed

Figure 8. Isopycnic centrignation of FeLV-R for varying periods of time. F-422 cells ($3.0x10^8$) at $2x10^6$/ml were incubated 16 h. with 4µci/ml of ^3H uridine. Virus was pelleted directly (no sucrose barrier) from clarified cell supernatant, resuspended by sonication in a Branson ultrasonic cleaner in the TNE 7.5, and divided into 3 aliquots: a, b, and c. Each was layered onto a 5.0 ml gradient of 40 to 15% w/w sucrose gradient and centrifuged in a SW 50.1 rotor for (a) 45 min., (b) 2.5 h., and (c) 15 h., at 45,000 r.p.m. The gradients were fractionated by dripping. Ten µl of each fraction was TCA precipitated and assayed for radioactivity as described in Materials and Methods.

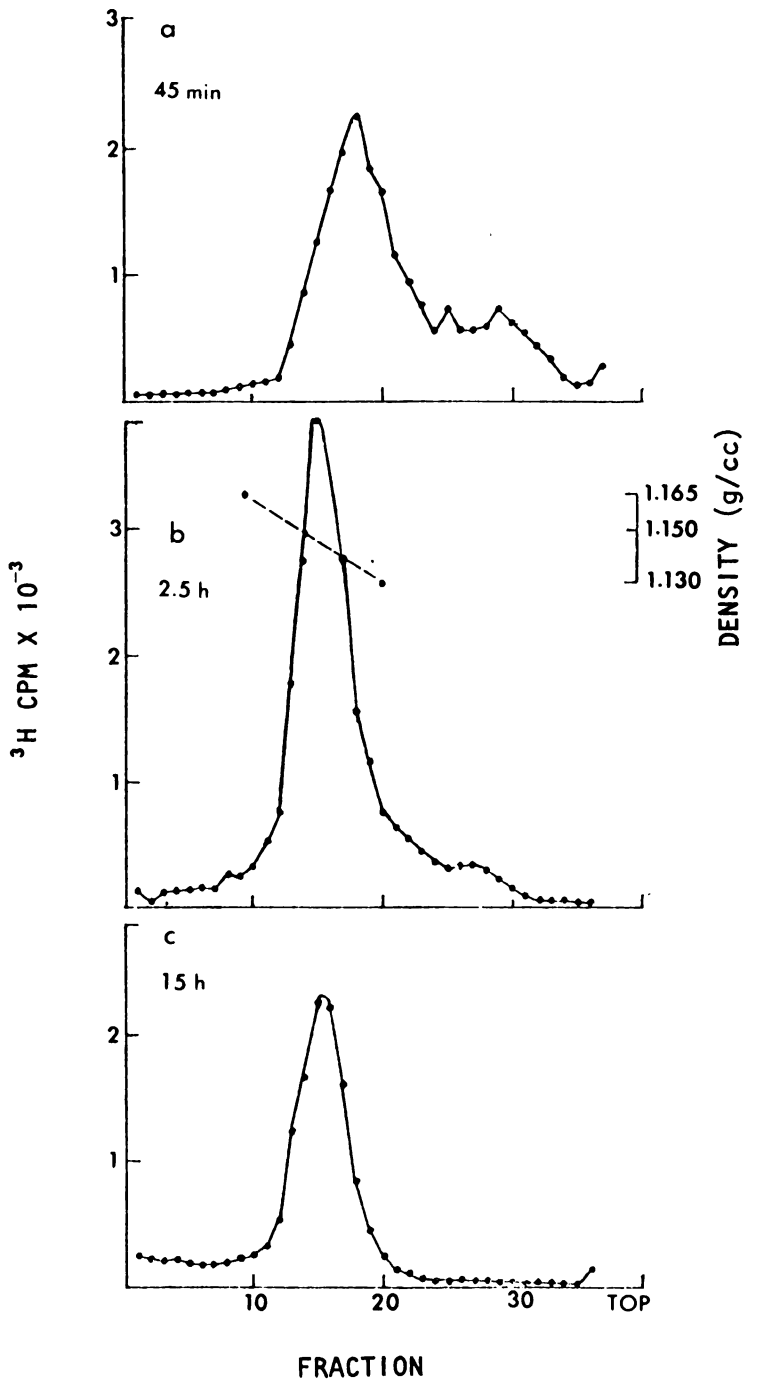
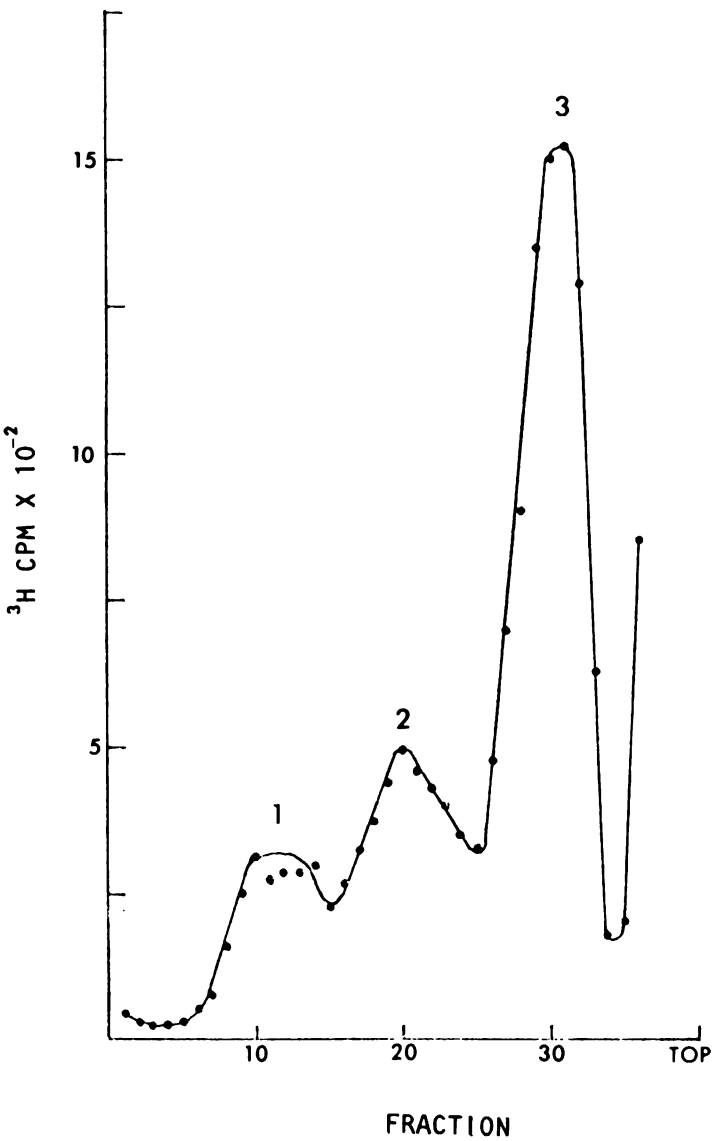

a
45 min
b
2.5 h
c
15 h
1.165
1.150
1.130
DENSITY (g/cc)
^{3}H CPM X 10^{-3}
10
20
30
TOP
FRACTION

Figure 9. Velocity sedimentation of FeLV-R. F-422 cells from experiment described in figure 8 were resuspended in fresh medium with no isotope for a second 16 h. period. Virus was pelleted directly (no sucrose barrier) from clarified cell supernatant, resuspended by sonication in a Branson ultrasonic cleaner in TNE 7.5 and centrifuged 15 min. through a 35-15% w/w sucrose gradient. The gradient was fractionated by dripping. Ten µl of each fraction was TCA precipitated and assayed for radioactivity as described in Materials and Methods. Peaks were numbered 1 (fastest sedimenting), 2, and 3 (slowest sedimenting).


3
15
10
5
^{3}H CPM X 10^{-2}
2
1
10
20
30
TOP
FRACTION

vesicular contaminant) contained radioactivity roughly equivalent to that in the combined first and second peaks.

The peaks were labeled 1, 2, and 3 and pooled separately. Two further experiments were performed. (1) Aliquots from each peak were isopycnically centrifuged 2.5 hr. (Fig. 10a,b,c). Radioactive particles from peaks 1 and 2 sediment to a density of 1.14 g/cc whereas material from peak 3 has a broad density range clearly less than 1.14 g/cc, peaking at approximately 1.125 g/cc. Note that the position of the radioactive peak in figure 10c is such that if it were proportionately integrated into either figure 10a or b, a prominant trailing shoulder would be seen. (2) The RNA from each peak was extracted and analyzed on 2.0% polyacylamide -0.5% agarose gels. The results are summarized in table 4. Note that peaks 1 and 2 contain 50-60S viral RNA whereas peak 3 does not. Peak 3 contains very little 4S RNA.

Infection of Crandell CCC Cells

Jarrett (66) reported that feline embryonic cells can be infected with a multiplicity of 10^3 particles/cell in a 2 h. period and that progeny virus production peaked at 24-48 h. post-infection. The rate of virus production by F-422 cells was unknown. The rate of 40 particles/cell/h. for MLV (10) was used as an approximation and judging from the rate of viral protein production, 3-4 mg protein/liter/24 h. (52) for F-422 cells, the rate of 40 particles/cell/h. for F-422 cells did not seem to be an unreasonable estimation. Since oncornavirus infectivity is known to decrease with the increasing age

Figure 10. Isopycnic centrifugation of FeLV obtained from velocity sedimentation gradient. Fractions comprising peaks 1, 2, and 3 from the gradient described in figure 9 were pooled separately. An aliquot from each pool was isopycnically centrifuged on a 5.0 ml 40-15% w/w sucrose gradient for 2.5 h. at 45,000 r.p.m. in a sw 50.1 rotor. a. Material from peak 1. b. Material from peak 2. c. Material from peak 3. Gradients were fractionated and assayed for radioactivity as described in Materials and Methods.

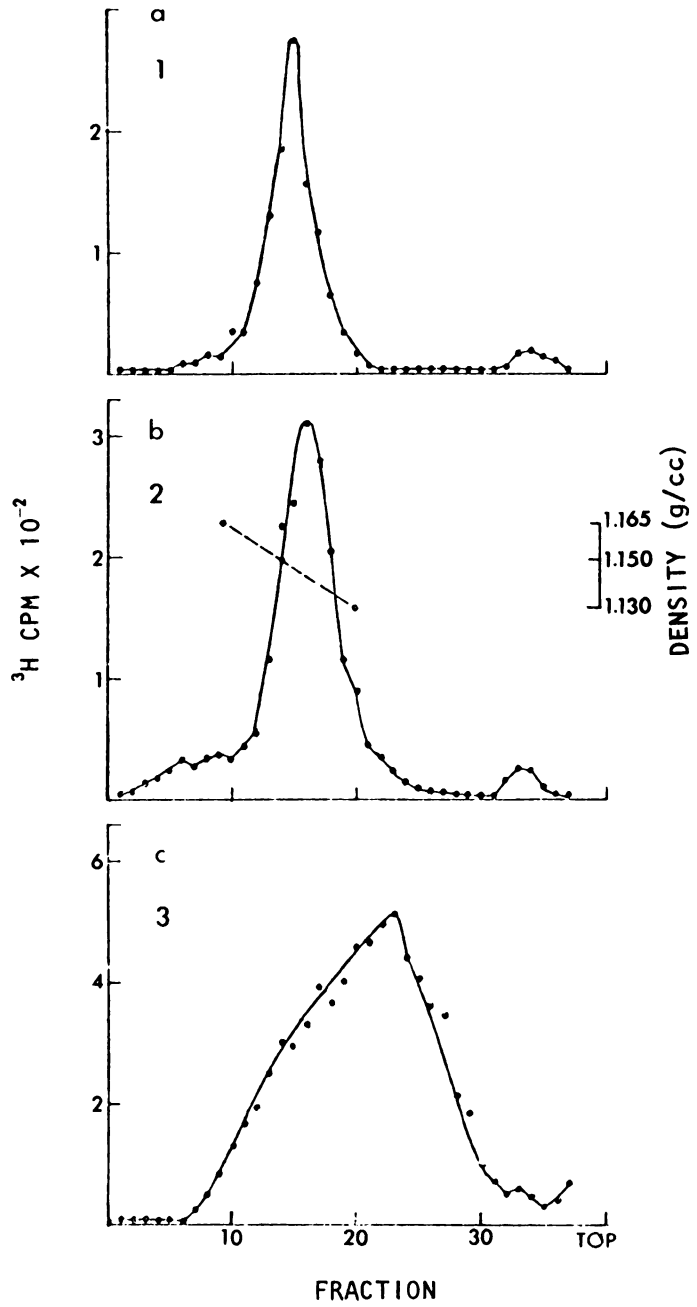

a
1
2
1
b
2
3
2
1
1.165
1.150
1.130
DENSITY (g/cc)
^{3}H CPM X 10^{-2}
c
3
6
4
2
10
20
30
TOP
FRACTION

Table 4.--RNA content in constituents resolved by velocity sedimentation.

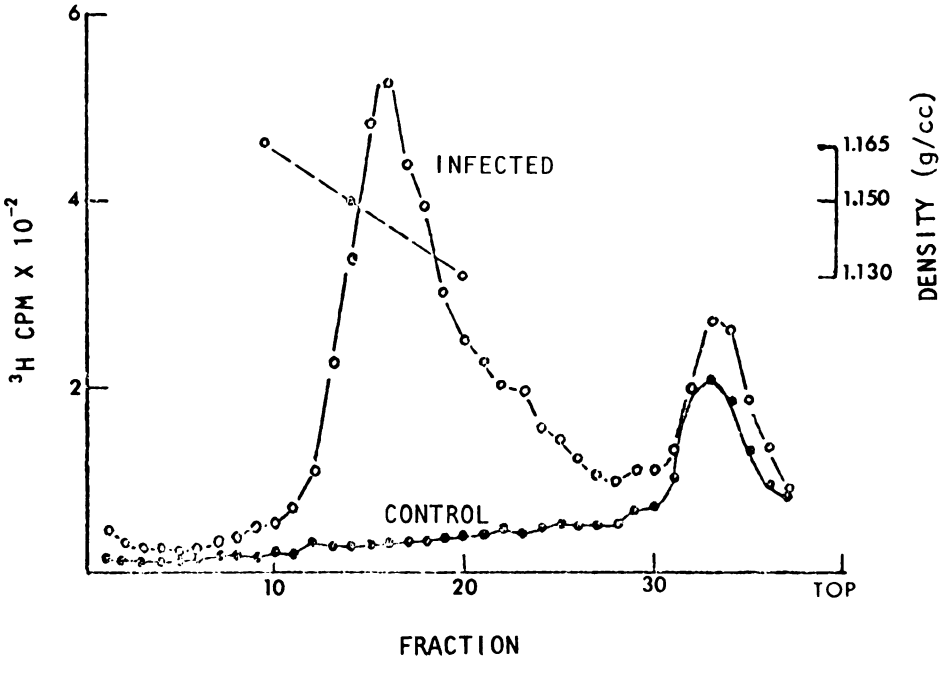
RNA	Peak 1		Peak 2		Peak 3	
	Cpm	%	Cpm	%	Cpm	%
Total	19,210	100	35,500	100	11,070	100
35-60S	1,470	7.7	4,170	11.7	100	.9
10-35S	7,290	37.9	13,230	37.3	8,300	75.0
4-10S	10,450	54.4	18,100	51.0	2,670	24.1

of virus (7), virus was freshly produced and rapidly purified under sterile conditions of infection.

To determine if CCC cells could be infected the experiment as described in figure 11 was performed. Control (uninfected) flasks were mock infected and handeled in parallel to infected flasks. Flasks (control and infected) were assayed for virus production at 24-36 h., then after the first, fourth, and seventh passages corresponding to 2, 11, and 31 days post infection. The results of the fourth passage (11 day) assay are shown in figure 11. Results for the 24-36 h. flask and the seventh passage flask are similar having 71% and 47% respectively the number of counts in the viral peak of the fourth passage flask. Infected cells were frozen (52) and stored at -80°C and in liquid nitrogen.

To determine the eclipse period for infection, i.e., the interval between infection and first appearance of progeny virus, two separate but identical experiments were done. CCC cells were infected as described in Materials and Methods for two flasks. The first flask was incubated with 80µci ^{3}H uridine in 5 ml medium for 6 hr. periods beginning at 12 h., 20 h., 28 h., 36 h., and 44 h. post-infection. For each 6 hr. period, virus was purified, isopycnically centrifuged and the gradient fractionated for radioactivity assay. The second flask and an uninfected control flask were incubated with 80µci ^{3}H uridine in 10 ml medium from 12 to 44 hrs. post infection. From these, virus was purified, isopycnically centrifuged, and the gradient fractionated and assayed as described above. In no case was a ratioactive peak at the density of 1.14 g/ml observed. Because all

Figure 11. Infection of Crandell CCC cells with FeLV-R. Feline kidney cells (Crandell CCC) in a monolayer were exposed to a suspension of FeLV-R pelleted from the supernatant of permanently infected feline thymus tumor F-422 cells (see Materials and Methods). After 4 passages (11 days) a 75 cm^2 monolayer of the infected Crandell cells was exposed to 80µci ^3H uridine in 5 ml media for 12 h. Labeled virus was precipitated from clarified supernatant fluid with 5% PEG and isopyinically centrifuged 2.5 h. at 45,000 r.p.m. in a 5 ml gradient of 40-15% w/w sucrose. The gradient was fractionated and assayed for radioactivity as described in Materials and Methods.


6
4
2
^{3}H CPM X 10^{-2}
INFECTED
CONTROL
1.165
1.150
1.130
DENSITY (g/cc)
10
20
30
TOP
FRACTION

infected cells were used for radioactive labeling, none was passaged, and hence future testing for virus production could not be done.

Effect of Cordycepin on Cell Viability

When F-422 cells are incubated continuously with 50µg/ml of cordycepin cell viability decreases from 90% to 50% by 4 h. and to less than 10% by 8 h. (data not shown).

Effect of Cordycepin on Virus Production

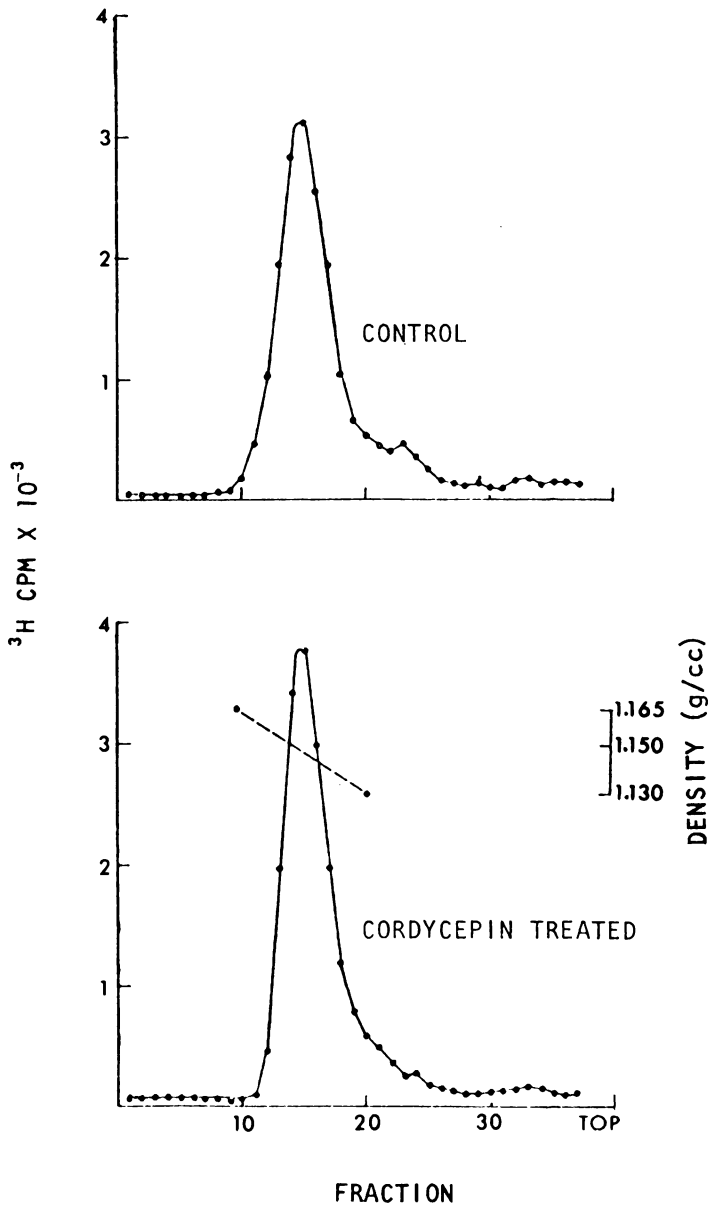
Cells were concentrated and pulsed with cordycepin at 50µg/ml, washed and pulsed with ^{3}H uridine and incubated in medium for 4.75 h. This is essentially the procedure used by Philipson et al. (87). As demonstrated in Figure 12b no inhibition of virus production is observed. In fact, radioactivity in virus from cordycepin cells represents 109% of the radioactivity in virus from control cells.

Kinetics of Incorporation of Uridine Into FeLV-R

It was useful for several reasons to study the kinetics of FeLV RNA synthesis and incorporation into released virions. (1) This is a necessary parameter to know before one can study the intracellular pulse-labeled viral RNA, part of a long-range study by this laboratory. (2) It was important to determine what minimum interval was needed after pulse-labeling cells before analytic quantities of viral RNA could be obtained. (3) It was important to compare the feline leukemia virus system with the murine and avian leukemia virus systems previously studied (6, 10).

Permanently infected F-422 cells synthesize virus continuously and presumably at a constant rate. Basic assumptions for these

Figure 12. Effects of cordycepin on FeLV-R production. Sixty million F-422 cells were incubated with 50μg cordycepin in 1 ml of medium for 15 min., washed 2X with 4 ml medium, then pulsed 15 min. with 50μci ^{3}H uridine in 1 ml, diluted to $2x10^{6}$ cells/ml and incubated 4.75 h. Labeled virus was precipitated from clarified supernatant fluid with 5% PEG and isopycnically centrifuged 2.5 h. at 45,000 r.p.m. in a 5 ml gradiant of 40-15% w/w sucrose. The gradient was fractionated and assayed for radioactivity as described in Materials and Methods. Control cells were treated identically except that cordycepin was omitted from the first step.


4
3
2
1
CONTROL
^{3}H CPM X 10^{-3}
4
3
2
1
CORDYCEPIN TREATED
1.165
1.150
1.130
DENSITY (g/cc)
10
20
30
TOP
FRACTION

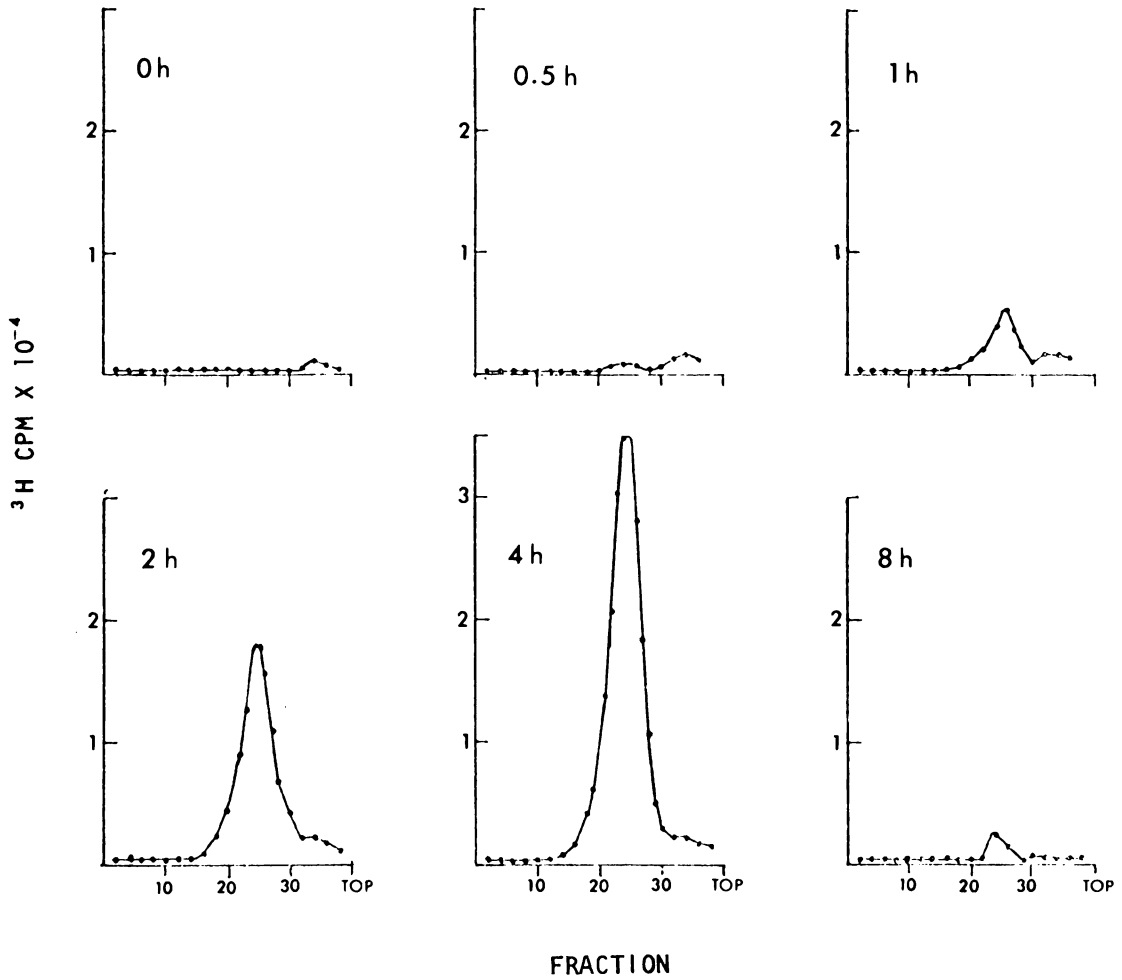
studies were that (1) acid precipitated uridine counts represent RNA, (2) viral RNA was synthesized at a constant rate and transported into released virions at a constant rate, and that this rate remained constant during cell manipulations, (3) viral RNA is synthesized at the same rate as cell RNA, and (4) viral RNA is synthesized from the same precursor (soluble) pool as cell RNA.

Baluda and Nayak (10) and Bader (6) have measured two aspects of viral RNA labeling kinetics: (1) a minimum interval between the addition of labeled uridine into a cell culture and the release of labeled RNA in virus, and (2) an average interval, i.e., the average time (half-life) that labeled viral RNA remains in the cell before being released in virus.

Continuous Labeling, Minimum Interval.--Baluda and Nayak (10) reported a minimum interval for labeling AMV RNA in avian myeloblasts to be within 2 h. of adding radioactive uridine to the cells, Bader (6) reported this interval to be 2 h. for RSV-RAV, and 70 min. for Rauscher MLV.

To determine this interval in the permanently infected feline thymus tumor cell, F-422, for FeLV-R RNA, the method of Baluda and Nayak (10) was used. F-422 cells, which normally grow to concentrations of $2\text{-}3\text{x}10^6$/ml, were concentrated to $10\text{x}10^6$/ml and incubated in the presence of ^{3}H uridine. Periodically equivolume samples were removed from the incubation mixture, the virus was purified, and the RNA quantitated by the number of counts in the purified virus. Two experiments were done as described in figure 13. A definite virus peak, 2.4% of the 4 h. peak, was seen for virus purified 30 min. after

Figure 13. Incorporation of ^{3}H uridine into purified virions with continuous labeling. F-422 cells at 10^{6}/ml were incubated with 4µci ^{3}H uridine/ml. At 0, 0.5, 1, 2, 4, and 8 h., 6.5 ml samples were taken. Virus was purified by ammonuim sulfate precipitation and isopycnic centrifugation, and the gradients were fractionated and assayed for radioactivity as described in Materials and Methods.


0h
0.5 h
1h
2h
4h
8h
^{3}H CPM X 10^{-4}
FRACTION
10
20
30
TOP

addition of labeled uridine. In both experiments, virus was detected at 30 min. Making the assumptions stated above, this clearly establishes a shorter minimum interval than those described for AMV, RSV-RAV, or MLV (6, 10). A second observation was made in this experiment. As noted in figure 13, virus counts decrease after 4 h., down to 5% at 8 h. of what is seen at 4 h. This suggested that the conditions of this experiment, perhaps, the artificially high cell density, resulted in something destroying viral integrity such that either (1) virus no longer purified at its normal density, or (2) virus no longer contained acid precipitable RNA. The fact that no radioactive peaks were seen in any other position in the gradient argues against the first possibility.

To test the second possibility, ^{14}C uridine labeled virus was purified from another source and added exogenously to a culture mocking the labeling conditions described in figure 13. The results are shown in table 5. The total counts in progressive aliquots surprisingly did not stay constant, as one would expect if viral integrity were being preserved, or decrease, as one would expect if viral integrity were being destroyed. While no cause for the vanishing labeled virus could be found, it was presumed to result from the artificially high cell concentration. Further experiments were done by keeping the cells at their normal, growing concentrations ($2\text{-}3x10^6$/ml) except for brief periods of pulse labeling.

To determine what portion of the minimum interval measured (30 min.) for FeLV-R is due either to (1) a delay of ^{3}H uridine into the cell, or (2) a delay of RNA synthesis from the cellular precursor

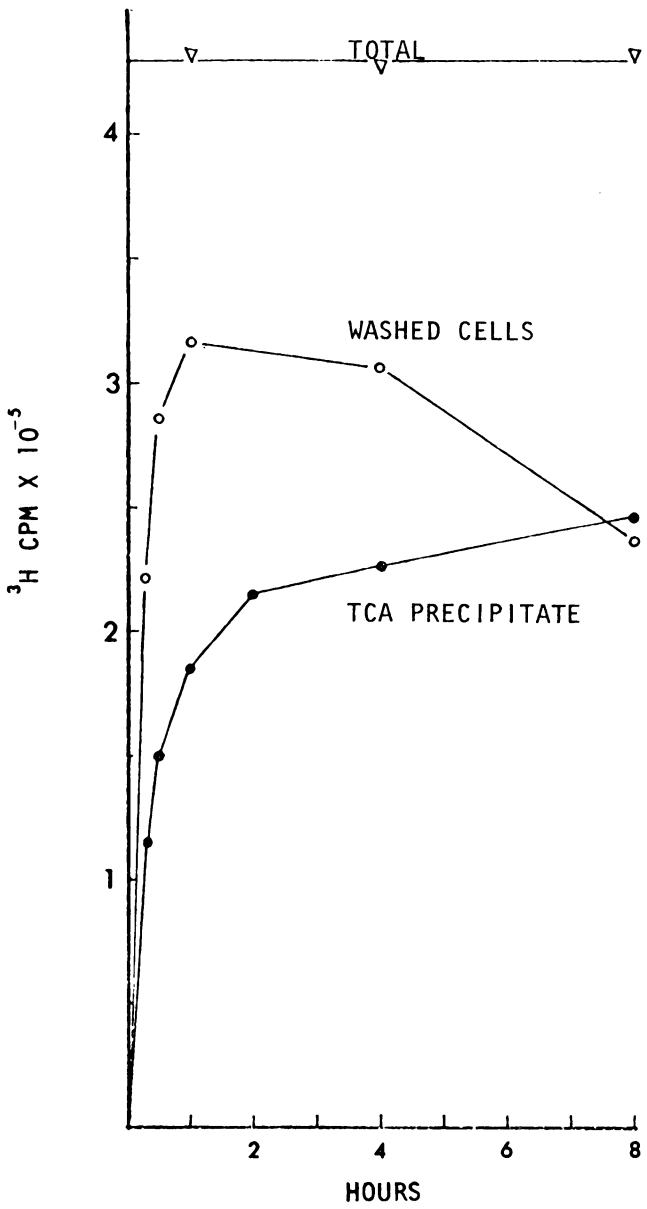
Table 5.--Behavior of exogenously added ^{14}C uridine labeled FeLV-R in tissue culture.

h. after adding exogenously labeled virus	% of maximum peak	
	experiment 1	experiment 2
0	60	23
1	88	40
2	100	40
4	97	. .
5.5	. .	40
6	88	. .
8	. .	100
10.5	70	. .

pool (uridine and its phosphorylated products) the following experiment was done (Fig. 14). Cells at 2-3 x 10^6/ml were incubated with ^{3}H uridine, and periodically the total amount of intracellular uridine and total TCA precipitable material were measured. The results indicate that as early as can be detected (<5 min.) both radioactive uridine and precipitable counts are seen, and by 15 min. after the beginning of labeling 69% of the maximum intracellular radioactivity is seen. Thus, if any delay into released virion RNA results from a delay of ^{3}H uridine uptake into the cell or from a delay during ^{3}H uridine to the site of synthesis, it can be no greater than 5 min. Additional observations are made from this experiment. (1) By 1 h. 74% of the total uridine introduced into the cell culture becomes taken up by the cells. (2) The TCA soluble (precursor) pool is maximum at 1 hr., representing 42% of the intracellular radioactivity.

Pulse Labeling, Average Interval.--Baluda and Nayak (10) measure the average interval for AMV RNA to be between 3 and 4 h. For the purpose of determining the average interval that viral RNA remains intracellular before being released in a virion, certain labeling conditions had to be established. (1) Cell concentrations must be such that labeled, released virions could be assuredly quantitated. (2) Cells must survive a short period (15 min.) of concentration for pulse-labeling for the purpose of maximum isotopic utilization. (3) Cells must survive repeated pelleting and resuspension for the purpose of monitoring virion (and hence virion RNA) release during the chase period. The following experiments confirmed that these conditions could be established.

Figure 14. Incorporation of ^{3}H uridine into the acid soluble pool and into RNA in F-422 cells. F-422 cells were incubated at 3×10^{6}/ml with 30 μCi/ml ^{3}H uridine. At 0, 0.25, 0.5, 1, 2, 4, and 8 h. after addition of the isotope, 0.1 ml samples were taken in triplicate. For each sample (1) the total radioactivity was obtained after solubilization in NCS tissue solubilizer, (2) the radioactivity in washed cells was obtained after solubilization and (3) the radioactivity in washed TCA precipitate was obtained after solubilization. NCS solutions were counted in 5.0 ml Aquasol.


TOTAL
WASHED CELLS
TCA PRECIPITATE
^{3}H CPM X 10^{-5}
1
2
3
4
2
4
6
8
HOURS

Cells were concentrated and pulse labeled 15 minutes then diluted to 2-3x10^6/ml and grown in medium without label (Fig. 15). At time intervals of constant size, equivolume aliquots were removed, and the total counts in the purified virus was measured. The results from this experiment show (1) a small number of counts is produced by 0.5 h. following the beginning of the pulse, a result which agrees with the continuous labeling experiment described above, and (2) counts in the virus peak continue to increase through the 6 h. aliquot. The experiment was repeated (Fig 16) except that aliquots were taken over a 24 h. period. These results show virus labeling levels at about 11 h.

Since viral integrity did not appear to be destroyed, the above experimental conditions were used for pulse-chase experiments in which cells were pulse labeled and pelleted at constant intervals in order to quantitate the virus produced in the entire cell supernatant during the chase period. The experiment is described in figure 17. A parallel experiment was performed (Fig. 18) in which cells were mock pulsed at a high density, and pelleted periodically but resuspended in medium containing ^{3}H uridine. The results of these experiments in combination, demonstrate that from pulse labeled cells, labeled virus is produced maximally between 4 and 5 h. following the beginning of the pulse. From continuously labeled cells, however, labeled virus production continues to increase over a 10 hr. period.

To determine the behavior of the soluble (RNA precusor) nucleotide pool in pulse-labeled cells, an experiment as described in figure 19 was performed. From this it can be seen that by 2 h. 56% of the TCA soluble pool is depleted.

Figure 15. Incorporation of ^{3}H uridine into purified virions after pulse labeling. F-422 cells, $468x10^{6}$, were incubated in 4 ml medium containing 200µci ^{3}H uridine, washed 2X with 5 ml medium containing 10µM uridine then incubated in 200 ml medium containing 1µM uridine. At 0.5, 1.0, 1.5, 2.5, 4.5, and 6.5 h. following the beginning of the pulse, 33 ml samples were taken. Virus was purified by PEG precipitation and isopycnic centrifugation, and the gratients were fractionated and assayed for radioactivity as described in Materials and Methods.

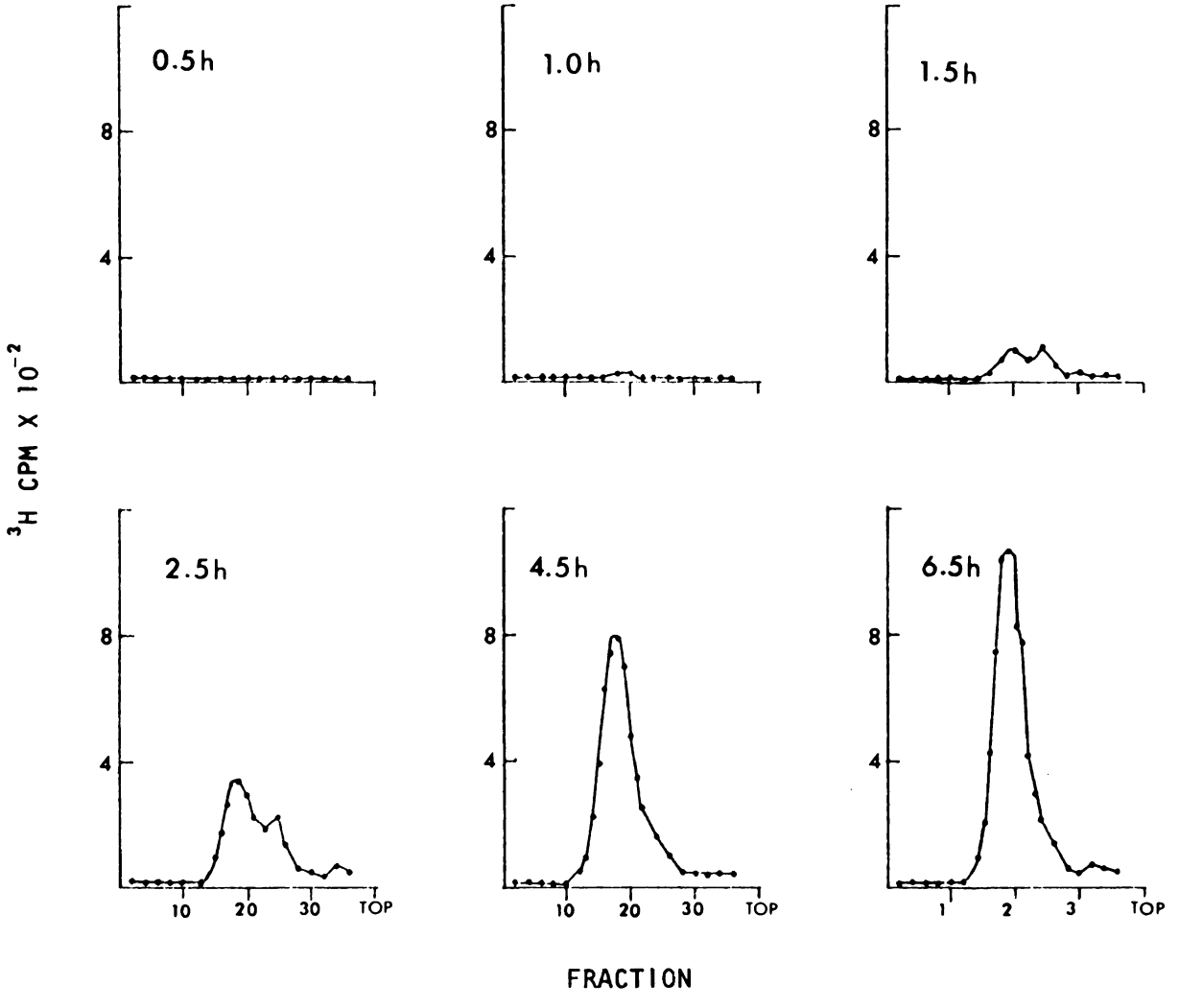

0.5h
1.0h
1.5h
2.5h
4.5h
6.5h
8
4
^{3}H CPM X 10^{-2}
10
20
30
TOP
1
2
3
FRACTION

Figure 16. Incorporation of ^{3}H uridine into virions after pulse labeling. Experiment was performed identically to that described in figure 14 except that samples were taken at 4, 6, 9, 11, 12.5, and 24 h. following the beginning of the pulse.

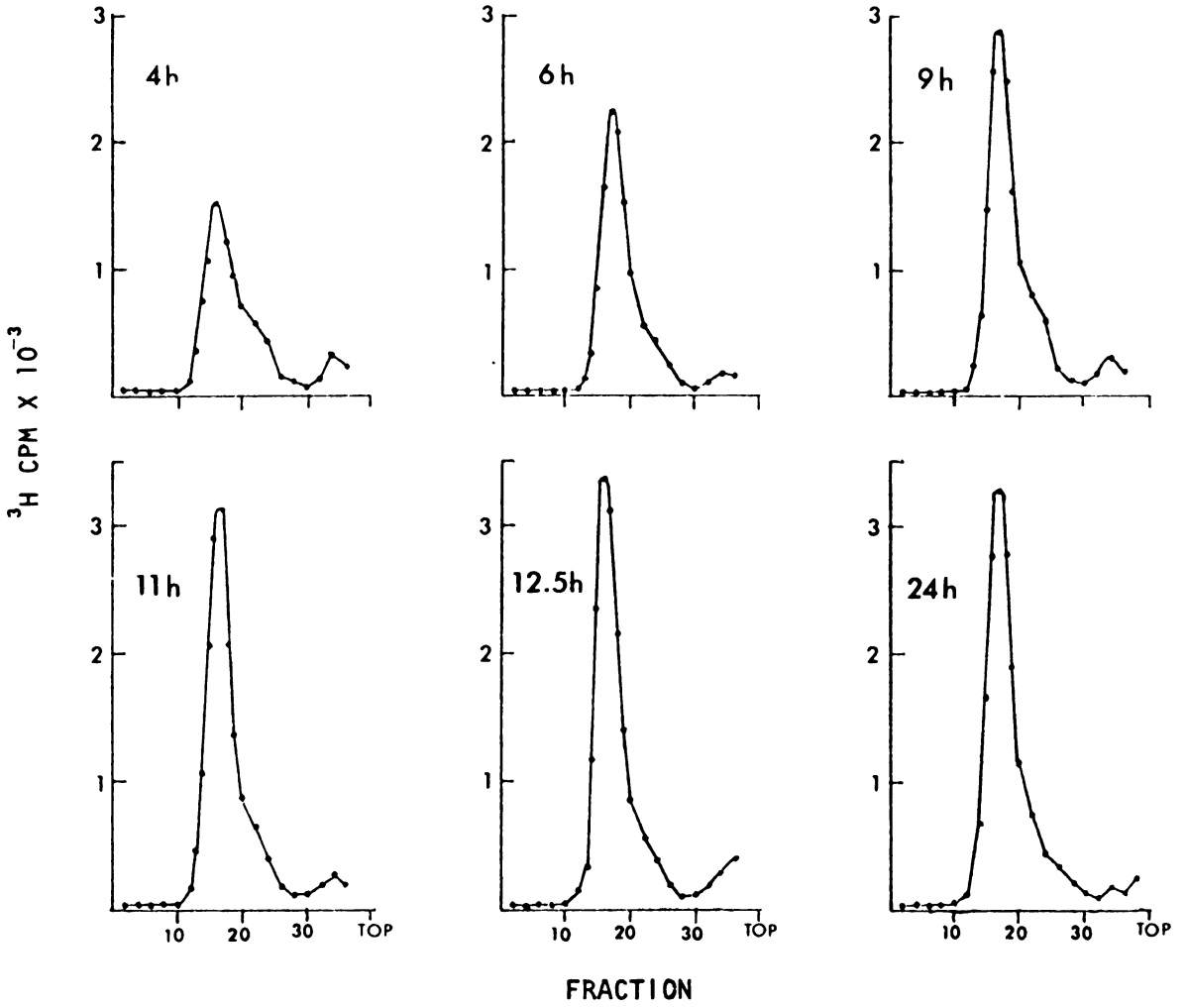

4h
6h
9h
11h
12.5h
24h
3
2
1
10
20
30
TOP
^{3}H CPM X 10^{-3}
FRACTION

Figure 17. Incorporation of ^{3}H uridine into purified virions after pulse labeling. F-422 cells, 66 x 10^{6}, were incubated in 1 ml of medium with 50 µCi ^{3}H uridine for 15 min., washed 2X with 5 ml medium containing 10 µM uridine. Cells were then incubated at 2 x 10^{6} cells/ml in medium containing 1 µM uridine. At hourly intervals, cells were pelleted and resuspended to 2 x 10^{6} cells/ml in fresh medium. Virus was purified by PEG precipitation and isopycnic centrifugation, and the gradients were fractionated and assayed for radioactivity as described in Materials and Methods.

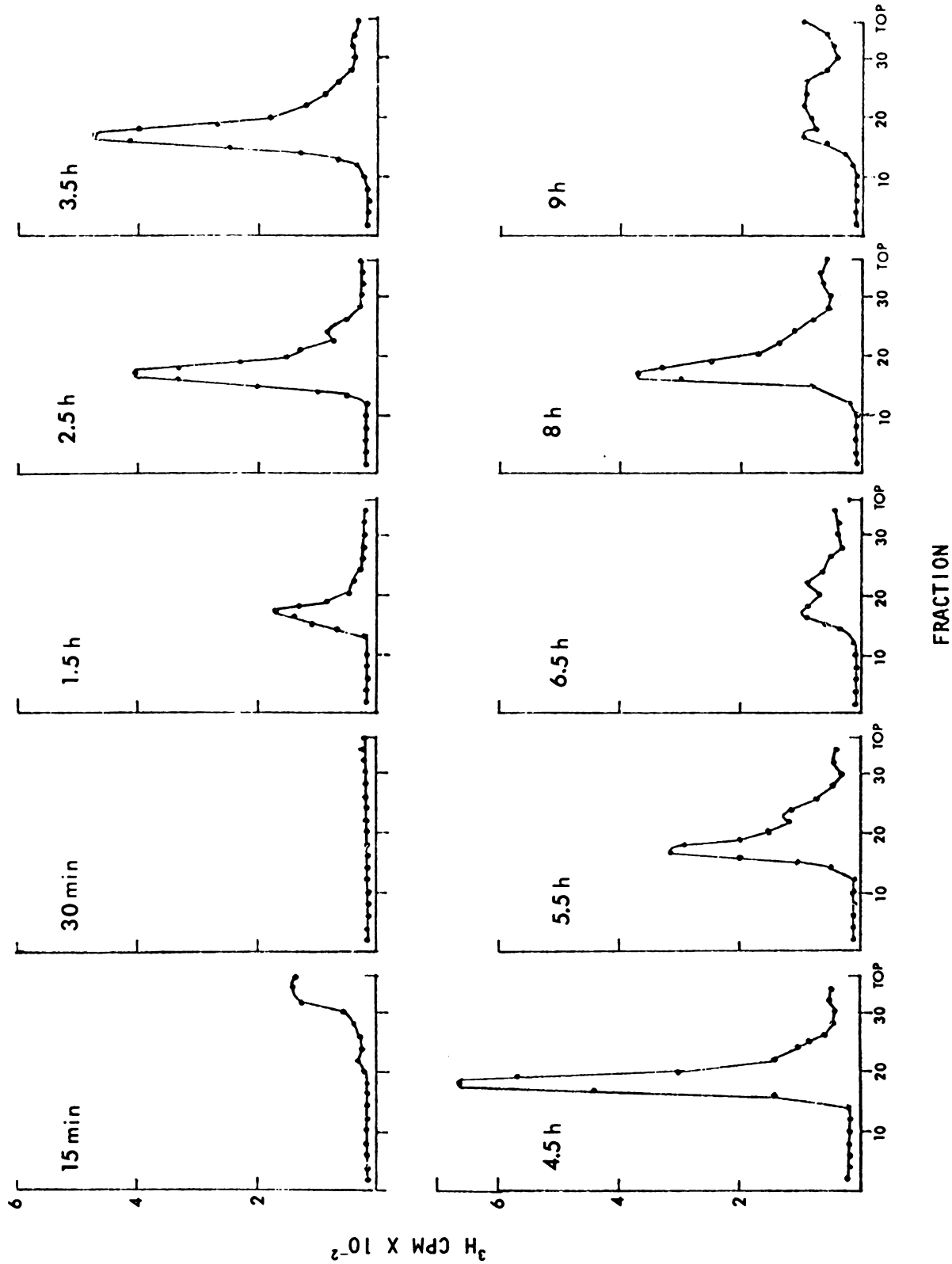

15 min
30 min
1.5 h
2.5 h
3.5 h
4.5 h
5.5 h
6.5 h
8 h
9 h
TOP
FRACTION
^{3}H CPM X 10^{-2}

Figure 18. Incorporation of ^{3}H uridine into purified virions, with continuous labeling. F-422 cells, 66 x 10^{6}, were incubated in 1 ml medium for 15 min. (to simulate pulse labeling described in figure 17), washed 2X with 5 ml medium, then resuspended to 2 x 10^{6} cells/ml with 1 µCi/ml ^{3}H uridine. At hourly intervals, cells were pelleted and resuspended to 2 x 10^{6}/ml fresh medium containing 1 µCi/ml ^{3}H uridine. Virus was purified by PEG precipitation and isopycnic centrifugation, and the gradients were fractionated and assayed for radioactivity as described in Materials and Methods.

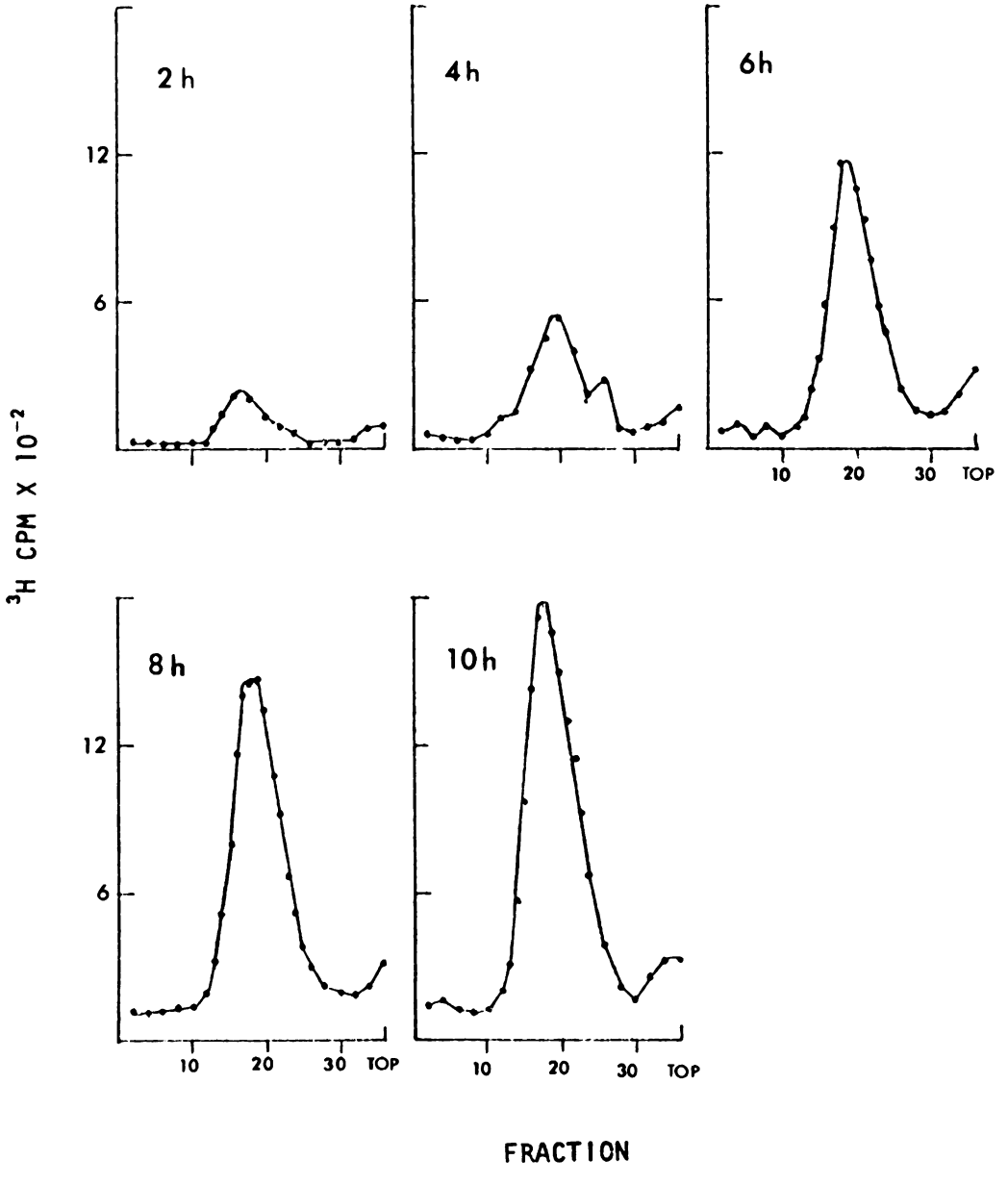

2 h
4h
6h
8h
10h
12
6
10
20
30
TOP
^{3}H CPM X 10^{-2}
FRACTION

Figure 19. Acid soluble pool in F-422 cells after pulse labeling with ^{3}H uridine. F-422 cells, 50 x 10^{6}, were incubated 15 min. in 1 ml medium containing 10 µCi ^{3}H uridine. Cells were then washed 2X with medium containing 10 µM uridine, and resuspended to a concentration of 2 x 10^{6} cells/ml in medium containing 1 µM uridine. 0.2 ml aliquots were taken in duplicate at 0.5, 0.75, 1.0, 1.5, 2.5, 4.5, and 5.5 h. following the beginning of pulse labeling. Radioactivity was measured in (1) washed whole cells after NCS tissue solubilization, and (2) washed TCA precipitate after solubilization. NCS solutions were counted in 5 ml Aquasol. The TCA soluble pool was determined by subtraction.

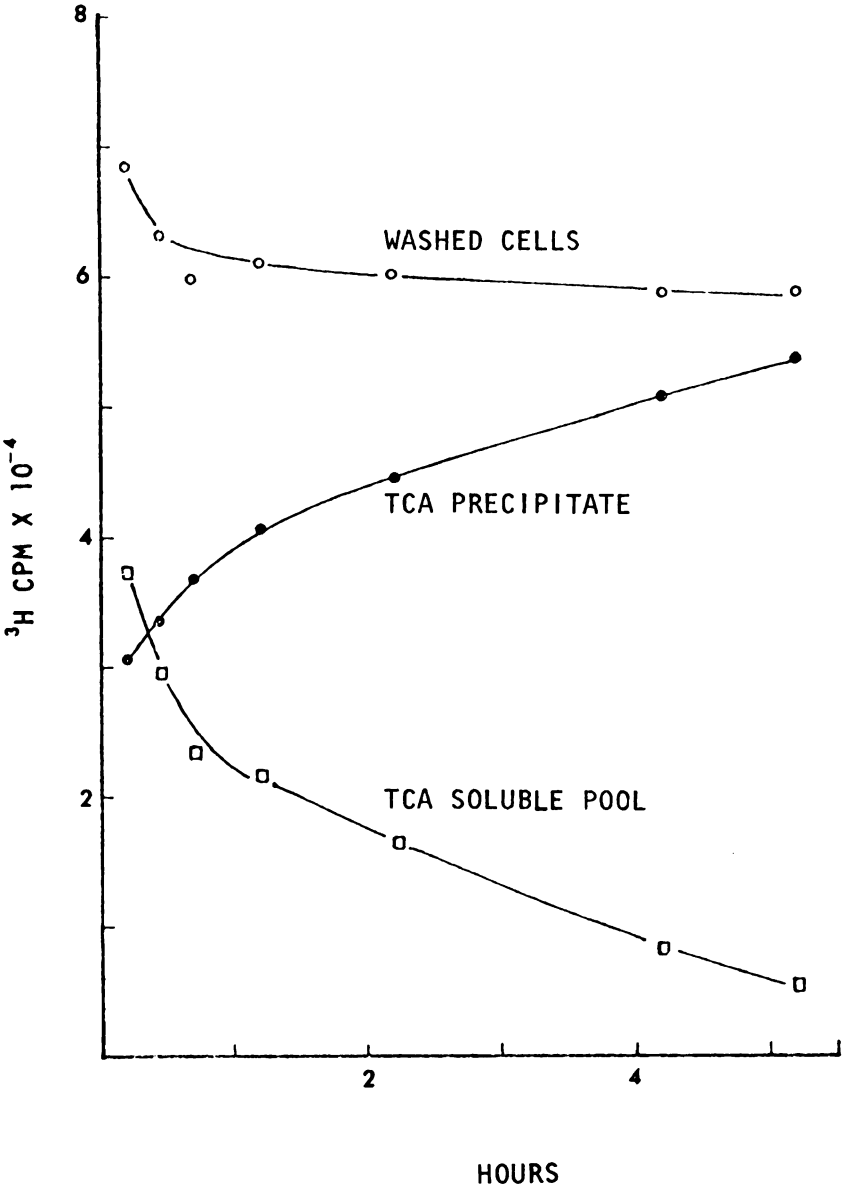
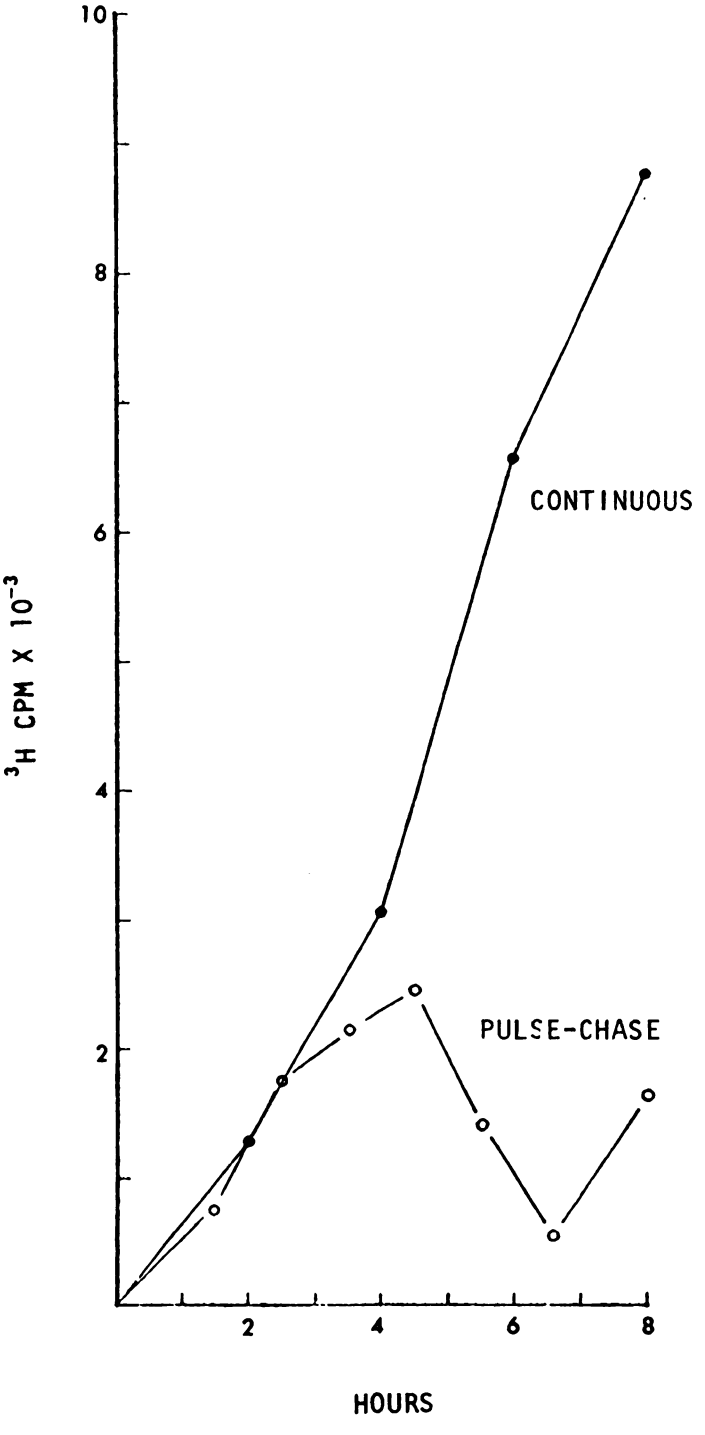

8
6
4
2
^{3}H CPM X 10^{-4}
WASHED CELLS
TCA PRECIPITATE
TCA SOLUBLE POOL
2
4
HOURS

Figure 20. Incorporation of ^{3}H uridine into FeLV during pulse-chase and continuous labeling. Total counts in the 7 peak fractions for each gradient depicted in Figures 17 and 18 was plotted against time. Time is measured from the beginning of labeling in each experiment.


10
8
6
4
2
^{3}H CPM X 10^{-3}
CONTINUOUS
PULSE-CHASE
2
4
6
8
HOURS

DISCUSSION

Electron Micrographs of F-422 Cells and FeLV-R

Electron micrographs of thin-sectioned feline thymus tumor cells reveal particles external to the cell as well as budding from the cell which fit the size and qualitative descriptions of the FeLV-R particles (89). High magnification of the particles from a preparation by thin sectioning have the same dimensions (100 mμ diameter) as particles negatively stained and prepared from an isopycnically centrifuged preparation having a density of 1.14 grams/cc. This argues that particles purified isopycnically are the same particles identified as virus in the thin section. Particles not fitting the exact description for FeLV were sometimes seen and this leaves open the possibility of cell vesicular contamination. Aggregates of virus particles were seen but this may be an artifact developing during the purification process.

Synchronization of F-422 Cells

All attempts to synchronize F-422 cells proved unsuccessful.

1. Thymidine Excess.--Cells appeared to be toxic to levels of thymidine ranging from 2 mM to 0.2 mM by the criteria of cell viability and growth. Altering the blocking period within a range of

8 to 18 h. did not alter the toxic effect. F-422 cells take up thymidine as well or better than MOPC-21F cells. The cause of the toxicity is not known.

2. Serum-Free Medium.--F-422 cells did not grow in serum-free medium, and when serum was added or cells were suspended into fresh medium after 10-16 h. of incubation in serum-free medium, a lag period of 10 h. was observed before cells grew asynchonously.

3. Isoleucine-Free Medium.--Attempts to synchronize F-422 cells in isoleucine-free medium were hampered by an improperly formulated amino acid-free medium mixture from GIBCO. Experiments were not repeated with the corrected medium mixture now available.

4. BSA Gradients.--F-422 cells were isopycnically centrifuged for the purpose of fractionating a population into groups of cells, each group being at a specific stage in the cell cycle. Two groups were resolved, neither of which grew synchronously. Fractionating the cells by velocity sedimentation might have yielded more fruitful results. This experiment raises the question of what the upper band cells were.

Purification of FeLV-R

In view of the fact that mammalian cells produce vesicles (91) the question persists as to what extent cell vesicles contaminate purified virus preparations. It is conceivable that vesicles would contain cytoplasmic RNA thus giving rise to a particle having nearly the same density as virus. Usually a two-step procedure is used for purifying RNA tumor viruses. (1) Salt precipitation using ammonium

sulfate (27, 90) or potassium tartrate (14), followed by isopycnic centrifugation, or (2) pelleting (6, 17, 22, 33) followed by isopycnic centrifugation. At times velocity sedimentation is used followed by isopycnic centrifugation (22). In spite of this, apprehension that cell vesicles contaminate virus preparations persists.

Because electronmicrographs of the feline thymus tumor cell (F-422) hint at the existence of vesicular material and because of a trailing shoulder on iscopycnically purified virus a series of experiments as described in figures 9 and 10 and table 4 were performed.

From the results in figures 9, 10, and table 4 the following conclusions are made.

1. Peaks 1 and 2 of figure 9 are virus particles since each contains 50-60S viral RNA. Perhaps the leading peak is aggregated virus.
2. Peak 3 contains no 50-60S RNA, and is therefore not virus. It does contain RNA which may possibly be ribosomal RNA although no distinct 18S and 28S peaks were seen. Peak 3 which sediments much slower than virus is a good candidate for cell vesicles. However, no 4S RNA was seen either, which is surprising if peak 3 does represent cell vesicles.
3. Material in peak 3 (vesicle particles?) partially contaminates the virus peak when the preparation is isopycnically centrifuged 2.5 h. (the condition described in figure 10). From figure 10, 52% of the peak 3 counts would be in the 1.14 g/ml density region which would be collected as purified virus (fractions 11-21).

4. Material in peak 3 is the source of the trailing shoulder normally seen in 2.5 h. isopycnically centrifuged virus preparations (under the conditions described).
5. A velocity sedimentation purification step is recommended to purify virus from particulate contamination which is presumed to be cell vesicular material.

It will be noted that in part II of this thesis that FeLV-R for RNA studies was purified simply by pelleting the virus twice through a 20% w/w sucrose solution. Under conditions in which RNA degradation can be ruled out, no 18S or 28S RNA is ever seen in these preparations and indeed very little RNA in the 10S to 40S region is seen. Contamination from the putative cell vesicle as described above does not seem to exist on the basis of RNA present in the purified virion. Perhaps vesicles sediment through the sucrose too slowly to pellet under the conditions used.

The kinetic studies, discussed later, in which the rates of virus production were measured, were done without knowledge of the putative cellular contaminant. The effects of such a contaminant, however, were minimized since radioactive counts in the trailing shoulders were ignored.

Infection of Crandell CCC Cells

While FeLV, like other leukemia viruses, does not transform the cell it infects and replicates in and hence is not a virus with which to study *in vitro* transformation, it is of interest for studying replication. It does cause leukemia, a malignancy. And in this broad sense it does transform normal cells to malignant cells. In the

narrow common usage of the term, "transformation" refers to the visible alteration of a fibroblast (or other cell type) in vitro brought on by infection with a virus.

There were two reasons for infecting a normal cell line with FeLV. (1) It was not possible to obtain an uninfected thymocyte (thymic lymphocyte) cell line to use as control cells during experimentation either from (a) primary cultures of fetal thymus tissue in our laboratory, or (b) an established cell line from another source. And hence infecting another established cell line (ca. Crandell-CCC) would be an alternative solution. There was the unfortunate possibility that infected CCC-H cells would not produce FeLV at the same high rate as F-422 if infected. This would be a disadvantage. (2) It was of great interest to have an oncornavirus system in which the latent period between infection and appearance of progeny virus was extremely short, for example, less than 8 h., in order to study the obscure events (e.g., the necessity for DNA synthesis) during the infection process.

At the time our laboratory received the Crandall-CCC cells (1971) they had been grown for over 5 years in tissue culture at Cornell. During this time there was close monitoring by electron microscopy for virus production. Efforts to induce an endogenous virus with thymidine analogues were unsuccessful. It appeared to be a good candidate for an uninfected control cell.

Since this time, RD-114 has been induced from CCC-A cells (46, 72). These data open the possibility that endogenous virus was

induced by infection with FeLV-R. Possibly a mixture of RD-114 and Rickard-FeLV are being produced in the "infected" cell.

There is no apparent reason why cells did not become infected during the experiments to determine the eclipse period. It was an error not to prepare a simultaneous parallel flask for continuous passage and for future assays of virus production.

Effects of Cordycepin on Virus Production

Philipson, et al. (87), demonstrated that cordycepin (3' deoxyadenosine) inhibits almost totally the appearance of mRNA into cellular cytoplasmic polysomes, and in adenovirus infected cells, cordycepin prevents the accumulation of ^{3}H adenosine into polysomal RNA late in the infection by 85-90%. Since cordycepin does not inhibit the synthesis of large heterogeneous nuclear RNA (HnRNA) (thought to be messenger precursor RNA), and since adenovirus mRNA is synthesized in the nucleus, and possesses poly(A) apparently identical to cellular mRNA, it was concluded that poly(A) is necessary for the processing of HnRNA into mRNA or for the transport of mRNA to the cytoplasm.

Because oncornavarius RNA possesses poly(A) resembling very closely the poly(A) found on mammalian mRNA, an assumption was made that FeLV RNA synthesis and transport might be similarly affected by cordycepin. That is, it was predicted that FeLV RNA synthesis and/or transport would be inhibited by cordycepin, and the manifestation of this would be an inhibition in the production of labeled progeny virus.

The effects of cordycepin were surprisingly not as predicted. Cells incubated continuously in 50 μg/ml of cordycepin suffered mildly

at 4 h. a 50% viability when compared to 90% viability in control cells. By 8 h. cells were only 10% viable. Cordycepin is clearly toxic to cells at 50 µg/ml.

After 4.75 h. of incubation, as much radioactivity could be found in virus from cordycepin treated cells as from control cells. While this was not predicted, possible explanations might include: (1) Cellular polysomal and adenovirus-specific polysomal inhibition as reported by Philipson, et al. (87), was measured within 45 min. after cordycepin treatment, whereas the FeLV detected in these experiments was labeled over a 5 h. period. The effects of cordycepin might have been reversed by this time. One would still expect a depressed production of virus, but a minor depression may not be detected within the sensitivites of this assay system. (2) Viral RNA, while possessing poly(A) may not be affected in the same way as adenovirus mRNA and cell mRNA. Poly(A) may serve an entirely different function in oncorna viral RNA. Controls on processing and transport may not be the same. (3) Viral RNA may, during cordycepin treatment, be synthesized, processed and transported without acquiring a poly(A) segment. It is known that some of the viral RNA does not possess poly(A) (55, 67).

Kinetics of Incorporation of Uridine Into FeLV-R

For FeLV-R it was concluded that the minimum interval between the uptake of radioactively labeled uridine and the appearance of radioactive viral RNA in the released virion is 30 min. or less. This is considerably less than the 70 min. measured for MLV (6) or the 2 h. measured for AMV (10) and RSV-RAV$_1$ (6). Since ^{3}H uridine

concentrations were similar in all experiments (AMV, MLV, RSV-RAV_1, FeLV) the shorter interval for FeLV may relfect a more efficient labeling system or possibly a more rapid viral synthesizing system. It is interesting to note that East, et al., reported a 30 min. minimum labeling interval for MLV-SD subsequent to the above studies (33).

The average interval for FeLV-R was measured to be between 4 and 5 h. This is longer than the 3-4 h. half-life interval measured for AMV (10), but the methods differed between the two determinations.

An exogenously added, differentially labeled FeLV in the experiments described in figures 15 and 16 would have demonstrated more conclusively that viral integrity was preserved under the conditions of the experiment.

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Properties of Feline Leukemia Virus:

III. Analysis of the Ribonucleic Acid

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ABSTRACT

The interval between the uptake of radioactive uridine into feline thymus tumor cells (Rickard, F-422) and its release into virions (Rickard strain of feline leukemia virus, FeLV) was measured. Labeled virus is found within 30 min after adding radioactive uridine to the cells. Production of labeled virus reaches a maximum at 4 to 5 h following pulse labeling. RNA from FeLV resolves into three size classes when analyzed by electrophoresis on 2.0% polyacrylamide-0.5% agarose gels: a 6.2×10^6 to 6.8×10^6 dalton mol wt (50 to 60S) class, a 1×10^5 dalton mol wt (8S) class, and a 2×10^4 dalton mol wt (4 to 5S) class. These respectively make up 52 to 76%, 2 to 5% and 7 to 21% of the total RNA and respectively represent 1, 3 to 10, and 23 to 122 molecules per virion. The 8S RNA in FeLV has not previously been reported. The 50 to 60S RNA from virus harvested after 4 h of labeling electrophoretically migrates faster and sediments more slowly than the same RNA harvested after 20 h of labeling. This was concluded to be the result of conformational changes in the molecule. Fifty to 60S RNA dissociates into subunits cosedimenting with 28S ribosomal RNA when analyzed by velocity sedimentation through 99% dimethyl sulfoxide (DMSO). Aggregates intermediate between 50 to 60S ($s_{25,\ DMSO} = 7.09$) and 28S ($s_{25,\ DMSO} = 4.22$) suggest that conditions other than 99% DMSO alone are needed for complete dissociation of 50 to 60S RNA.

INTRODUCTION

The RNA tumor viruses from all species studied to date possess in common a high molecular weight, single-stranded RNA which is an aggregate of smaller molecules thought to be held together by hydrogen bonding (2,14,15,18,19,21,29,37,38). This aggregate can be denatured into its subunits by heat, urea, formamide, formaldehyde or DMSO, (2,14,15,17,18,21,22,29,37,38) and subunits of two major sizes result: a large 28 to 35S subunit which possesses stretches of polyadenylic acid [poly(A)] showing characteristics similar to the poly(A) tract on mammalian messenger RNA (25,27,32,42), and small tRNA-like subunits which can be aminoacylated (20,22). In addition to the aggregate there are smaller amounts of 36S, 28S, 18S, and 4 to 10S RNA present in the virion (10,11,14,15,18,19,21, 29,33,37,41). In immature virus these may be preassembled subunits which eventually become aggregated (10,11).

When this study was initiated, characterization of FeLV RNA was limited to one report (29). Based primarily on velocity sedimentation studies, it was shown that FeLV possesses a uniquely large proportion of native 35S and 4 to 10S RNA molecules when compared to other oncornaviruses. This discrepancy made it necessary to further characterize FeLV RNA under conditions which minimize degradation with an analytic system giving superior resolution (gel electrophoresis). These experiments were undertaken 1) to characterize the interval between the uptake of radioactive uridine into infected cells and the release of radioactive virus, 2) to characterize the

native RNA molecules in FeLV of varying ages with respect to molecular weight and percentage composition, and, 3) to determine the molecular weight of the denatured high molecular weight subunit.

MATERIALS AND METHODS

Source of cells and viruses. The permanently infected feline thymus tumor line, F-422, which produces the Rickard strain of feline leukemia virus (FeLV) (40), and grows in suspension culture, was obtained from Dr. C. G. Rickard (Cornell University). Cells were propagated as previously described (26). The Kansas-Manhattan strain of Newcastle disease virus (NDV) was obtained from Dr. R. P. Hanson (University of Wisconsin).

Adaptation of F-422 cells for monolayer growth. Cells which adhere to a flask surface were selected by periodically replacing media on a stationary flask of cells. A monolayer of adhering thymocytes is detached by vigorous shaking. Progeny cells can either form new monolayers in a flask or roller bottle, or be grown in spinner suspension culture.

Isotopic labeling of FeLV RNA. Cells were incubated in 36 to 200 ml volumes in medium containing 10% fetal calf serum and either ^{3}H uridine (40 Ci/mM), ^{14}C uridine (57 m Ci/mM) or both as described in the Results. New England Nuclear Corp. (NEN), Boston, Mass. was the source for all isotopes.

Purification of FeLV. For studying kinetics of viral labeling, virus was purified as follows. Cells were pelleted at 1000 rpm (300 x g) for 5 min in an International PR-6 centrifuge, and cellular debris was pelleted from cell supernatant at 10,000 rpm (16,000 x g) for 10 min in a Sorvall GSA rotor. Virus was precipitated from clarified supernatant in 50% (wt/vol) ammonium sulfate or 5% (wt/vol)

polyethylene glycol for 16 h at 4 C, and the precipitate was pelleted at 10,000 rpm for 20 min in a Sorvall GSA rotor and resuspended in 0.5 ml TNE-7.5 buffer (0.01 M Tris, pH 7.5, 0.1 M NaCl, 0.001 M EDTA). Virus was layered onto a 5.0 ml gradient of 15 to 40% (wt/wt) sucrose made up in TNE-7.5 and isopycnically centrifuged for 2.5 h at 50,000 rpm (240,000 x g) in a SW 50.1 rotor. Gradients were fractionated by dripping through the bottom of the tube.

For studying viral RNA, FeLV was purified as follows. Clarified cellular supernatant was layered over 8 ml of 20% (wt/wt) sucrose made up in TNE-7.5, and centrifuged at 25,000 rpm (84,000 x g) in a SW 27 rotor for 3 h. The viral pellet was resuspended into 1 ml of TNE-7.5 by 15 sec of sonication in a 150 Watt Branson ultrasonic cleaner (Branson Instruments Company, Stamford, Conn.), layered onto 4.5 ml of 20% (wt/wt) sucrose in TNE-7.5 and pelleted at 45,000 rpm (190,000 x g) in a SW 50.1 rotor for 1.5 h.

RNA extraction. The viral pellet was resuspended in 0.3 ml TNE-9 buffer (0.1 M Tris, pH 9.0, 0.1 M NaCl, 0.001 M EDTA) by 15 sec of sonication. The suspension was made 1.0% SDS by adding 10% SDS in TNE-9, and an equal volume of pronase (Calbiochem) at 500 μg/ml in TNE-9 (self-digested 2 h at 37 C) was added to make a final concentration of 250 μg/ml. The solution was incubated 5 min at 37 C before extracting 3 times with an equal volume of TNE-9 saturated phenol. When necessary, RNA was precipitated with or without carrier (5 A_{260}/ml Torula grade B RNA, Calbiochem) in 67% ethanol for 16 h at -20 C.

Preparation of cytoplasmic RNA markers. Cytoplasmic RNA was prepared from ^{3}H uridine labeled feline thymus tumor cells by the method of Erikson (22). Murine cytoplasmic RNA was a gift from Ron Desrosiers (Michigan State University).

Preparation of ^{32}P-NDV RNA. The lyophilized source of NDV was passaged once in 9-day-old chick embryos. Allantoic fluid after 48 h contained 5 x 10^{9} pfu/ml as assayed on baby hamster kidney cells (12). Radioactive labeling, virus purification and RNA extraction methods were modified procedures of Duesberg (16). Eight-day-old chick embryos were inoculated with $H_3\ ^{32}PO_4$ (1 mc/egg) into the allantoic cavity. After 24 h incubation at 38 C, 10^{3} to 10^{4} pfu of NDV were inoculated via the same route. After 48 h incubation (embryos were usually dead) allantoic fluid was collected, frozen, thawed, and clarified at 10,000 rpm, 10 min, in a Sorvall GSA rotor. Virus was pelleted through 8 ml 20% (wt/wt) sucrose made up in TNE-7.5 buffer at 25,000 rpm for 4 h in a SW 27 rotor at 4 C. The viral pellet was resuspended into 0.5 ml TNE-7.5 buffer by 15 sec of sonication and layered onto a 5.0 ml gradient of 65% (wt/wt) sucrose in D_2O to 20% (wt/wt) sucrose in TNE-7.5. Virus was isopycnically centrifuged 15 h at 35,000 rpm (110,000 x g) in a SW 50.1 rotor at 4 C. The virus was collected visually from a dripping needle, diluted to 5 ml in TNE-7.5, pelleted at 45,000 rpm in a SW 50.1 rotor at 4 C, and frozen at -76 C. RNA was extracted as described for FeLV RNA. Twenty to 30% of the trichloracetic acid (TCA) precipitable counts sedimented as 50S (30).

Polyacrylamide-agarose gel electrophoresis. The method of Peacock and Dingman (39), as described by Bunting (9) was modified for use in tubes to facilitate accurate fractionation of low percentage gels in a Gilson gel fractionator. Ten tubes, 18 cm x 0.5 cm inside diameter, each containing 3.0 ml of 2.0% polyacrylamide-0.5% agarose were prepared as follows. 0.16 grams of agarose (Biorad) was refluxed 15 min with stirring in 22.6 ml water and cooled to 48 C. Simultaneously, 3.2 ml of 20% acrylamide-bis acrylamide solution [20%(wt/vol)water solution of cyanogum 41, Fisher] was combined with 3.2 ml of Peacock's 10-fold concentrated electrophoresis buffer (0.89 M Tris, 0.89 M boric acid, and 0.025 M EDTA in water) and warmed to 48 C. Within 1 min, 1) 1.0 ml of a 1.6% (wt/vol) ammonium persulfate solution was mixed into the agarose solution, 2) 2.0 ml of a 6.4%(wt/vol) 3-dimethylaminopropionitrile solution was mixed into the acrylamide-bisacrylamide buffer solution, 3) all solutions were mixed well together and dispensed with a syringe and needle into vertical tubes that had been treated with Photoflo (Kodak), and dried. Gels were allowed to polymerize for 1 h at room temperature, capped with parafilm and stored at 4 C. Storage for up to 6 months seemingly caused no detrimental consequences. Just prior to use, gels were slightly displaced with a Gilson gel piston and sliced transversely with a razor blade, forming a flat surface for the RNA sample. The gel was then retracted into the tube. Parafilm was used to cap the bottom end of the tube and 12 to 15 holes were made with a needle to allow for current flow.

Gels were pre-electrophoresed 1 h at 150 V. Electrophoresis was done at 150 V constant voltage at 4 C for approximately 3.0 h. Gels were fractionated into 2 mm fractions with the Gilson gel fractionator.

Velocity sedimentation of RNA through aqueous sucrose gradients. RNA was centrifuged through a 5.0 ml linear gradient of 20 to 50% (wt/wt) sucrose (ribonuclease free, Schwarz-Mann) made up in TNE-7.5. Centrifugation was for 80 min, at 45,000 rpm and 4 C, or 70 min at 45,000 rpm and 20 C when the sucrose contained 0.1% SDS, in a SW 50.1 rotor. Gradients were fractionated by dripping.

Velocity sedimentation of RNA through 99% dimethyl sulfoxide. RNA was centrifuged through a 5.0 ml linear gradient of 5 to 20% (wt/wt) sucrose, ribonuclease free, made up in 99% dimethyl sulfoxide (DMSO). Ninety-nine percent DMSO, 0.001 M EDTA, was prepared using reagent grade 99.2% DMSO (Fisher), TNE-7.5 and a 0.2 M EDTA stock solution. The pH of the final DMSO solution was brought to pH 8 using 1.0 N HCl. Centrifugation was for 13 to 16 h (exact number of revolutions given in the data) at 45,000 rpm in a SW 50.1 rotor at 25 C. Gradients were fractionated by dripping.

Oligo (dT)-cellulose chromatography. Oligo (dT)-cellulose was prepared essentially as described by Gilham (24) and the chromatographic method was essentially that of Aviv and Leder (1). RNA sedimenting with a $s_{25,\ DMSO}$ of 4.22 S on DMSO gradients was precipitated in 67% ethanol after making the pooled fractions 0.1 M NaCl. The precipitate was dried in a nitrogen stream, redissolved in high salt buffer (0.01 M Tris, pH 7.4, 0.5 M NaCl, 0.001 M EDTA, and 0.2% SDS) and applied to a column (0.5 x 6 cm) previously equilibrated with this buffer. Poly(A)(-)

RNA was eluted by washing the column with high salt buffer. Poly (A) (+) RNA was eluted with low salt buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA, and 0.2% SDS).

Radioactivity assay. Entire fractions or samples of fractions from isopycnic gradients were dripped or spotted, respectively, on 2.3 cm Whatman paper filter discs, air dried precipitated 20 min at 0C in 5% TCA, washed one min with 5% TCA, and one min with acetone, air dried, and counted in toluene-POPOP in a Packard Tricarb liquid scintillation spectrometer. Entire fractions or partial fractions from velocity sedimentation gradients were assayed as described above, or counted directly in 5 ml Aquasol (NEN). Gel fractions were digested 1 h at 50 C in 0.1 ml NCS tissue solubilizer (Amersham/Searle) then counted in 5 ml Aquasol.

RESULTS

Kinetics of viral labeling. When cells infected with oncornaviruses are labeled with a continuous source of radioactive uridine, radioactive RNA from progeny virus appear as early as 2 h for Rous sarcoma virus-Rous associated virus, (RSV-RAV_1) (3), 1.5 h for avian myeloblastosis virus (AMV) (4), 80 min for Rauscher murine leukemia virus (MLV) (3) and 30 min for Sohner-Dmochowski murine sarcoma virus (SD-MSV) (18). Continuous labeling experiments using F-422 feline thymus tumor cells (Fig. 1) demonstrate that radioactive virus is detected as early as 30 min after addition of the radioactive RNA precursor. This minimum interval is similar to the 30 min interval for SD-MSV (18).

When infected cells are pulse-labeled 15 min with radioactive uridine, radioactive RNA is release into progeny virus at a maximum rate of 5 h for RSV-RAV_1 and MLV (3), and between 3 and 6 h for AMV (4). Pulse-chase experiments during which 56% of the intracellular nucleotide precursor pool disappears by 2 h (D. Brian, data not shown) demonstrate that production of labeled virus reaches a maximum at 4 or 5 h following the beginning of the pulse (Fig. 2). These results with the feline leukemia virus are similar to those for RSV-RAV_1, MLV (3) and AMV (4).

Figure 1. Incorporation of ^{3}H uridine into purified virions during continuous labeling. $_{3}F$-422 cells at 10^{7} cells/ml were incubated with 4 uCi ^{3}H uridine/ml. At 0, 0.5, 1, and 2 h, 6.5 ml samples were taken. Virus was purified by ammonuim sulfate precipitation and isopycnic centrifugation, and the gradients were fractionated and assayed for radioactivity as described in Materials and Methods.

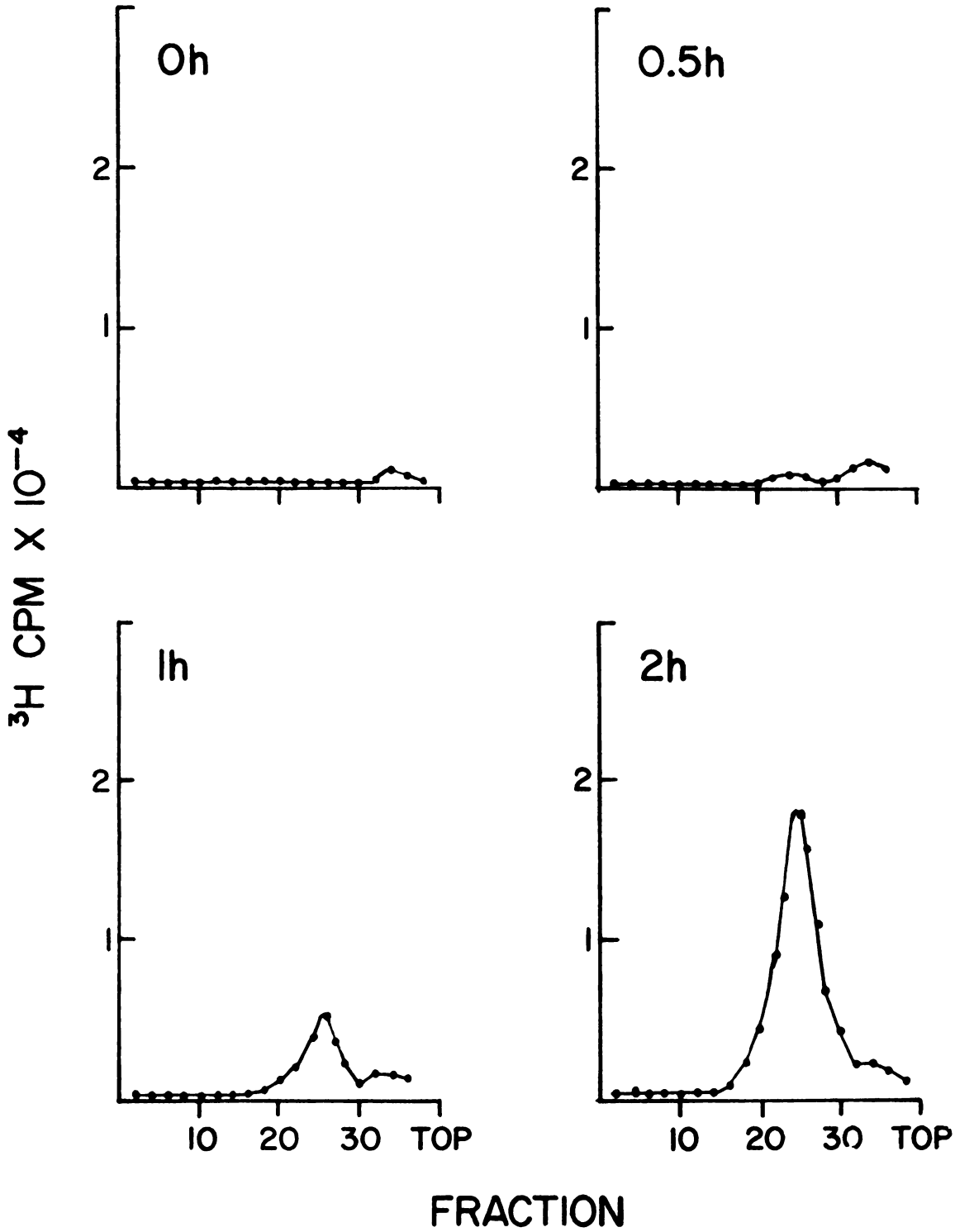
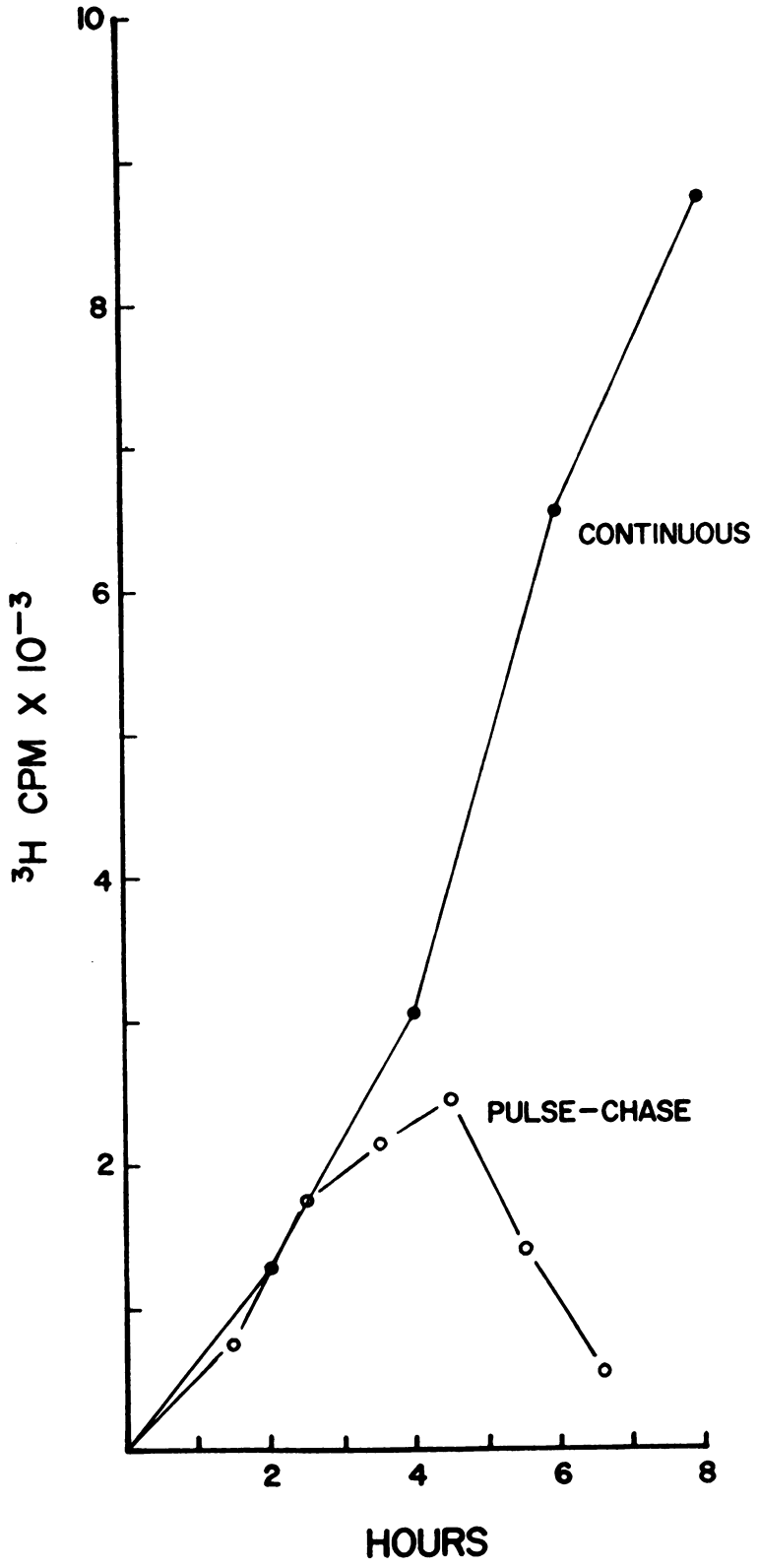

0h
0.5h
1h
2h
2
1
^{3}H CPM X 10^{-4}
10
20
30
TOP
FRACTION

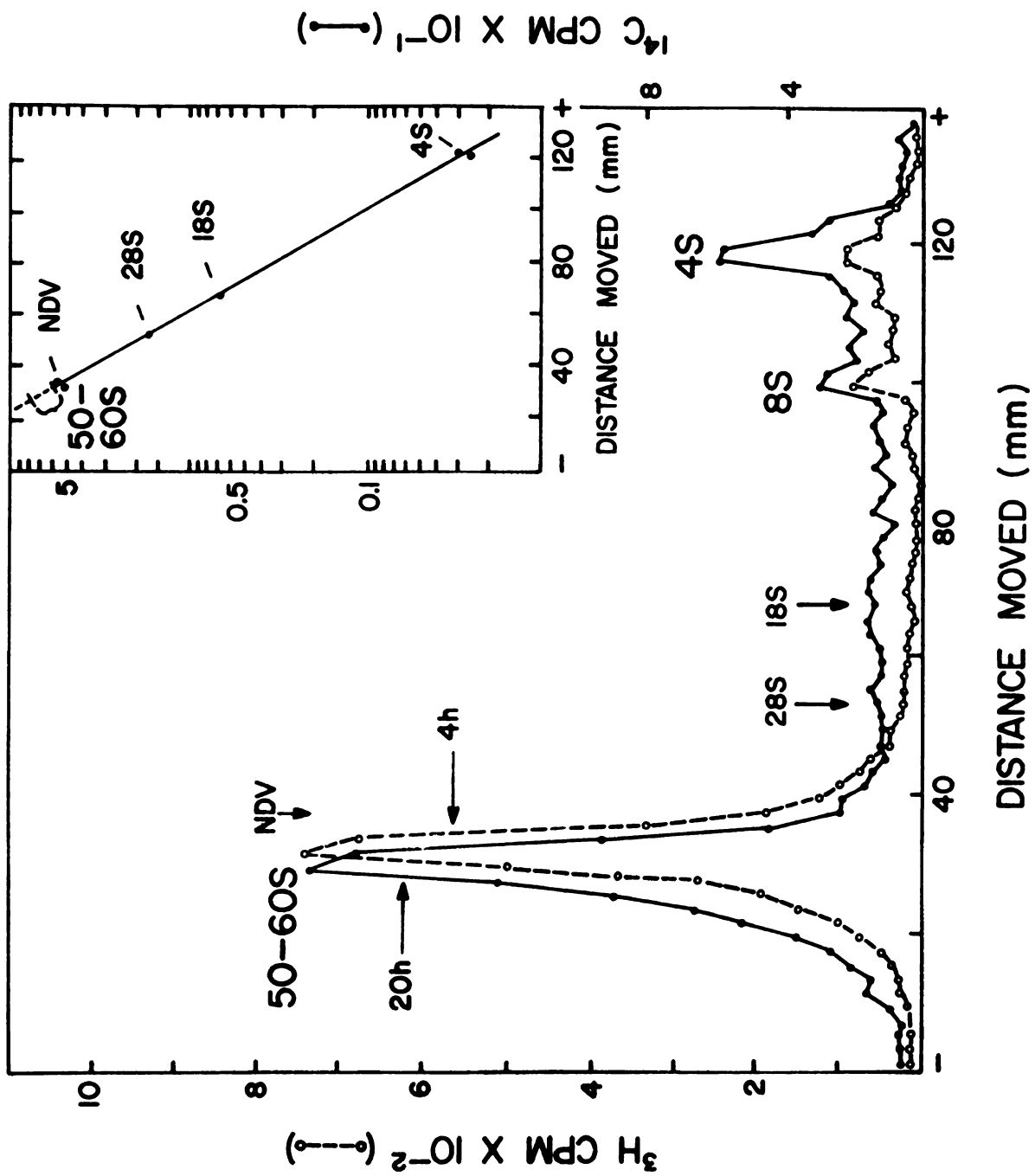
Figure 2. Incorporation of ^{3}H uridine into FeLV during pulse-chase and continuous labeling. Pulse-chase labeling (o). F-422 cells (6.6×10^{6}) were pulse-labeled 15 min in 2 ml medium with 50 uCi ^{3}H uridine, washed twice with 5 ml medium containing 10 uM uridine, and resuspended in 33 ml medium containing 1 uM uridine. Cells were periodically pelleted and resuspended in fresh medium containing 1 uM uridine. Virus from each collection was purified and its radioactive content was assayed as described in Materials a d Methods. Continuous labeling (●). F-422 cells (6.6×10^{6}) were incubated in 33 ml medium with 1 uCi/ml ^{3}H uridine. Periodically cells were pelleted and resuspended in 33 ml fresh medium containing 1 uCi/ml ^{3}H uridine. Virus from each collection was purified and its radioactivity content was assayed as described in Materials and Methods.


10
8
6
4
2
^{3}H CPM X 10^{-3}
CONTINUOUS
PULSE-CHASE
2
4
6
8
HOURS

Gel electrophoresis and velocity sedimentation of native viral RNA. Velocity sedimentation of FeLV RNA in aqueous sucrose gradients does not resolve the 4 to 10S species (19,29). A recent gel electrophoretic study of FeLV RNA focuses only on the high molecular weight species and its subunits (46).

In an effort to resolve the entire RNA content of FeLV into size classes and to obtain an approximation of corresponding molecular weights and percentage composition, electrophoresis was done on combination gels of 2.0% polyacrylamide-0.5% agarose (39). Figure 3 illustrates the results obtained when virus is uridine labeled for 4 h or 20 h and the RNA is extracted and electrophoresed. In each case three molecular weight size classes are identified: a 6.2×10^6 to 6.8×10^6 dalton mol wt class, a 1×10^5 dalton mol wt class and a 2.5×10^4 dalton mol wt class. These make up 52 to 76%, 2 to 5% and 6 to 12% of the total RNA respectively, with the remainder being heterogeneously dispersed throughout the gel, and they represent respectively 1, 3 to 10, and 23 to 122 molecules per virion. From sedimentation data, these correspond respectively to 50 to 60S, 8S and 4 to 5S classes. The sedimentation coefficient of the 1×10^5 dalton mol wt class was not measured, but its electrophoretic mobility appears identical to the 8S species reported in the murine sarcoma virus (20,33), and is therefore called 8S. Molecular weights were determined by reference to feline cellular 4S tRNA, and 18S and 28S rRNA run in parallel gels under identical conditions, and to differentially labeled 50S NDV RNA (30) included in all gels with FeLV RNA. Under

Figure 3. Coelectrophoresis of native FeLV RNA, feline thymus tumor cell rRNA and tRNA, and NDV RNA on 2.0% polyacrylamide-0.5% agarose gels. ^{14}F-422 cells at 2 x 10^6/ml were incubated 20 h with 2.5 uCi ^{14}C uridine and 4 h (the last 4 h of the ^{14}C uridine labeling period) with 20 uCi/ml ^{3}H uridine to provide doubly labeled virus as a source of FeLV RNA. Fifty microliters of phenol-SDS-pronase extracted RNA from the purified FeLV was used to solubilize ethanol precipitated ^{32}P NDV RNA marker (see Materials and Methods). Final solution was then made approximately 10% sucrose and 0.005% bromophenol blue and electrophoresed 3 h. Insert: ^{32}P NDV RNA and ^{3}H uridine labeled feline thymus tumor cell 28S, 18S ribosomal and 4S tRNA were coelectrophoresed on parallel gels. Four S tRNA and 50S NDV RNA positions on the two gels were superimposed.

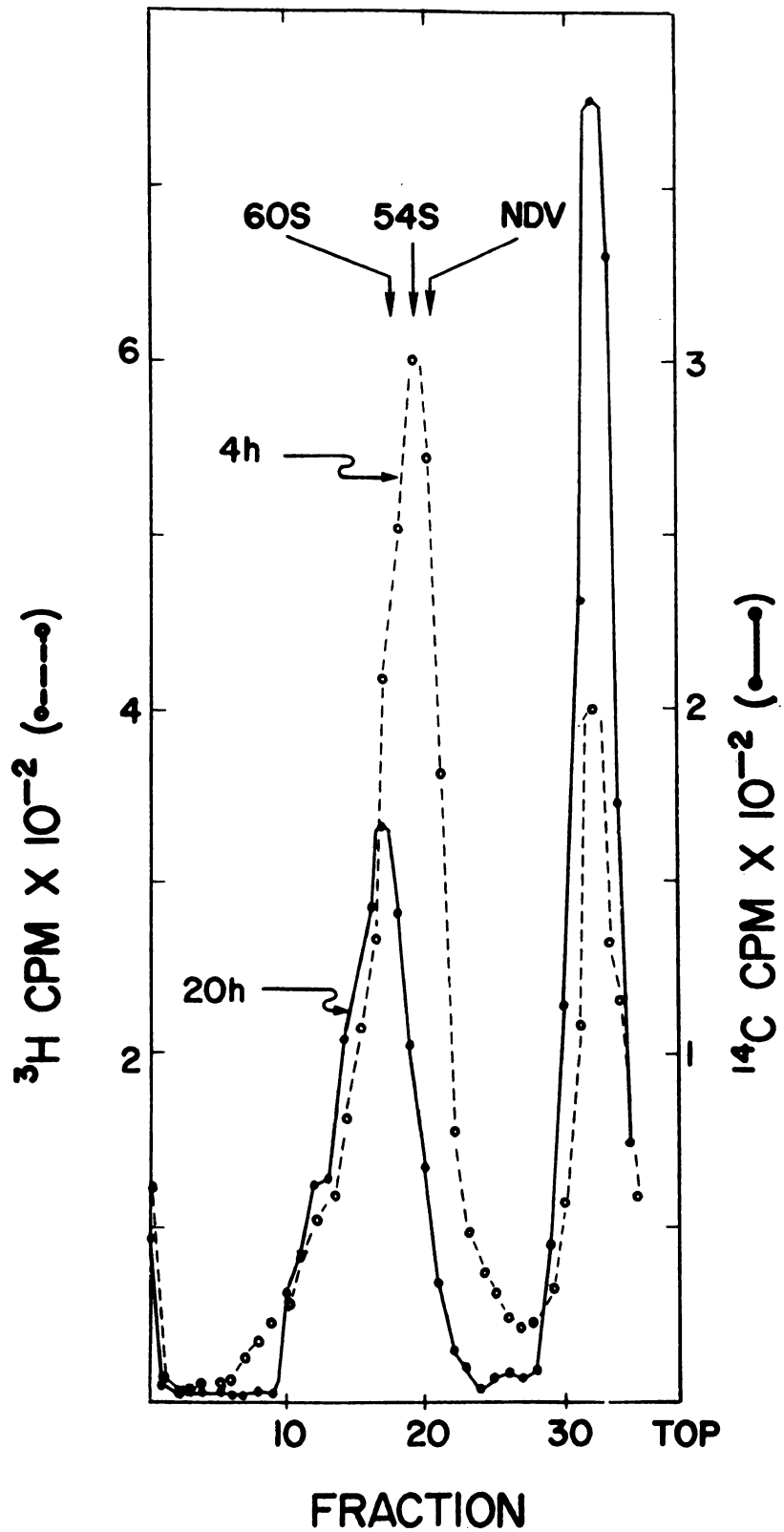

^{14}C CPM X 10^{-1} (—●—)
^{3}H CPM X 10^{-2} (---○---)
DISTANCE MOVED (mm)
DISTANCE MOVED (mm)
NDV
50-60S
4h
20h
28S
18S
8S
4S
NDV
28S
18S
4S
50-60S
5
0.5
0.1

these electrophoretic conditions a straight line relationship existed for migration distances versus molecular weights for all marker molecules (see insert, Fig. 3). The electron microscopically determined molecular weight of 5.2×10^6 to 5.6×10^6 daltons (31) was used for NDV. To use this graph for molecular weight estimates of 50 to 60S viral RNA mol wt one must assume that a straight line can be projected for up to 3 fractions beyond NDV (insert, Fig. 3). Shoulders on the 4S and 8S peaks suggest that other minority species might be present which would require gels with a smaller pore size for resolution.

Velocity sedimentation in sucrose gradients (Fig. 4) resolves FeLV RNA into a broadly sedimenting 50 to 60S class and a group of unresolved molecules sedimenting betwen 4 and 10S. The sedimentation coefficient of 50 to 60S is determined by the method of Martin and Ames (36) in reference to differentially labeled 50S NDV RNA included in the gradient.

One constant observation is the existence of much (25 to 50%) TCA precipitable RNA in the 4 to 10S region of velocity sedimentation gradients (Fig. 4). This percentage is similar to the findings of Jarrett, et. al., in which phenol extracted FeLV RNA in analyzed on sucrose gradients (29). By contrast, 4 to 10S RNA on gels represents less than 20% of the total (Fig. 3). Making the sucrose solution 0.1% SDS did not decrease the occurrence of small molecular weight RNA in the gradients.

Figure 4. Velocity sedimentation of FeLV RNA and NDV RNA. FeLV RNA was labeled and prepared as described in Fig. 3. Ethanol precipitated ^{32}P NDV RNA was solubilized with FeLV in extraction buffer before sedimenting. Sedimentation was as described in Materials and Methods. Fractions were counted directly in Aquasol.


60S
54S
NDV
4h
20h
6
4
2
3
2
1
10
20
30
TOP
FRACTION
^{3}H CPM X 10^{-2} (•—•)
^{14}C CPM X 10^{-2} (•—•)

Effects of labeling time on viral RNA. In three out of three experiments in which virus was doubly labeled, 4 h with ^{3}H uridine and 20 h with ^{14}C uridine, the 50 to 60S RNA of 20 h RNA electrophoretically migrated 0.5 to 1.0 fraction slower than the 50 to 60S RNA from 4 h virus, giving an average difference of 0.7 fractions (Table 1). This represents an apparent difference in molecular weights of 0.5 to 1 million daltons in this region of the gel (insert, Fig. 3). By comparison to reference RNA molecules, mean molecular weight estimates of 6.2×10^{6} daltons and 6.8×10^{6} daltons are derived for the 4 h and 20 h high molecular weight molecules respectively (Table 1).

This apparent size difference in the 50 to 60S RNA between 4 h and 20 h labeled virus is confirmed and measured by velocity sedimentation in aqueous sucrose gradients (Fig. 4). The results of two experiments are summarized in Table 1. The mean sedimentation coefficients are 50.4S for 4 h viral RNA and 59.6S for 20 h viral RNA. ^{32}P NDV 50S RNA was an internal marker for each gradient.

These results confirm the findings by East, et. al. (19) in which FeLV RNA collected after 2 h of labeling had a sedimentation coefficient of 50S while after 20 h of labeling had a coefficient of 58S. The explanation given by East, et.al. (18), is that a structural modfication of the RNA, either by a joining of subunits, or an altered secondary structure to an already assembled molecule, gives the older form of the viral RNA (20 h) a higher sedimentation coefficient. If this explanation is correct, then one should see a more slowly sedimenting 50 to 60S molecule for virus produced over any 2 (19) to 4 h

TABLE 1. Electrophoretic migration and velocity sedimentation of FeLV 50-60S RNA relative to NDV 50S RNA

	Interval of virus labeling (hours)			
	0→20	0→4	4→8	20→24
1. Number of fractions FeLV RNA migrated slower than NDV RNA	2[b]	1.3[c]	1.2[c]	1.3[b]
2. Number of fractions FeLV RNA migrated faster than 20 hr. FeLV RNA	-	.7	.8	.7
3. Apparent average molecular weight (daltons) estimated from gel position	6.8×10^6	6.2×10^6	6.2×10^6	6.2×10^6
4. Sedimentation coefficient	59.6[a]	50.4[d]	50.7[c]	56.5[a]

[a] average of 5 experiments

[b] average of 4 experiments

[c] average of 3 experiments

[d] average of 2 experiments

period, not just the first period following addition of isotope. To test this, cells which had undergone 20 h of labeling with ^{14}C uridine, and 4 h with ^{3}H uridine, were resuspended in fresh medium (with no isotopes) for an additional 4 h period. Hence, ^{14}C uridine labeled viral RNA becomes incorporated into the virion during the 20 to 24 h interval post-labeling from an intracellular pool of ^{14}C-labeled viral RNA. Likewise the ^{3}H-labeled RNA becomes incorporated during the 4 to 8 h interval post-labeling.

The 50 to 60S RNA incorporated during the 4 to 8 h and 20 to 24 h post-labeling periods electrophoretically comigrate (Fig. 5). Averages of several experiments show them respectively migrating 0.8 and 0.7 fractions faster than 0 to 20 h RNA (Table 1). Sedimentation analysis appears to confirm the electrophoretic data since an average of 50.7S and 56.5S are found for 4 to 8 h and 20 to 24 h RNA respectively as compared to 59.6S for 0 to 20 h RNA (Table 1).

Recent studies with RSV demonstrate the existence of unassembled 35S subunits in newly formed virus particles, particles 3 min to 1 h in age (10,11). Such subunits were sought in 1 h FeLV. F-422 cells adapted for adherence to flasks were used to facilitate rapid medium changes. Adapted cells were grown to concentrations of 1×10^{6} ml in spinner culture and then placed in a roller bottle to which most of the cells adhered forming a near-monolayer in 8 h. Unattached cells were removed by pipetting, and adhered cells were incubated with tritiated uridine for 16 h, following which, collections of cell supernatant were made at 1 h intervals. Figure 6 demonstrates the coelectrophoresis of

Figure 5. Coelectrophoresis of FeLV RNA and NDV RNA. Cells from the experiment described in Fig. 3 were resuspended in fresh medium (no labeled uridine) for 4 h. All other steps were carried out as described in Fig. 3 except that FeLV RNA was ethanol precipitated with carrier before dissolving in electrophoresis buffer along with ^{32}P NDV RNA.

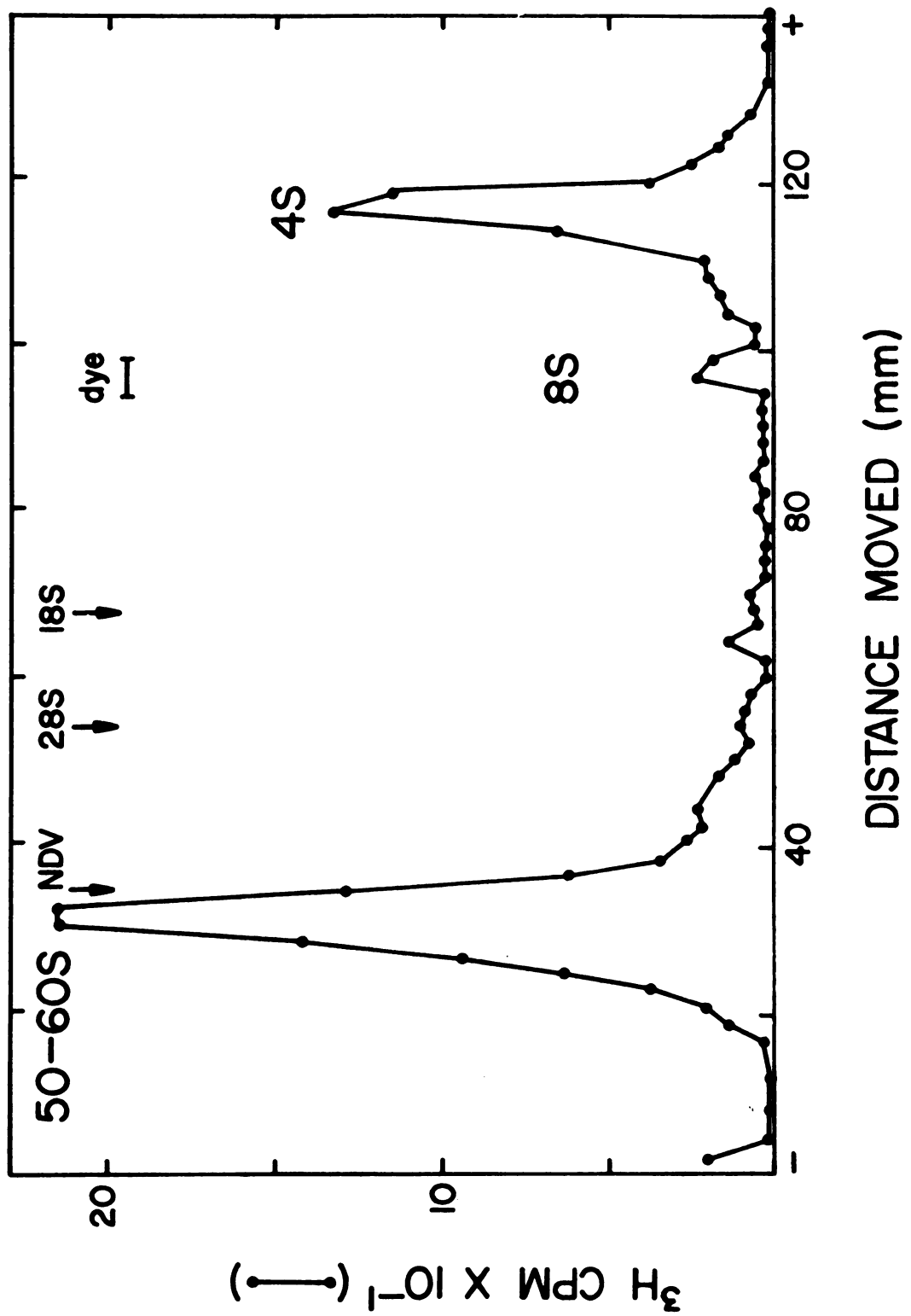
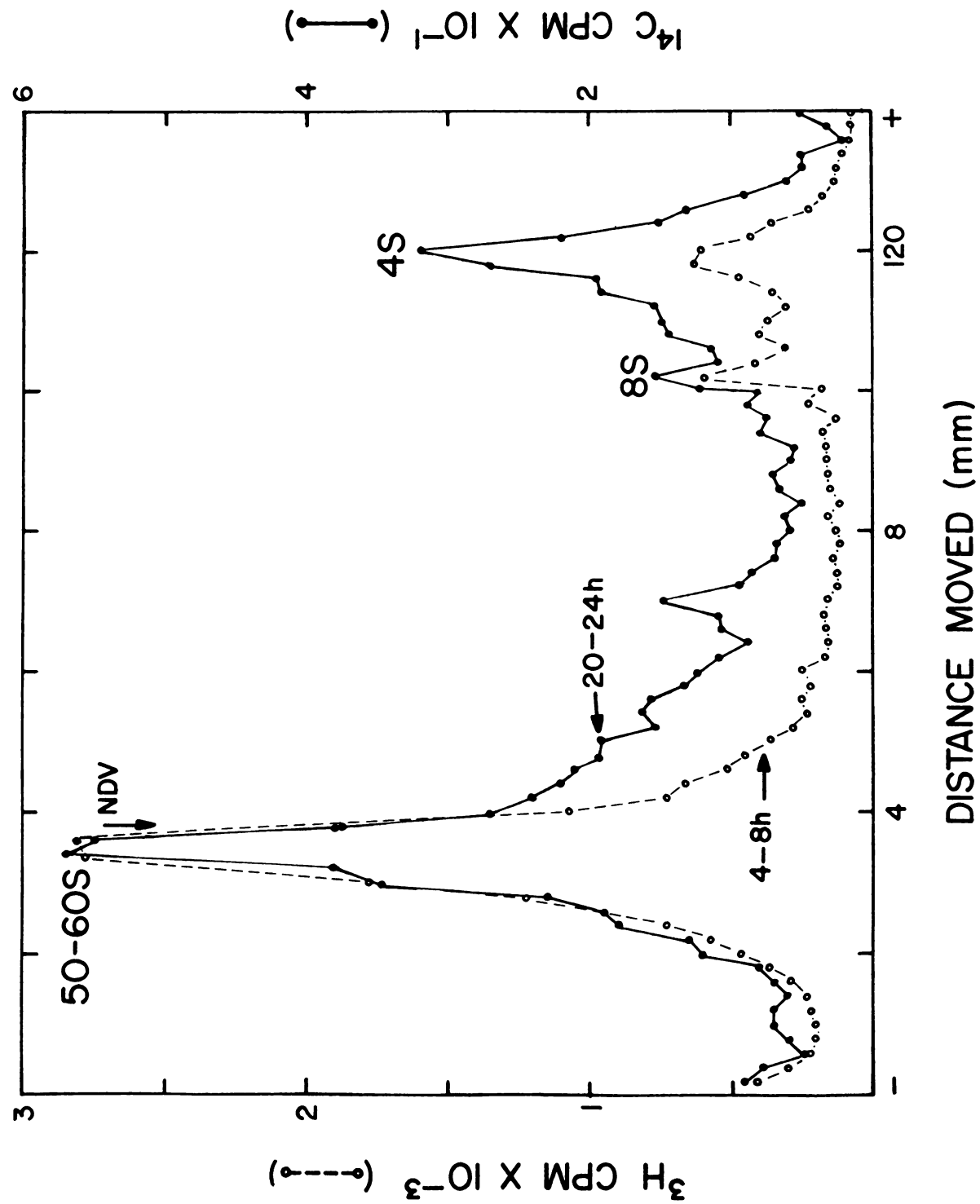

50-60S
NDV
28S
18S
dye
4S
8S
20
10
0
40
80
120
+
DISTANCE MOVED (mm)
^{3}H CPM X 10^{-1} (●——●)

Figure 6. Coelectrophoresis of FeLV RNA and NDV RNA. FeLV labeled 1 h with ^{3}H uridine was prepared separately by incubating 4×10^{7} F-422 cells in monolayer with 200 uCi uridine for 16 h, after which a series of 8 1-h collections were made. Cell supernatant was kept at 0 C until all collections were made. Virus was purified immediately. Virus purification and RNA extraction as well as ^{32}P NDV RNA preparation was as described in Materials and Methods. Electrophoresis was as described in Fig. 3.


^{14}C CPM X 10^{-1} (●——●)
6
4
2
4S
8S
120
+
20–24h
NDV
50–60S
4–8h
8
4
–
3
2
1
^{3}H CPM X 10^{-3} (●---●)
DISTANCE MOVED (mm)

^{3}H-labeled 1 h viral RNA prepared from monolayer cells with ^{32}P NDV RNA. No 35S subunits were found in the 1 h virus (Fig. 6). The 4S, 8S and 50-60S RNA species were present in the amounts of 21, 3 and 58% respectively.

Velocity sedimentation of viral RNA through 99% DMSO. To determine whether differences in size (length) or integrity of the 50 to 60S RNA subunits might explain the observed differences in sedimentation and migration behavior of the 4 h and 20 h 50 to 60S molecules, the following experiment was done. Total RNA from virus double-labeled 20 h with ^{14}C uridine and 4 h with ^{3}H uridine was denatured in 90% DMSO, a treatment known to completely denature single-stranded and double-stranded RNA (44) and to dissociate high molecular weight RNA from oncornaviruses into its subunits (2,14). Denatured viral RNA was then analyzed by velocity sedimentation in sucrose gradients made up in 99% DMSO. The rationale was that 1) if subunits are identical in length then superimposible sedimentation profiles will be seen since the sedimentation coefficient of RNA in DMSO is a direct function of its molecular weight (44) and 2) if subunits contain discontinuities, for example from ribonuclease nicks, its fractured integrity will become apparent by a heterogeneous sedimentation pattern (14). Twenty-eight S RNA with a molecular weight of 1.8×10^{6} daltons (35) which sediments in 99% DMSO at 25 C with a sedimentation coefficient of 4.22S (14) was used as a marker to determine the $s_{25,\ DMSO}$ for FeLV RNA subunits by the method of Martin and Ames. The molecular weight was calculated

from the sedimentation coefficient using Strauss' equation:

$$s_{25,\ DMSO} = 0.052\ M^{0.31}$$

Three observations were made. 1) Under conditions of complete denaturation (Fig. 7A), 4 h viral RNA sediments as two major peaks: a small molecular weight peak coincident with cellular tRNA, and a large molecular weight peak nearly coincident with 4.22S (25, DMSO) rRNA (4.3S), with a corresponding mol wt of 1.9×10^6 daltons. The 1.9×10^6 dalton mol wt species is known to be the large subunits of 50 to 60S RNA since the latter RNA purified on aqueous velocity sedimentation gradients denatured on DMSO gradients to molecules primarily this size (D. Brian, data not shown) and because they contain poly(A) (Fig. 8) a property of oncornavirus large RNA subunits (27,32,42). It was not determined what fraction of the 4S peak is contributed by 50 to 60S denaturation products. The 4S and 8S RNA species are not resolved by these sedimentation conditions. 2) Under conditions of complete denaturation (Fig. 7A) the high mol wt 20 h RNA differs from the high mol wt 4 h RNA in two respects. a) It sediments more slowly, 3.9S (25, DMSO) as compared to 4.3S (25, DMSO) with a corresponding mol wt of 1.2×10^6 daltons. b) It is broader, presumably containing a population of molecules more heterogeneous in size. The slower sedimenting peak comprises 50% of the 20 h RNA as compared to 20% for the 4 h RNA. 3) When ethanol precipitated viral RNA is dissolved and heat treated (56 C, 15 min) in anything less than 90% DMSO, complete dissociation may not occur (Fig. 7B, C, D). Fig. 7D demonstrates the resolution of two RNA peaks in RNA treated 56 C, 5 min in 80%

Figure 7. Velocity sedimentation of FeLV RNA and murine cytoplasmic RNA through 99% DMSO. Gradients were made, centrifuged, fractionated and assayed as described in Materials and Methods. The position of 28S rRNA (4.22S in 99% DMSO at 25 C) was determined in separate gradients run under identical conditions. ^{14}C-labeled 20 h FeLV RNA and ^{3}H-labeled 4 h FeLV RNA were prepared as described in Fig. 3 and ethanol precipitated with carrier. Pelleted precipitate was dried in a nitrogen stream, solubilized in water with 0.001 M EDTA, 0.1% SDS, made 90% DMSO, and treated 5 min at 58 C before layering onto the gradient. Centrifuged 43.192 x 10^6 revolutions. B. Same as A except that RNA was heated 56 C, 5 min in 80% DMSO. Centrifuged 40.510 x 10^6 revolutions. C. Same as A except that RNA was heated 56 C, 5 min in 84% DMSO. Centrifuged 33.879 x 10^6 revolutions. D. Same as A except that RNA was heated 56 C, 5 min in 80% DMSO. Centrifuged 42.517 x 10^6 revolutions.

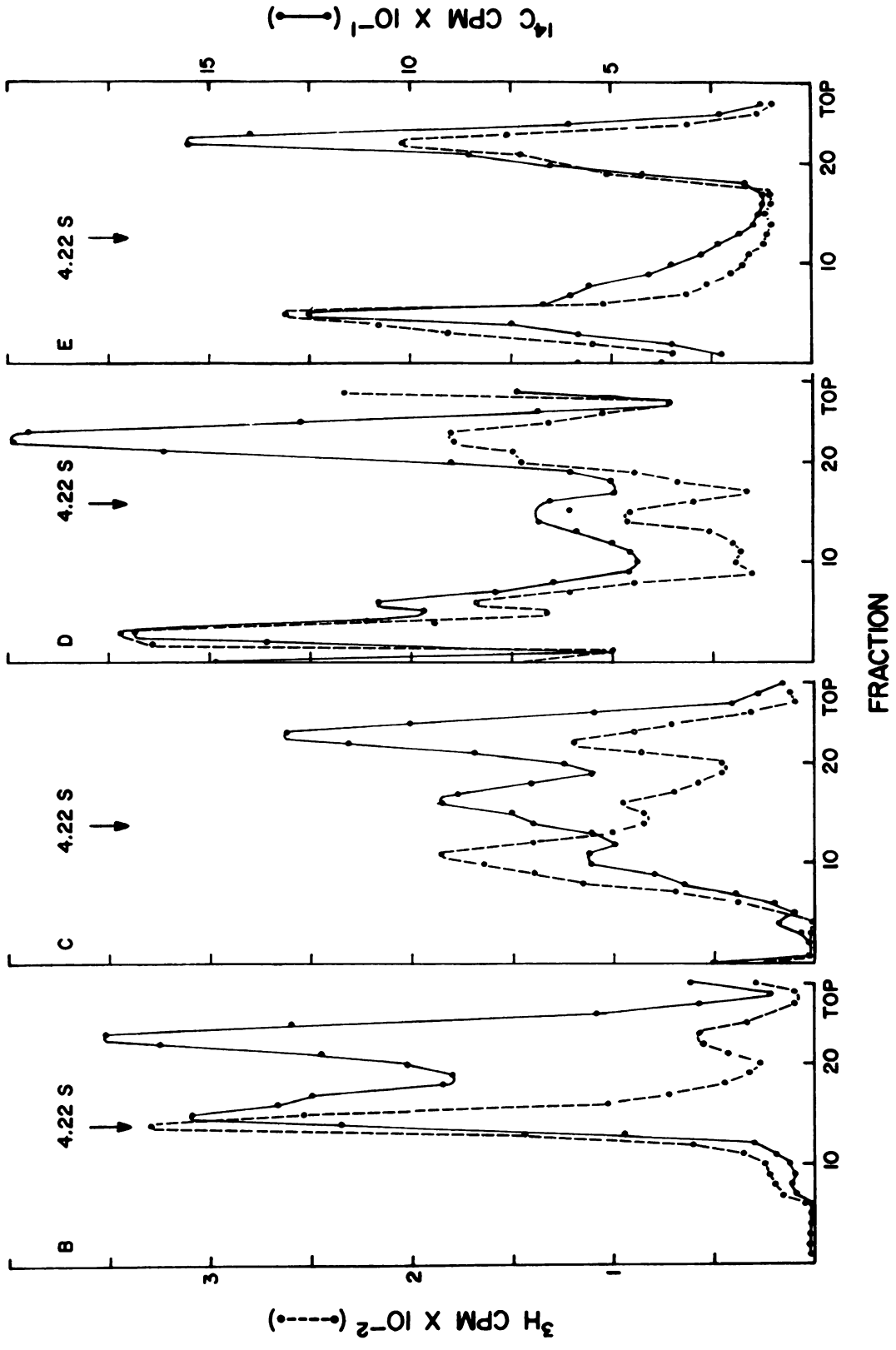
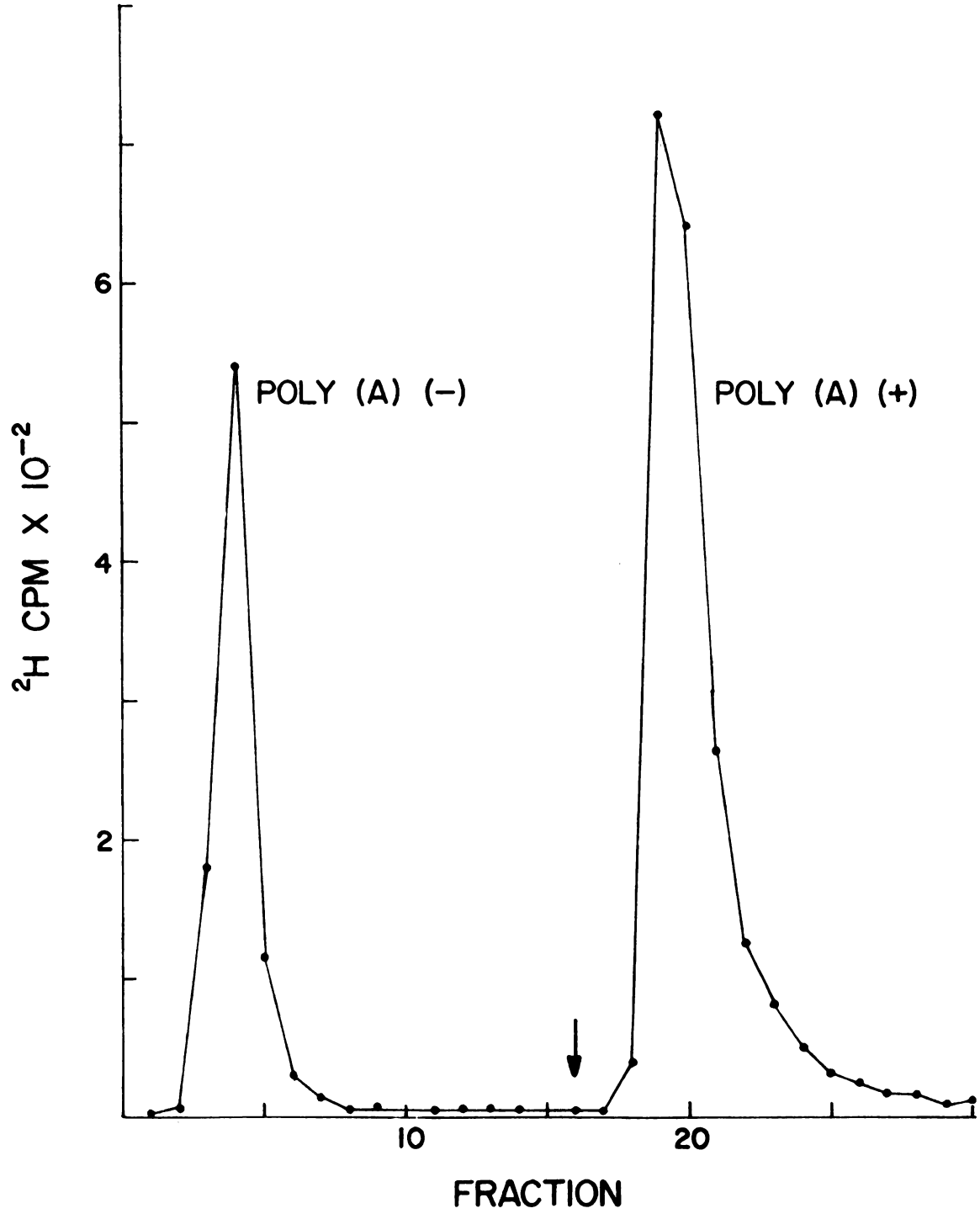

^{14}C CPM X 10^{-1} (•——•)
^{3}H CPM X 10^{-2} (•----•)
FRACTION
TOP
4.22 S
B
C
D
E

Figure 8. Oligo(dT)-cellulose chromatography. F-422 cells in suspension at 3 x 10^6/ml were incubated 16 h with 4 uCi/ml ^{3}H uridine. Virus was purified and RNA was extracted as described in Materials and Methods. RNA was ethanol precipitated with carrier, dissolved in water with 0.001 M EDTA, and 0.1% SDS, made 90% with 99% DMSO, heat dissociated at 56 C for 5 min then sedimented through 99% DMSO, 40.113 x 10^6 revolutions. The 4.22S peak (profile was very similar to Fig. 7A, data not shown) was collected, made 0.1 M NaCl, and ethanol precipitated with carrier. The resultant precipitate was dried in a nitrogen stream, dissolved in high salt buffer and chromatographed on oligo(dT)-cellulose as described in Materials and Methods. Elution with low salt buffer started at fraction 16.


POLY (A) (−)
POLY (A) (+)
^{2}H CPM X 10^{-2}
6
4
2
10
20
FRACTION

DMSO prior to centrifugation. The faster peak sediments at 7.09S (25, DMSO) with a corresponding molecular weight of 7 x 10^6 daltons using Strauss' equation. This assumes Strauss' equation holds true for molecules considerably larger than 2 x 10^6 dalton mol wt tobacco mosaic virus RNA (44). The 7.09S (25, DMSO) molecule therefore appears to be undenatured 50 to 60S viral RNA. At times when RNA from the same extract as sedimented in Fig. 7A and 7D is heat treated 56 C, 5 min in 80% (Fig. 7B) or 85% (Fig. 7C) a peak corresponding to the 1.2 to 1.9 dalton subunit peak begins to appear. Peaks sedimenting faster than 4.3S (25, DMSO) are not seen with heat treatments (56 C, 5 min) in 90% DMSO, and dissociation is assumed complete under these conditions (A. Thomason, data not shown).

Oligo (dT) cellulose chromatography. The 1.2 x 10^6 to 1.9 x 10^6 dalton mol wt subunits of viral RNA were confirmed to be 50 to 60S RNA subunits on the basis that 69.5% bound to oligo (dT) cellulose in 0.5 M NaCl (Fig. 8). Under these conditions rRNA and tRNA do not bind (1). Recovery from oligo (dT) cellulose columns is complete.

DISCUSSION

The minimum interval for labeling FeLV in F-422 cells with an RNA precursor (uridine) is 30 min. This short interval of virus production is similar to the SD-MSV system described by East et al. (18) but shorter than the 80 min interval for MLV described by Bader (3) and for the 1.5 and 2 h interval for AMV and RSV-RAV, and MLV (3) and AMV (4). It remains to be determined whether the shorter interval for FeLV is a reflection of a higher rate of virus production, more efficient uridine incorporation, or merely a reflection of total cellular metabolic rate differences. Too few counts were present to determine what RNA species were present in the 30 min virus.

Analysis of FeLV RNA by gel electrophoresis yields three size classes of RNA: 1) a 6.2×10^6 to 6.8×10^6 dalton mol wt class sedimenting in 0.1 M NaCl with a 50 to 60S sedimentation coefficient and comprising 52 to 76% of the total viral RNA, 2) a 1×10^5 dalton mol wt class not yet reported in feline leukemia or sarcoma viral RNA, corresponding to the 8S RNA reported in MSV RNA (20,33), comprising 2 to 5% of the total RNA, and 3) a 2.5×10^4 dalton molecular weight class sedimenting with 4S cellular tRNA in 0.1 M NaCl and comprising 6 to 12% of the total viral RNA. Assuming that the 50 to 60S molecule exists at the rate of one per virion, then the 8S molecule is present at the rate of 3 to 10 per virion, and the 4 to 5S molecule is present at the rate of 23 to 122 per virion. Species sedimenting as 18S, 28S or 34S are not detectable and in this respect the Rickard strain of FeLV differs from that used by Jarrett et. al., (29). The absence of 18S and 28S RNA argues that there is no cell vesicular contamination. Perhaps the cellular material does not traverse

the sucrose barrier rapidly enough to be pelleted during virus purification.

The apparent molecular weight of 6.2×10^6 to 6.8×10^6 daltons for the 50 to 60S RNA is based on the demonstration by Peacock and Dingman that a straight line relationship exists between the electrophoretic migration distance of a single-stranded RNA molecule and the log of its molecular weight (39). It was measured under conditions in which a straight line was found among 5.2 to 5.6×10^6 dalton mol wt NDV RNA, 1.8×10^6 dalton mol wt 28S feline rRNA, 0.5×10^6 dalton mol wt 18S feline rRNA, and 0.2×10^5 dalton mol wt tRNA, but assumes that a straight line can be projected up to 3 fractions beyond the NDV marker RNA (insert, Fig. 3). Using Spirin's equation (43):

$$M = 1550 \times s^{2.1}$$

the molecular weight for a 50 to 60S molecule is 9.7 to 11.7×10^6 daltons. Applying this equation to an aggregate molecule may lack validity since the equation was derived from data using single-stranded molecules ranging from 0.3 to 2.1×10^6 daltons. The measured sedimentation coefficient of 50 to 60S agrees closely with 50 to 58S for FeLV-R measured by East et al. (19). It does not agree with the 74S measured by Jarrett, et. al. (29). It appears therefore that a significant different exists between Rickard strain of FeLV and other (A, B and C) strains which have been studied (46).

RNA from virions purified isopycnically yields larger proportions of both 8S and 4S RNA (16 and 35% respectively) and a relatively smaller proportion of 50 to 60S RNA (17%) (D. Brian, data not shown). Presumably intravirion RNA degradation occurred during the lengthy

purification process (2). While 8S and 4S species were minimally present in virus purified only by pelleting, the question remains whether 8S and 4S RNA species present are degradation products of 50 to 60S RNA. Nucleotide analysis (7) may answer this question. Notable is that small mol wt RNA species are absent in MLV prepared similarly (2). Pelleting virus a second time through sucrose presumably purified them from ribonucleases present in the serum (R. Patterson, personal communication) since this step improved the yield of undegraded RNA. Noteworthy also is that 4 to 8S RNA routinely composes a larger fraction (25 to 60%) of the total RNA when total RNA is analyzed by sucrose gradient centrifugation as compared to gel analysis (8 to 17%). Recovery of counts on both gels and gradients is equivalent. It appears, therefore, that degradation occurs during sucrose gradient centrifugation, a process that is not inhibited by making the gradients 0.1% SDS.

The electrophoretic mobility and the sedimentation coefficient of the 50 to 60S molecule are variable depending upon the length of time over which the virus was labeled. From virus labeled for 20 h, high molecular weight RNA electrophoretically migrates with a mean molecular weight of 6.8×10^6 daltons, and sediments in 0.1 M NaCl with a mean sedimentation coefficient of 59.6S. From virus labeled for 4 h the corresponding mol wt and sedimentation coefficient are 6.2×10^6 daltons and 50.4S. These data confirm and extend findings of East et al., in which the sedimentation coefficients for the Rickard strain of FeLV were measured to be 58 and 50S respectively. A similar phenomenon was observed in RSV (10,11). In

agreement with the maturational hypothesis, i.e., that the modification giving rise to a faster sedimenting molecule occurs within the virion after budding, our data demonstrate that virus labeled over any 4 h period, not just the first, possesses the faster electrophoretically migrating (slower sedimenting) molecule. Virus labeled from 4 to 8 h, and from 20 to 24 h following addition of label to the cells have electrophoretically estimated molecular weights of 6.2×10^6 daltons in each case, but mean sedimentation coefficients of 50.7S and 56.5S respectively. Electrophoretic data alone suggest, and sedimentation data does not rule out that RNA in virus labeled during the first 4 h is identical or very similar in size and arrangement to RNA from virus labeled over any 4 h period. The hypothesis favored by others (10,11,18) to explain the maturational modification says that subunit structures assemble into a larger molecule. In immature (60 min) RSV, subunits from 15S to 60S are found which apparently assemble into the larger aggregate of 68S (11). No subunits within the range of 8S and 50S are found in FeLV collected at 60 min intervals. Any assembly within FeLV would therefore involve two or more 52S aggregates to form a 59S aggregate (18). This would yield a molecule of 21×10^6 using Spirin's equation, with a resultant s_w of 1088. This is a molecule far larger than any found in FeLV, and therefore the above hypothesis seems implausible. An alternative explanation was sought by observing FeLV subunits under complete denaturation.

The 60 to 70S molecules of RSV apparently retain their aggregate structure while possessing nicks which are revealed only after the

aggregate has been denatured (2). Conceivably, nicks within the loops of an aggregate molecule could allow the ends to fold more tightly by way of secondary bonds and thereby create a slower electrophoretically migrating (faster sedimenting) molecule compared to the unnicked aggregate. Such a model would explain the faster sedimenting older (and presumably more nicked) 50 to 60S viral aggregate structure.

Sedimentation of completely denatured FeLV RNA through gradients of 99% DMSO suggests such a qualitative difference. Large subunit RNA from 20 h virus sediments more slowly and more heterogeneously than subunits from 4 h RNA (Fig. 7A) suggesting it harbors nicks before denaturation. The proposed hypothesis is therefore a favorable one and the apparent difference in molecular weights between 4 h and 20 h 50 to 60S RNA more likely represents a conformational difference.

Sixty-nine percent of the 4.22S (25, DMSO) RNA binds to poly (dT) cellulose under conditions which will bond only poly(A) containing RNA, and will not bind rRNA or tRNA (1). This confirms these are subunits of the large 50 to 60S previously shown to possess poly (A) stretches (27,32,42). It also argues that most of the 4.22S (25, DMSO) subunits are intact. Finally, it establishes a procedure whereby large subunit RNA can be prepared free of small subunit (4S RNA) and rRNA contamination for analytic purposes.

It is noteworthy that denatured FeLV sediments in DMSO at the same rate as 28S rRNA. Its molecular weight therefore is approximately 1.9×10^6 daltons, data which agrees with the results of East et al., in which heat denatured FeLV 50 to 60S RNA subunits sediment in aqueous sucrose gradients with 28S rRNA. This contrasts sharply with the 5.35S

(25, DMSO) subunit found in RSV (14) for which the corresponding molecular weight is 3.1×10^6 daltons. It also contrasts sharply with the results of Whaley and Jarrett et. al., (29,46) in which subunits of 74S FeLV RNA sediment as 34S molecules, and had electrophoretically determined molecular weights of 2.2 to 2.6×10^6 daltons. This further substantiates a possible difference between Rickard's strain of FeLV and other strains (A, B and C) studied (46).

From our data the 50 to 60S, 6.2×10^6 to 6.8×10^6 dalton mol wt, RNA molecule would possess 3 to 4 1.9×10^6 dalton mol wt subunits. This agrees with the number of subunits calculated for RSV (14).

Strong forces apparently form the aggregate 50 to 60S molecule since heat treatment in combination with 90% DMSO seems to be required for complete denaturation. Ninety-nine percent DMSO at 25 C alone seems ineffective for complete RNA dissociation since these are the conditions under which the RNA is subjected during centrifugation and in certain cases (Fig. 7D) no dissociated subunits are seen. Aggregate structures intermediate between 60 to 70S and 34S occur in AMV RNA during controlled denaturing conditions (45) and therefore it is not surprising to find a similar phenomenon for FeLV RNA. What is surprising is that any aggregate at all survives the 99% DMSO gradient (44).

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APPENDIX A

METHOD FOR POLYACRYLAMIDE-AGAROSE GEL ELECTROPHORESIS

APPENDIX A[1]

METHOD FOR POLYACRYLAMIDE-AGAROSE GEL ELECTROPHORESIS

The method of Peacock and Dingman (39) as described by Bunting (9) for making combination polyacrylamide-agarose gels was modified for use in gel tubes. Reagents used were made up as follows.

1. Acrylamide--N,N'-Methylenebisacrylamide (Cyanogum 41, Fisher)

   200 g. Cyanogum 41

   water to 1 liter

   Filtered. Stored in brown glass bottle at 4°C.

2. Tris-EDTA-Borate Buffer, pH 8.3 (Peacock's 10X Buffer).

   216 g Tris-(hydroxymethyl)-amino methane

   110 g boric acid

   18.6 g disodium ethylenediaminetetraacetate

   water to 2 liters

   Solution is filtered and stored in a glass bottle. Solution is ten times working concentration.

3. DMAPN, 6.5% (catalyst).

   16 ml 3-dimethylaminopropionitrile concentrated reagent (Eastman Organic Chemicals)

   water to 250 ml

   Stored in brown bottle at 4°C.

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[1]List of references are on page 146.

4. Ammonium Persulfate, 1.6% (catalyst).

   0.16 g ammonium persulfate

   water to 10 ml

   Prepared fresh just before using.

5. Agarose, (Biorad) electrophoresis grade.

6. 40% sucrose -1 mM EDTA-Bromphenol Blue Dye Solution (for sample dilution).

   20 g sucrose

   0.5 ml 0.1 M Na_2EDTA

   0.05 g bromphenol blue dye

   water to almost 100 ml Adjust pH to 6.2 with 5N NaOH.

   Water to 100 ml. Freeze in 0.3-0.4 ml aliquots.

Gels were made by the following protocol.

Protocol for making polyacrylamide-agarose gels.

Gel	Agarose	Water	20% Acryl-Bis Solution(19:1)	6.4% DMAPN	10X Buffer	1.6 % Ammonium Persulfate
1.5%-0.5%	0.16 g	23.4 ml	2.4 ml	2.0 ml	3.2 ml	1.0 ml
1.75%-0.5%	0.16 g	23.0 ml	2.8 ml	2.0 ml	3.2 ml	1.0 ml
2.0-0.5%	0.16 g	22.6 ml	3.2 ml	2.0 ml	3.2 ml	1.0 ml
2.5-0.5%	0.16 g	21.8 ml	4.0 ml	2.0 ml	3.2 ml	1.0 ml
3.0-0.5%	0.16 g	21.0 ml	4.8 ml	2.0 ml	3.2 ml	1.0 ml
3.5-0.5%	0.16 g	20.2 ml	5.6 ml	2.0 ml	3.2 ml	1.0 ml

1. Reflux agarose in water 15 min. with constant stirring (stirring bar).

2. Combine 20% Acryl-Bis solution (19:1) with 10X buffer.

3. Cool agarose solution to 48°C; warm Acryl-Bis and buffer to 48°C.

4. Within one minute:

   a. Add ammonium persulfate to agarose solution.

   b. Add DMAPN to Acryl-Bis-buffer solution.

   c. Combine all together, mix thoroughly, pour gels.

5. Allow to polymerize at 20°C for 1 h.

Gel tubes [18 cm. x 0.8 cm. outside diameter (0.5 cm inside diameter)] were rinsed with Photoflo (Kodak), dried, stoppered at one end with parafilm, and mounted vertically. A 30 ml syringe with a 5 inch cannula was used to rapidly fill the tubes. The quantity in the above protocol (32 ml) will fill 10 tubes. Filled tubes were stored for longer than 6 months at 4°C with no noticeable detrimental consequences.

Just prior to use gels were slightly (2-3 mm) displaced with a Gilson gel piston and sliced transversley with a razor blade forming a flat surface for the RNA sample. The gel was then retracted into the tube. Parafilm was used to cap the bottom end of the tube and 12-15 holes were made with a needle to allow for current flow. Gels were preelectrophoresed for 1 h. at 150 volts.

All RNA analyzed by electrophoresis was radioactively labeled. The concentrations of RNA preparations were estimated to be well within the limits of 500 µg RNA/ml, and sample sizes within the limit of 10 µg RNA as advised by Bunting (9). RNA samples were dissolved in either extraction buffer or electrophoresis buffer, mixed 1:1 (sometimes 10:1, in which case a small sucrose crystal was also added) with bromphenol blue dye solution, and loaded onto the gel in 50 µl or less. Resolution between 8S and 4S RNA species was not obtained when sample sizes approached 100 µl.

Electrophoresis was carried out at 150 volts constant voltage at 4°C using a Polyanalyst (Buchler Instruments). Gels were fractionated into 2 mm fractions with the Gilson gel fractionator. Each fraction

was digested 1 h. at 50°C in 0.1 ml NCS tissue solubilizer (New England Nuclear) then counted in 5 ml Aquasol.