cerebral passage series. The virus(es) produced a lethal effect in the animals 6 to 14 days following inoculation. Identification of the mouse passage virus was established by serum neutralization tests in tissue culture and in suckling mice. Further identification was established by direct fluorescent antibody staining of the mouse passaged virus after one passage in tissue culture. It is suggested that the growth of RS virus in brain of suckling mouse with resultant illness and death may have practical value in the evaluation of prospective vaccines.

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Concentration of Complement Fixing Viral Antigens.* (30692)

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Pereira and Valentine(1), Wilcox and Ginsberg(2), and many others showed that cell-associated infective virus could be concentrated by slow speed cell sedimentation prior to release from the virus-bearing cells. Others(3,4) used this procedure to concentrate neo-antigens(5). In this note we describe its application for the routine preparation of high titered complement fixing (CF) antigens for a great variety of viruses.

The following preparations were made using standard virus inocula and standard virological and tissue culture procedures. No steps were taken to define carefully maximum virus propagation. The results, therefore, should not be regarded as optimal but rather as indicative of the usefulness of this technique.

Materials and methods. Cell cultures were obtained from Microbiological Associates, Inc. (Table I) and maintained on Eagle's Minimum Essential Medium in Earle's Balanced Salt Solution containing varying concentrations of Agamma Calf Serum (Hyland†), 4 mM of Glutamine, 100 Units of Penicillin and 100 γ of Streptomycin/ml.

Viruses were obtained from various sources; they were passed once or twice in our laboratory under recommended conditions in order to obtain standard seed stocks(6,7).

CF tests were done in the microtiter system (Cooke Engineering‡) as described by Sever (8): Antigens and control antigens were diluted serially in 0.025 ml volumes of Veronal buffer by carrying over 0.025 ml volumes of the antigens in calibrated spiral loops. Following addition of 0.025 ml of complement (containing 1.8 full units), the plates were incubated overnight at 4°C, after which the hemolytic system (0.05 ml of 1% sensitized sheep red blood cells) was added. The whole

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† Hyland Laboratories, Los Angeles, Calif.

‡ Cooke Engineering, Alexandria, Va.

TABLE I. Cell Cultures Employed for the Preparations Described.

Source	Species	Tissue	Reference	% agamma calf serum in maintenance medium
Primary cell cultures:				
AGMK	Cercopithecus	Kidney	—	3
MK	Macacus rhesus	"	—	3
HEK	Human	Embryonic kidney	—	3
MsE	Mouse	Embryo	—	1
MsK	"	Embryonic kidney	—	1
BK	Bovine	Kidney	—	3
Continuous cell lines:				
HeLa	Human	Carcinoma of cervix	Gey, G. O. *et al*, 1952(12)	5
KB	"	" " nasopharynx	Eagle, H., 1955(13)	10
WI-38	"	Amnion	Hayflick, *et al*, 1962(14)	3
BSC-1	Cercopithecus	Kidney	Hopps, *et al*, 1962(15)	3
NCTC 1469	Mouse	Liver	Hobbs, G. L., *et al*, 1957(16)	5
BHK-21	Hamster	Newborn kidney	MacPherson, I., *et al*, 1962(17)	10
MAF	Human	Embryonic skin and muscle	*	3
MA-104	Macacus rhesus	Kidney	*	3
MA-111	Rabbit	"	*	3
MA-112	Human	Whole embryo	*	3

* Obtained from the Dept of Research and Development, Microbiological Associates, Inc.

TABLE II. Infective Virus and CF Antigen Development in KB Cell Cultures Infected with Adenovirus Type 12 (Multiplicity of Infection 30).

Serum used for evaluation	Time of harvest (Days post inoculation)	Day after 4+ CPE	Infectivity titer*		CF titer†	
			20× cell pack	Supernate	20× cell pack	Supernate
Adenovirus	3		7.7	>5.4	80	<20
Group	5	1	7.9	6.9	160	<20
Reference	6	2	7.9	6.4	>160	<20
Serum	7	3	8.2	6.9	>160	<20
(Human pool)	8	4	8.5	6.9	80	20
	10	6	7.2	7.4	80	20

* $\log_{10}$ HEK ID50/ml (estimates on the basis of 2-3 tubes per 1 log dilution).
† 3+ or 4+ complement fixation.
Footnote: Titers represent means of 2 replicate preparations.

was incubated at 37°C for one hour and the tests were read as soon as the cells had settled out. The sera employed for CF testing were standard antiviral reagents of the CF laboratory of the Laboratory of Infectious Diseases, NIAID, National Institutes of Health. Such antisera were screened as a matter of routine for the absence of antibodies to viral antigens frequently found in "normal" sera. Titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement within a range of — to 4+.

For *infectivity titrations* 2 to 3 tubes of the appropriate indicator cell line were inoculated with 10-fold serial dilutions of the virus. The cultures were fed every 4-6 days; titrations were held until it was clear that the final endpoint had been reached.

Antigen concentration was achieved as follows: Inoculated cell cultures were harvested by shaking or scraping all the cells into the supernatant medium without prior freezing and thawing. The cells were then centrifuged gently for 10 minutes at slow speed (1000 rpm) following which most of the supernatant medium was carefully removed leaving only enough on the cell pack for the desired concentration. Removal of 90% of the super-

TABLE III. Infective Virus and CF Antigen Development in KB Cell Cultures Infected with Adenovirus Type 7, (Gomen; 257a) (Multiplicity of Infection 1:10).

Serum used for evaluation	Time of harvest (Days post inoculation)	Day after 4+ CPE	Infectivity titer*		CF titer†	
			20× cell pack	Supernate	20× cell pack	Supernate
Adenovirus	7	0	8.9	7.2	128	<8
Group	8	1	9.9	7.4	128	<8
Reference	9	2	9.4	8.4	128	8
Serum	10	3	8.4	8.9	256	8
(Human pool)	11	4	8.9	8.7	256	16
	12	5	7.9	8.4	256	16
	13	6	8.4	8.4	256	32
	14	7	7.7	7.9	128	16

* $\log_{10}$ HEK ID50/ml (estimates on the basis of 2-3 tubes per 1 log dilution).
† 3+ or 4+ complement fixation.
Footnote: Titers represent means of 2 replicate preparations.

TABLE IV. CF Antigen* Concentration of Herpesviruses[18] by Slow Speed Cell Sedimentation of Cultures Harvested at Maximum CPE.

Virus	Host tissue	MOI	n	Concentration factor	Crude	SN	Cell pack
Herpes Simplex	HeLa, MA-104	1/1000	5	10×	<4*	<4*	16–32*
Varicella	MAF, HEK, WI-38	NT	3	10×	<4	<4	32
Salivary gland (human)	MAF, WI-38	1/1000	15	20×	4–8	<4	64
Salivary gland (mouse)	MsE	1/1000	2	20×	4	<4	>64

* 3+ complement fixation; mean of n replicate preparations.
n: No. of replicate preparations.
Crude: Virus-cell suspension frozen and thawed 3 times.
SN: Supernatant medium following centrifugation for 10 min at 1000 rpm of crude suspension before freezing and thawing.
Cell pack: Cell concentrate after removal of SN.
MOI: Multiplicity of infection (number of infective doses/cell).
NT: Not titrated.

natant medium, for instance, gave a concentration of 10×, resulting in a 2-3% cell suspension. The cell pack was then frozen and thawed 3 times and homogenized for one minute in a Virtis "45" homogenizer at maximum speed. Only occasional preparations had trace amounts of anticomplementary activity at low dilutions.

Time of harvest was important. For all the lytic viruses this was at the time when 4+ CPE was observed or shortly thereafter. Incubation beyond this period resulted in the antigen being released from the cells into the entire medium. In the case of viruses without noticeable cytopathic effect the optimal harvest time was achieved by following CF antigen development at daily intervals.

Results. Table II illustrates the development of Adenovirus Type 12 infectivity and CF antigen in KB cell cultures under the experimental conditions described. Results of a similar experiment with Adenovirus Type 7 are given in Table III. We obtained comparable results with cell pack antigen preparations made of almost every one of the 31 adenovirus serotypes as well as with a number of intratypic strains.

Similarly, concentration of CF antigens by slow speed sedimentation of the infected cell cultures could be demonstrated with a great variety of viruses (Tables IV, V, and VI), and in most cases infective virus content was concentrated as well (Table VII).

Discussion. The procedure used for concentration of the various virus antigens described here was extremely simple; instead of dispersing the antigens into the medium and then concentrating them by ultracentrifugation they were sedimented in unruptured cells following which the antigens were dispersed into a small volume of medium by homogenizing the virus laden cells.

TABLE V. CF Antigen* Concentration of Myxoviruses by Slow Speed Cell Sedimentation of Cultures Harvested at Maximum CPE.

Virus	Host tissue	MOI	n	Concentration factor	Crude	SN	Cell pack
Influenza	MK	NT	4	10×	—	<8*	8–16*
Mumps	HeLa, KB	1/100–1	6	20×	8–16*	<8	32–64
Sendai	MK, MA-111	NT	6	10×	4–8	<4	32
Parainfluenza Type 2	MA-104, MA-111	1/10	2	20×	<4	<4	> 8
SV-5	MA-104, MA-111	1/50	3	10×	4–8	<4	>32
Parainfluenza Type 3	KB, MA-104	1/10	3	10×	4–8	<4	16–32
Respiratory syncytial	MA-112	1/10	4	20×	<4	<4	> 8
" "	HeLa	1/10	3	20×	16	8–16	128
Measles	HeLa	1/50	4	20×	4–8	<4	64–128

* 3+ complement fixation; mean of n replicate preparations.
MOI: Multiplicity of infection (No. of infective doses/cell).
n: No. of replicate preparations.
Crude: Virus-cell suspension frozen and thawed 3 times.
SN: Supernatant medium following centrifugation for 10 min at 1000 rpm of crude suspension before freezing and thawing.
Cell pack: Cell concentrate after removal of SN.
NT: Not titrated.

TABLE VI. CF Antigen* Concentration of Various Viruses by Slow Speed Cell Sedimentation of Cultures Harvested at Maximum CPE.

Virus	Host tissue	MOI	n	Concentration factor	Crude	SN	Cell pack
Polyoma	MsE, MsK	1/10	4	10×	4*	<4*	16–32*
SV-40 (#776)	AGMK, BSC-1	10–100	6	20×	4–8	<8	64
SV-40 (#777)	BSC-1	10	1	10×	–	4–8	16–32
Polio Type 1	HeLa	>100	1	10×	–	<4	64
Polio Type 2	HeLa	>100	1	10×	–	<4	16–32
Vaccinia	HeLa	10	4	20×	–	<8	>64
Milkers' nodule virus	BK	1/10	1	10×	–	<4	>32
Reovirus Type 3	MA-104, BSC-1	1/10–10	6	10×	<4	<4	32–64
LCM†	AGMK	NT	2	20×	<4	<4	32
MHV	NCTC-1469	NT	2	20×	8–16	8	32
Rubella[19]	BHK-21	1/100–1/10	5	20×	<2	<2	4–8

* 3+ complement fixation; mean of n replicate preparations.
† Inoculated with 10% LCM mouse brain suspension and harvested 7 days later.
MOI: Multiplicity of infection (No. of infective doses/cell).
n: No. of replicate preparations.
Crude: Virus-cell suspension frozen and thawed 3 times.
SN: Supernatant medium following centrifugation for 10 min at 1000 rpm of crude suspension before freezing and thawing.
Cell pack: Cell concentrate after removal of SN.
NT: Not titrated.

Pereira and Valentine(1) concentrated cell associated infective adenovirus by slow speed sedimentation and removal of part of the supernatant fluid from the virus-containing cells and, using essentially the same procedure, Schmidt *et al*(9) succeeded in obtaining satisfactory varicella CF antigen preparations. Sarma *et al*(10) concentrated avian leucosis and Rous sarcoma virus antigens which fixed complement with sera from Rous tumor bearing hamsters. They obtained antigens ("COFAL" antigens) in chick embryo cultures infected with avian leucosis virus by removing supernatant medium from healthy appearing monolayer cultures and thereby concentrating the antigen containing cells. Also, by employing the cell packing procedure Hoggan *et al*(3) and Sabin *et al*(4) detected, in acutely infected cell cultures, the presence of complement fixing virus specific non-virion "T" antigens(5) of Adenovirus Type 12 and SV-40 virus. It is important to distinguish

TABLE VII. Infectivity* Concentration by Slow Speed Cell Sedimentation.

Virus group	Virus	Host TC	n	Conc factor	Crude	SN	Cell pack	Indicator host system
Cytomegalo	Salivary gland (mouse)	ME	3	20×	5.5	—	6.0	MsK
					2.0	—	3.0	"
					4.5	—	5.0	"
Myxo	Mumps	HeLa	4	20×	5.2	—	7.2	AGMK
					7.7	—	>8.7	"
					6.7	—	7.7	HeLa
					6.2	—	7.7	"
	Parainfluenza 3	MA-104	1	20×	5.2	5.7	6.2	MK(Rh)
	" 3	KB	2	10×	6.2	6.7	7.2	"
					—	5.2	6.7	"
	Respiratory syncytial	HeLa	3	20×	5.7	—	7.7	AGMK
					6.2	—	7.7	"
					6.2	—	7.7	"
	Measles	HeLa	2	20×	6.2	—	7.2	HeLa
					6.7	—	7.2	AGMK
Papova	Polyoma	MsK	2	10×	6.7	—	7.7	MsK
					6.7	—	>7.0	"
	SV-40	BSC-1	3	20×	7.7	7.7	>8.7	AGMK
					7.7	6.2	>8.8	"
					8.2	7.2	8.7	"
Picorna	Polio Type 1	HeLa	1	10×	—	8.2	10.7	"
	" " 2	"	1	10×	—	8.2	10.2	"
	GDVII	HK	1	20×	5.2	5.2	6.7	HK
Pox	Vaccinia	HeLa	4	20×	7.7	6.7	8.7	AGMK
					6.7	6.7	8.2	"
					6.2	6.2	8.2	"
					6.7	6.7	7.5	"
Reo	Reo Type 3	MA-104	3	10×	7.7	6.7	8.2	"
					6.2	6.2	7.7	"
					7.2	6.2	7.7	"
	Rubella	BHK-21	3	20×	5.2		>5.7	" †
					3.2		4.7	" †
					4.7		5.7	" †

* $\log_{10}$ ID50/ml (estimates on the basis of 2-3 tubes per 1 log dilution).
† By interference with ECHO 11 CPE (10-100 ID50/ml).
n: No. of replicate preparations.
Crude: Virus-cell suspension frozen and thawed 3 times.
SN: Supernatant medium following centrifugation for 10 min at 1000 rpm of crude suspension before freezing and thawing.
Cell pack: Cell concentrate after removal of SN.

these neo-antigens from the virion antigens described herein; for whereas little or no neo-antigen was usually found at the time of maximum CPE, maximum amounts were found when the cells were harvested before the appearance of CPE.

The Laboratory of Infectious Diseases of NIAID and the Section on Infectious Diseases, Perinatal Research Branch of NINDB have for a number of years been occupied with efforts to produce "diagnostic antigens" for the numerous viruses known or suspected to cause human illness. Although the effort met with considerable success(11) many of the antigens were produced in unpredictable fashion and only after much costly manipulation; in some cases satisfactory antigens could not be produced at all. The usefulness to diagnostic virology of the "cell pack" procedure for producing viral antigens therefore seems apparent.

Summary. Complement fixing antigens produced in cell culture by a variety of viruses were concentrated by slow speed sedimentation of undisrupted virus infected cells and removal of the supernatant medium. In most cases, concentration of virus infectivity was demonstrated as well.

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Animal Plasma as Substrate for Synthetic Fibrinolysis Inducers.* (30693)

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Fibrinolytic, caseolytic and esterolytic activity is generated in human plasma in which certain synthetic organic compounds are dissolved and incubated. There is a close relationship between the chemical structure of a compound and its fibrinolysis-inducing capacity(1,2,3). All studies were carried out in human plasma. Attempts to evoke fibrinolytic activity in animal plasma with synthetic organic compounds gave either equivocal results or failed completely with the methods used(3,4). Animal plasma with a fibrinolytic system sensitive to synthetic organic compounds similar to that of human plasma would be of great assistance in the development of synthetic thrombolytic agents. This need, together with the availability of compounds more potent than the previously reported structures, prompted us to reinvestigate the use of animal plasma.

Material and methods. Plasma: Citrated plasma obtained from citrated blood (one part sodium citrate U.S.P. 3.8%, four parts blood) by spinning for 5 minutes at 1480 *g* was frozen in lots at —25°C until use within 3 weeks.

Buffered saline: One part barbital acetate buffer(5) pH 7.4 to 4 parts NaCl 0.85%.

Thrombin solution: 1000 units thrombin (Parke Davis) dissolved in 5 ml 50% glycerol.

Test of animal plasma for fibrinolytic activation by synthetic organic compounds: 3-isopropyl-6-methylsalicylic acid Na (o-thymotic acid Na) is dissolved to make a 20 mM concentration in a 0.4% solution of bovine fibrinogen Armour in buffered saline pH

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