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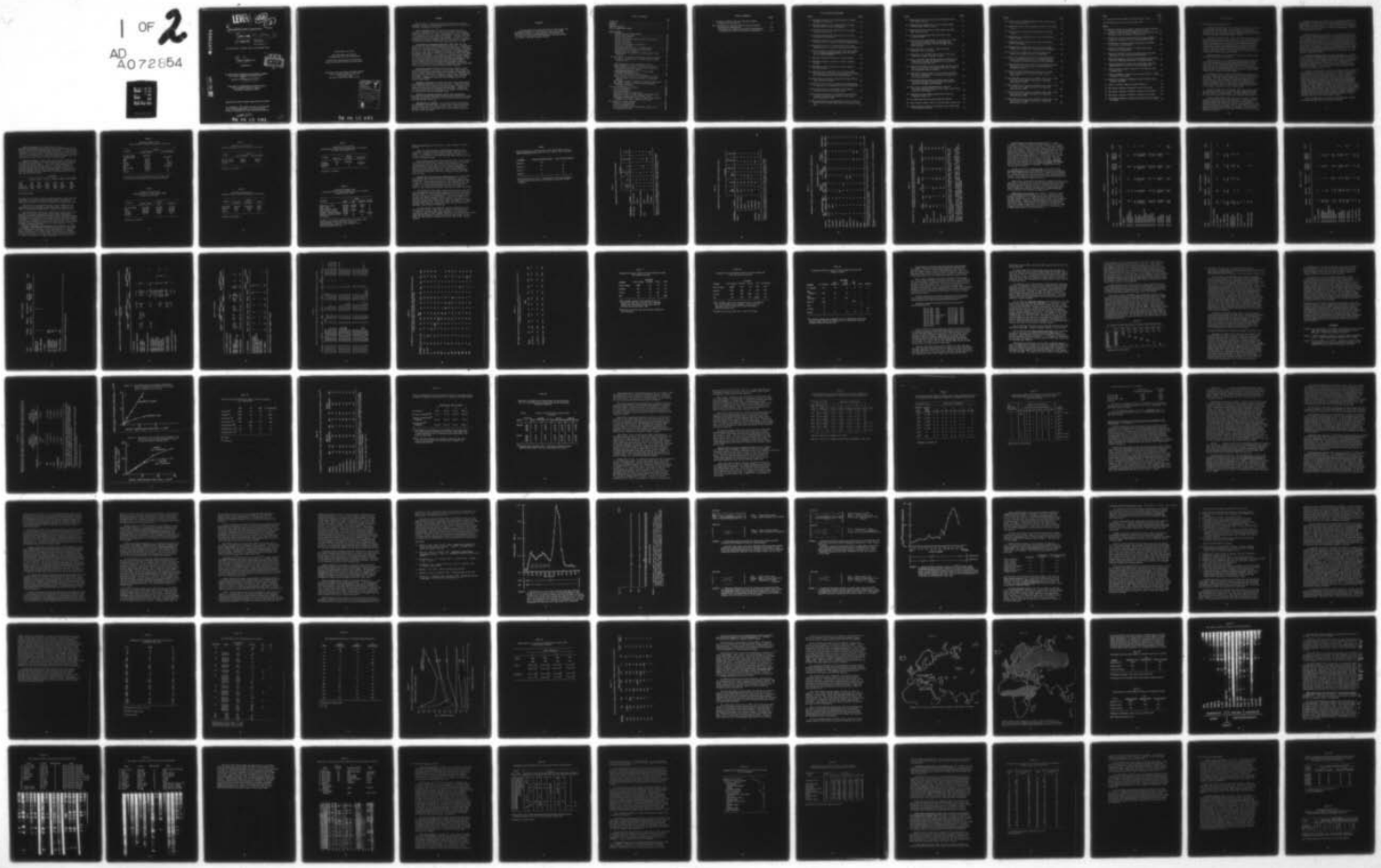

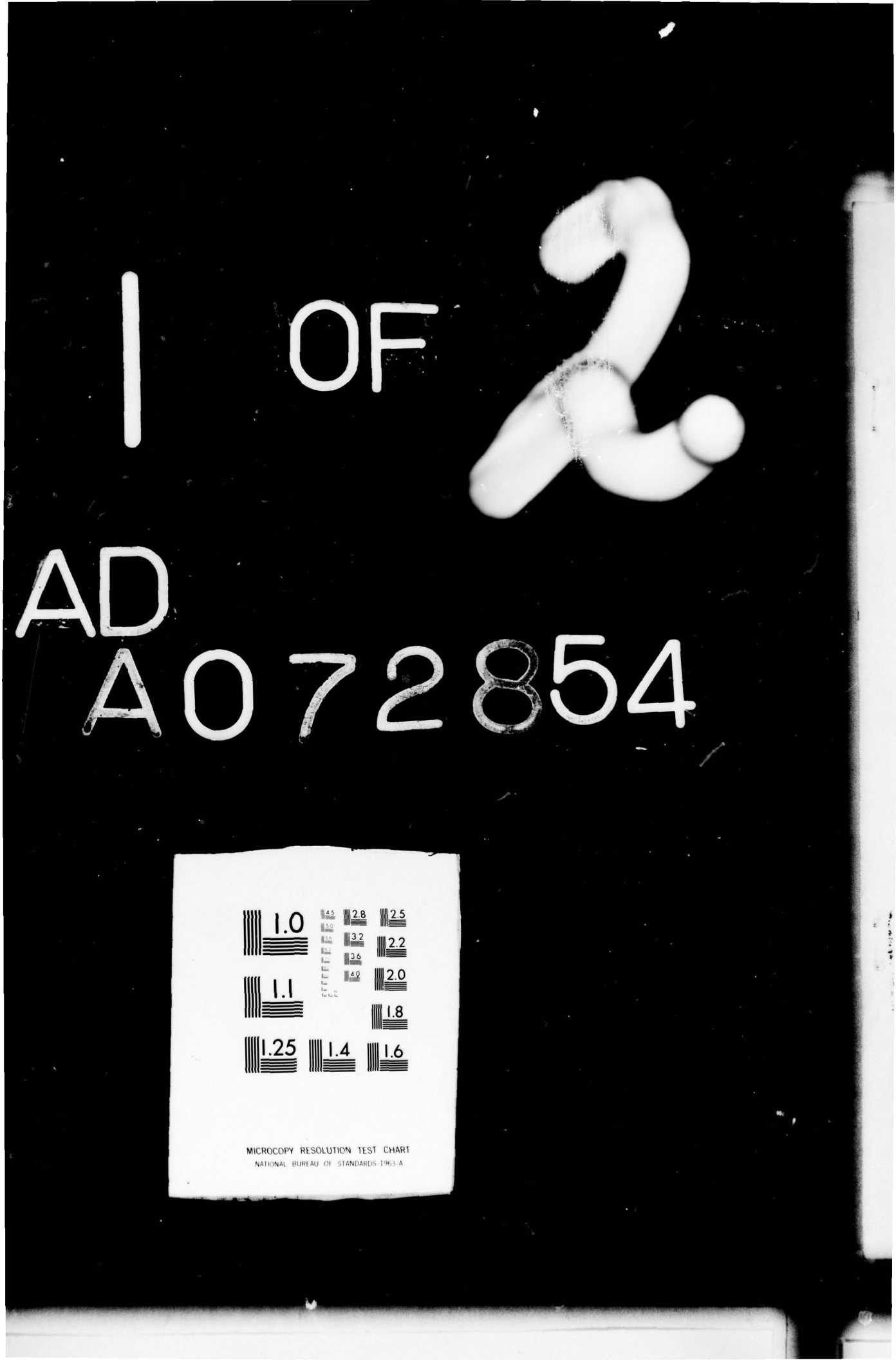
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(10) by

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Annual Report for 1978

Yale Arbovirus Research Unit
International Center for Arboviruses

World Health Organization Collaborating
Center for Arbovirus Reference and Research

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SUMMARY

Major effort of the reference center was devoted to rapid diagnosis and more sensitive methods for detection of antibody and antigens. Findings of note were:

Virus identification. Viruses were studied from Japan, Australia, Seychelles, France, Senegal, Ethiopia, Kenya, Brazil, Venezuela, and the U.S.A. Three presumably new viruses including a new orbivirus were identified from Ethiopia. Keystone virus was recognized for the first time in New England. A revision of the rhabdovirus family was completed and included study of 37 serotypes. Identification studies of 76 viruses were carried out in 1978.

Development of techniques and models. The ELISA test was refined for use with purified bunyavirus, flavivirus, and alphavirus whole virion antigen to give a sensitive specific test. ELISA was applied to virus identification, serosurvey, and serologic diagnosis. The test is rapid and is completed in one day. The Aedes pseudoscutellaris cell line was tested in the laboratory and in the field in Nigeria and Malaysia. It is nearly as sensitive as the mosquito for detection of dengue virus, which produces a cytopathic effect. RNA segment analysis of the Kemerovo and bluetongue groups of orbiviruses demonstrated the high sensitivity of this technique. Kemerovo RNA gel patterns correlate with geographic origin, and virus from a migratory bird captured in Egypt had a very similar RNA pattern to that of the U.S.S.R. human and tick isolates.

Serologic surveys. Broadly based surveys of arbovirus and arenavirus antibody in man were carried out with Lassa antigen in Liberia; Congo-Crimean hemorrhagic fever antigen in Yugoslavia; and with a variety of arbovirus antigens in Guam, U.S.A., Colombia, and New Guinea. Study of sera of laboratory workers indicated high prevalence of inapparent infection with Pichinde virus of the Arenaviridae family.

Diagnosis of disease. A fatal case of La Crosse encephalitis was diagnosed in Westchester County, New York. A Jamestown Canyon virus infection was recognized in Connecticut and a Tacaribe virus laboratory infection was documented serologically. Arboviruses were not incriminated in arthrogryposis in Canada; chlamydia were not implicated in Korean hemorrhagic fever.

Duration of antibody following yellow fever vaccination. World War II veterans presumably vaccinated in 1943 with 17D vaccine retained neutralizing antibody in at least 62% of sera when tested more than 30 years later.

Distribution of reagents. The reference center distributed 528 ampoules of reference sera, viruses, and antigens during 1978, as well as mosquito and vertebrate cell lines. Viruses of 149 different types were distributed; this is the largest variety in any year so far requested.

FOREWARD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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I. Identification of virus strains.

Strains from Venezuela (G. Calderon and J. Casals). Strains Ven-PAr 30318, Ven-PAr 30475, Ven-PAn 33382, Ven-PAn 33571, Ven-PAn 33574, Ven-PAn 33733, Ven-PAn 33706 and Ven-PAn 34958 were submitted for identification by Dr. R. Walder, Instituto Venezolano de Investigaciones Cientificas (IVIC), in 1977.

During the course of 1977, immune mouse sera and ascitic fluids were prepared for each strain as well as sucrose-acetone antigens, generally from brain tissue and in certain instances from liver tissue. During the year, two of the strains were identified by complement-fixation test: Ven-PAr 30318, was considered to be a strain of Una, and Ven-PAn 33382 was identified as a strain of Itaqui. The remaining strains were placed in group A, except Ven-PAn 33706 which could not be placed in any arbovirus antigenic group with the polyvalent reagents used.

Work during the current year, also by CF, has characterized the additional viruses, as follows.

Immune sera for strains Ven-PAn 34598 and Ven-PAr 30475 were tested in increasing two-fold dilutions against dilutions 1:8, 1:16, and 1:32 of several group A antigens, as shown in Table 1. The result of this test shows that virus Ven-PAr 30475 was a strain of Una, or an agent extremely close to it. Strain Ven-PAn 34958 was close to both VEE type Florida and to VEE type Mucambo; at any rate, a strain in the VEE complex.

In another CF test, strain Ven-PAn 34958 was compared with VEE type Pixuna, VEE type Mucambo and VEE, with the results shown in Table 2. The only immune Mucambo serum available failed to react with any of the antigens, including the homologous; the results with it have been omitted. From the combination of results in Tables 1 and 2, it seems that strain Ven-PAn 34958 is closer to VEE virus than to other members of the VEE complex; it is, therefore, considered to be a strain of VEE.

Strains Ven-PAn 33571 and Ven-PAn 33574, were indistinguishable by CF; the former was in addition, indistinguishable from Ven-PAn 34598 in a single CF test. Pending confirmation these two isolates are also considered to be strains of VEE (Table 3).

Strain Ven-PAn 33706 did not fall in an arbovirus antigenic group, as was stated in the 1977 YARU Report. Subsequent investigations showed the strain to be closely related to mouse hepatitis virus; most likely the virus is a contaminant picked up in this laboratory at the time, early in 1978, when mouse hepatitis broke out in the animal quarters. It remains to be determined whether the strain can be re-isolated from the original suspension submitted from IVIC.

Strain Catatumbo. This virus had been submitted in 1975 by Dr. Ronald Mackenzie, Venezuela; it had been partly identified as a group C agent, possibly Caraparu. Additional CF testing with this agent, Table 4, showed that it was indistinguishable from Caraparu, and close to Ossa, as would be anticipated; Catatumbo is considered to be a strain of Caraparu.

Strain from France (J. Casals). Strain Fr-Brest ArT 13 was isolated from ticks, Onithodoros maritimus, in Brittany, France by Dr. C. Chastel, Brest Medical School. An antiserum for this strain was submitted with the request that a relationship between this agent and Uukuniemi, found at the Brest laboratory, be ascertained. In a CF test in this laboratory employing the serum submitted by Dr. Chastel and reference reagents for Uukuniemi and Soldado viruses (Table 5) no relationship was found with Uukuniemi, instead serum Brest ArT 13 reacted with Soldado antigen at low titer, 1:8. The precise relationship between the latter two viruses has not been further elucidated at YARU.

Strains from Rutgers Medical School, New Jersey (J. Casals). Strains SVE-31973 and SVE3, were submitted for identification by Dr. V. Stollar. In the course of experiments in his laboratory with a strain presumed to be Sindbis virus, certain anomalous behavior was observed which prompted the request for identification. Materials supplied by Dr. Stollar were: virus stocks for strains SVE3-1973 and SVE3; and an antiserum for SVE3, designated Ab25, anti-E3, presumably a rabbit serum.

A single intracerebral (ic) passage in newborn mice was done in this laboratory at different times for each strain, in order to forestall any risk of cross-contamination. The inoculated mice in each case were sick or moribund in 24 or 48 hours. Crude CF antigens were prepared with brain tissue from the sick mice and from normal mice, in the form of a 10% suspension in physiologic saline, centrifuged at 1500 rpm for 10 minutes; the supernatant fluids, designated undiluted antigen were tested with reference reagents for EEE, Sindbis and Semliki viruses, with the result shown in Table 6. Serum SVE3 was not anticomplementary but since it gave a high titered non-specific reaction with normal brain crude antigen, its reaction with the SV antigens cannot be properly evaluated. However, as this serum gave only a limited, probably non-specific, reaction with the acetone-sucrose Semliki and Sindbis antigens, the high titer given with EEE antigen is valid.

The remaining results clearly show that SVE3-1973 and SVE3 are strains of EEE virus, not Sindbis or Semliki.

Strain from Kenya (W.D. Downs and W. L. Ooi). KT576/75 from Amblyomma variegatum of Kenya was referred by Dr. D. Simpson of the London School of Tropical Medicine. A liver antigen reacted with sera of Ganjam and Dugbe viruses and was negative with a battery of group A, group B, and other bunyaviruses. In reciprocal cross-CF tests, KT576/75 was indistinguishable from Dugbe virus and represents the first isolate of this virus from Kenya.

Strains from Brazil (R.E. Shope and O. Oliva). BrH 9956 and BrH 10344 were isolated from febrile human beings living along the trans-Amazon highway, Brazil. Vero cell fluids submitted by Dr. K. Dixon, WRAIR, were used as antigen. In the 1:2 dilution, antigens reacted with a phlebotomus fever grouping ascitic fluid and were negative with 29 other grouping fluids. A subsequent CF test with ascitic fluids of members of the phlebotomus fever group gave the following results:

	Antibody					
	Anhanga	Bujaru	Candiru	Icoaraci	Cedros	BeAn 100049
9956	0/0	0/0	64/8	0/0	0/0	0/0
10344	0/0	0/0	64/2	0/0	0/0	0/0
Homologous	8/≥40	16/≥40	32/≥40	8/≥40	32/≥40	32/≥40
Normal Vero	0/0	0/0	0/0	0/0	0/0	0/0

*antibody/antigen

The viruses are closely related to Candiru by the CF test, although they have not yet been tested with BeAn 213452 virus which is identical to Candiru by CF test but differs by neutralization test.

BrH 4327 virus was submitted by Dr. Dixon of WRAIR with the information that it belonged to the Simbu group. By CF test at Yale it cross-reacted reciprocally to titer with Oropouche virus.

BeAn 238758 virus from a rat in Brazil was tested earlier with groups A, B, C, Guama, Bunyamwera, Capim, Simbu, phlebotomus, Tacaribe, California, Sakhalin, Kemerovo, Palyam, Quaranfil, Patois, and Anopheles A as well as to 192 other strains in the WHO reference collection. It presented a 1:4 cross-reaction by CF with 2 strains of Congo virus. Attempts to repeat this reaction with 5 other strains of Congo virus by CF and by FA were uniformly negative. It thus remains as an ungrouped virus.

Viruses from Senegal and Seychelles (S.M. Buckley, R.E. Shope, J. Rodriques, and I. Mattos). HD 10674 and SS20 viruses from human sera suspected of being dengue were submitted by Dr. Y. Robin of Dakar. HD 10674 was isolated in Dakar and SS20 from an epidemic of suspected dengue in the Seychelles. Both viruses were confirmed as dengue by propagation in Aedes albopictus cell culture and in

Table 1

Complement-fixation Test
Identification of strains isolated in Venezuela

Antigen	Serum	
	Ven-PAn 34958	Ven-PAr 30475
Ven-PAn 34958	1024/32+	
Ven-PAr 30475		1024+/32+
Aura	32/32+	0
EEE	64/32	0
Mayaro	16/32+	16/32+
Mucambo	512/32+	8/32+
Una	32/16	1024+/32+
VEE-Florida	512/32+	0
WEE	32/32+	0
Control	0	0

Reciprocal of serum titer/reciprocal of antigen titer
0, no fixation at dilution 1:8 of serum and antigen

Table 2

Complement-fixation Test
Identification of strain Ven-PAn 34958
isolated in Venezuala

Antigen	Serum		
	Ven-PAn 34958	VEE	Pixuna
Ven-PAn 34958	512/256+	512/256	64/256+
VEE	256/128+	512/128+	64/128
Pixuna	128/128	128/64	512/128
Mucambo	128/128	128/64	64/128
Control	0	0	0

Footnote: see Table 1

Table 3

Complement-fixation Test
Identification of strains isolated in Venezuela

Antigen	Serum	
	Ven-PAn 33571	Ven-PAn 34958
Ven-PAn 33571	128/64+	128/64+
Ven-PAn 34958	128/64+	128/64+
Control	0	0

Footnote: see Table 1

Table 4

Complement-fixation Test
Identification of strain Catatumbo from Venezuela

Antigen	Serum		
	Catatumbo	Caraparu	Ossa
Catatumbo	64/128	64-128/128	32/256
Caraparu	64/64	128/128	32/32
Ossa	32/8	32/4	32/8
Control	0	0	0

Footnote: see Table 1

Table 5

Complement-fixation Test
Relationship between strain Fr-Brest ArT 13 isolated in France and Soldado virus

Antigen	Serum		
	BrArt 13	Soldado	Uukuniemi
Soldado	8/32	128+/128	0
Uukuniemi	0	0	128+/256+
Control	0	0	0

Footnote: see Table 1

Table 6

Complement-fixation Test
Identification of strains submitted by Dr. V. Stollar
Rutgers Medical School

Antigen	Serum			
	SVE3	EEE	Sindbis	Semliki
SVE3-1973, crude	128/32+	64/32+	16/32	0
SVE3, crude	128/32+	64/32+	8/16	0
Normal brain, crude	64/4+	0	0	0
EEE, sucrose-acetone	128/32+	128/32+	0	0
Sindbis, sucrose-acetone	16/16	0	256/32+	0
Semliki, sucrose-acetone	16/16	0	0	256/32+
No antigen	0	0	0	0

Reciprocal of serum titer/reciprocal of antigen titer.
Crude antigens: first dilution, undiluted.
Sucrose-acetone antigens: first dilution, 1:8.
Sera: first dilution, 1:8.

Aedes pseudoscutellaris cell culture, using a dengue-2 serum by FA test.

Direct FA was undertaken in Aedes pseudoscutellaris cells using FITC conjugates of mouse ascitic fluids. The titers were highest for DakH10674 with the dengue-2 conjugate (Table 7). A CF test using antibody to HD 10674 and SS20 confirmed that they are strains of dengue-2 virus.

Virus from Australia (R.E. Shope and W.H. Ooi). Aus Ch 16129 was reported in 1977 to be a member of the Simbu group. A plaque reduction neutralization test in Vero cells failed to show neutralization of Aus Ch 16129 virus by a Simbu grouping fluid prepared from the known members of the group; the homologous neutralization was greater than 256. This confirms previous CF test results showing this virus to be different from other Simbu group viruses.

Serological relationships of rhabdoviruses (C.L. Frazier and R.E. Shope). The initial results of a serological revision of rhabdoviruses were reported in the 1977 YARU Annual Report (pages 43 and 44). Thirty-seven rhabdoviruses were examined by CF. The 6 members of the VSV serogroup did not react with other rhabdoviruses. The cross-reactions within the group are shown in Table 8; the results do not differ from those previously described.

The 6 members of the rabies serogroup also did not react with other rhabdoviruses but did react inter se (except for kotonkan) as shown in Table 9. Table10 demonstrates the reactions seen among the minor serogroups of rhabdoviruses. Five minor serogroups are defined: Hart Park, Sawgrass, Kwatta, Mossuril, and Timbo. In addition the following rhabdoviruses showed no cross-reaction with any other rhabdoviruses: Gray Lodge, Barur, Joinjakaka, Kern Canyon, Klamath, Mount Elgon bat, Navaro, Oita 296, Porton S-1643, Yata, Keuraliba, Parry Creek, and Kimberley.

The fluorescent focus inhibition test was developed for the VSV serogroup viruses. This test is complete in 16 to 18 hours, is sensitive, and relatively cheap and simple. The results are displayed in Table 11, and a greater degree of cross-reactivity was noted among the VSV serogroup viruses than seen by other tests. At the same time there were no cross-reactions seen with 31 other rhabdoviruses.

Table 7

Direct fluorescent antibody results with Dak HD 10674 virus in Aedes pseudoscutellaris cells and mouse ascitic fluid conjugates

Conjugate	Titer with Dak HD 10674	Titer with homologous
Dengue-1	<2[a]	8
Dengue -2	32	32
Dengue-3	<2	16
Dengue-4	4	32

[a]end-point taken as 3 or 4 + fluorescence; cells were harvested on day 7 post inoculation; spot slides prepared with 40,000 cells/ml.

Table 8

Complement-fixation Reactions of Vesiculoviruses

Serocomplex	Antigen	Antibody: VSV Indiana	Cocal	VSV New Jersey	Piry	Chandipura	Isfahan
VSV Indiana	VSV Indiana	256[a]	4	0	0	0	0
	Cocal	8	64	0	0	0	0
VSV New Jersey	VSV New Jersey	0	0	32	0	0	0
Piry	Piry	0	0	0	128	16	8
	Chandipura	0	0	0	4	256	8
	Isfahan	0	0	0	0	4	256

[a]Reciprocal of hyperimmune mouse ascitic fluid titer at optimal antigen dilution in grid titrations; 2 units C'; overnight incubation; 0 = <4

Table 9

Complement-fixation Reactions of Rabies Serogroup Viruses

		Antibody					
Serocomplex	Antigen	Rabies HEP	Mokola	Lagos bat	Duvenhage	Obodhiang	Kotonkan
Rabies	Rabies Pasteur	1024[a]	512	4	4	0	0
	Rabies TR 5843	1024	512	64	16	0	0
	Mokola	64	512	64	0	64	0
	Lagos Bat	64	512	1024	64	16	0
	Duvenhage	256	128	64	64	0	0
Obodhiang	Obodhiang	0	512	0	0	256	0
Kotonkan	Kotonkan	0	0	0	0	0	64

[a]Reciprocal of hyperimmune mouse ascitic fluid titer at optimal antigen dilution in grid titrations; 2 units C'; overnight incubation; 0 = <4

Table 10

Complement-fixation Reactions of the Minor Serogroups of Rhabdoviruses

	HYPERIMMUNE ASCITIC FLUID										
	Hart Park Group			Sawgrass Group		Kwatta Group		Mossuril Group		Timbo Group	
ANTIGEN	Hart Park	Flanders	Mos-queiro[a]	Saw-grass	New Minto	Kwatta	BeAn 157575[a]	Mossuril	Kamese	Timbo	Chaco
Hart Park	512[b]	1024	0	0	0	0	0	0	0	0	0
Flanders	16	2048	0	0	0	0	0	0	0	0	0
Mosqueiro	8	64	64	0	0	0	0	0	0	0	0
Sawgrass	0	0	0	512	4	0	0	0	0	0	0
New Minto	0	0	0	0	512	0	0	0	0	0	0
Kwatta	0	0	0	0	0	8192	8	0	0	0	0
BeAn157575	0	0	0	0	0	8192	32	0	0	0	0
Mossuril	0	0	0	0	0	0	0	128	128	0	0
Kamese	0	0	0	0	0	0	0	64	256	0	0
Timbo	0	0	0	0	0	0	0	0	0	512	16
Chaco	0	0	0	0	0	0	0	0	0	8	32

[a]Mosqueiro and BeAn 157575 are unpublished viruses supplied for study by F.P. Pinheiro and A.P.A. Travassos da Rosa, Belem, Brazil.

[b]Reciprocal of ascitic fluid titer with optimal antigen dilution; 2 units C'; overnight incubation; 0 = <4

Table 11

Fluorescent Focus Inhibition Test Cross-reactions among Vesicular Stomatitis Group Viruses and Absence of Cross-reactions with other Rhabdoviruses

	Antibody						
Virus	VS-Indiana	Cocal	VS-New Jersey	Piry	Chandipura	Isfahan	Other rhabdoviruses
VS-Indiana	99[a]	97	27	0	42	51	<40
Cocal	100	100	2	6	76	98	<40
VS-New Jersey	84	0	100	46	92	40	<44
Piry	37	0	31	100	93	80	<50
Chandipura	0	0	18	100	100	100	<42
Isfahan	17	84	68	97	93	100	<50

[a]Percent reduction in fluorescent foci; hyperimmune mouse ascitic fluids reacted 1:10 with 25 to 220 fluorescent focus units of virus; CER cell monolayers; 16 to 18 hours secondary incubation.

[b]Rabies, Mokola, Lagos bat, Duvenhage, kotonkan, Obodhiang, Hart Park, Flanders, Gray Lodge, Kwatta, BeAn 157575, Mossuril, Kamese, Timbo, Chaco, Marco, Sawgrass, New Minto, Barur, Joinjakaka, Kern Canyon, Klamath, Mount Elgon bat, Navarro, Oita 296, BeAr 185559, S-1643, Yata, Keuraliba, Parry Creek, Kimberley.

Identification of viruses from Connecticut (A.J. Main, S. Brown, S. Buckley, S. Hildreth, and R. Wallis). During 1978 surveillance for arboviruses was carried out in Madison, Killingworth, and Clinton, Connecticut under auspices and support of the State of Connecticut. The collections of biting flies included mosquitoes, tabanids, blackflies and Culicoides (Table 12). In addition ticks and small mammals were trapped and processed for virus isolation attempts (Table 13). Twenty-three virus strains were isolated and identified (Table 14). Powassan virus was recovered from ticks and from a long-tailed weasel. Jamestown Canyon virus was isolated on 6 occasions from Aedes abserratus mosquitoes, WEE (subtype II) from Culiseta melanura, and Flanders virus 14 times from Culex and Culiseta. Identifications were carried out by CF test.

Isolate Ar 623-75 from Aedes aurifer and Ar 1274-76 from Chrysops obseletus appear to be identical by CF and neutralization test, and related to the California group. Hamsters were immunized by a single inoculation to these and to prototype California group viruses. The 21-day sera were tested by CF and neutralization test with the results shown in Tables 15 and 16.

These viruses cross-react with Keystone although the lack of serospecificity of Ar 623-75 and Ar 1274-76 and the low titer of these homologous systems precludes a definite identification until new sera are prepared. Keystone virus has not previously been recognized in Connecticut and its presumed vector, Aedes atlanticus, is not believed to be prevalent in Connecticut.

Alphaviruses from Colorado (R.E. Shope). CF and HI studies with Bijou Bridge and Fort Morgan viruses were carried out with reagents supplied by Dr. N. Karabatsos of CDC, Fort Collins, Colo. The Bijou Bridge virus was previously shown at CDC to be a member of the WEE complex. Studies at YARU confirmed the CDC findings and indicated a close relationship of Bijou Bridge to Mucambo virus, and of Fort Morgan to WEE subtype II (Tables 17, 18, and 19). WEE subtype II (strain 72-666) used in these tests is representative of WEE strains found in the eastern U.S.A.

Table 12

Biting flies collected in Connecticut during 1978 for virus isolation attempts

Area:	Madison	Madison	Killingworth	Clinton	Madison	Other
Site:	Site II	Site III	Site IV	Aquarius Stables	Mosquito Control	*
Number of Collections:	196	76	79	81	80	8
A. Females						
An. crucians		7				
An. punctipennis	68	83	19	73	10	1
An. quadrimaculatus	2			10		
An. walkeri	7	12		1		
Ae. abserratus	2587	24	135	275	118	9
Ae. aurifer	1	4	2	3	1	
Ae. canadensis	9345	584	1087	5101	414	4
Ae. cantator	186	49	27	1172	247	
Ae. cinereus	3216	166	111	334	106	2
Ae. excrucians group	1854	63	55	781	264	3
Ae. sollicitans		1		1	26	
Ae. sticticus				2		
Ae. thibaulti	19	9	15	4	1	
Ae. triseriatus	844	27	37	685	651	
Ae. trivittatus	17	8		6	4	
Ae. vexans	249	427	74	403	908	
Cx. pipiens	23	140	23	45	25	
Cx. restuans	153	784	105	103	111	3
Cx. salinarius	109	150	28	163	875	
Cx. territans	30	36	9	9	21	
Cs. inornata	1	3		1	1	
Cs. melanura	2456	395	366	183	5	176
Cs. morsitans	170	55	12	28	1	229

Table 12 continued

Area:	Madison	Madison	Killingworth	Clinton	Madison	Other
Site:	Site II	Site III	Site IV	Aquarius Stables	Mosquito Control	*
A. Females, continued						
Cq. perturbans	369	147	55	420	12	3
Ps. ferox	1					
Ur. sapphirina	172	321	107	70	222	
TOTAL CULICIDAE	21879	3495	2264	9900	4023	430
Cnephia spp.	24		1	4	2	
Prosimulium spp.	67		74	353	1	
Simulium spp.	1024	13	31	34	34	
TOTAL SIMULIIDAE	1115	13	106	391	37	
Culicoides spp.	77					
TOTAL	77					
Symphoromyia spp.	68	6		8	6	
TOTAL RHAGIONIDAE	68	6		8	6	

Table 12 continued

Area:	Madison	Madison	Killingworth	Clinton Aquarius	Madison Mosquito	Other *
Site:	Site II	Site III	Site IV	Stables	Control	
B. Males						
An. punctipennis	4	15	3	3	1	
Ae. abserratus	31			5	1	
Ae. canadensis	146	6	2	69	9	
Ae. cantator	1			6	12	
Ae. cinereus	282	192	27	44	26	
Ae. excrucians group	41	1		8	5	
Ae. sollicitans					1	
Ae. thibaulti		1	21			
Ae. triseriatus	117	4	10	37	20	
Ae. vexans	93	131	25	68	220	
Cx. pipiens	5	29	1	7	25	1
Cx. restuans	239	287	60	103	97	2
Cx. salinarius	11	34	4	39	124	
Cx. territans	264	89	25	22	62	
Cs. melanura	4991	348	120	66	22	13
Cs. morsitans	54	44	20	5	4	24
Cq. perturbans	45	13	3	1	1	4
Ur. sapphirina	90	201	43	42	603	
TOTAL CULICIDAE	6414	1395	364	525	1233	44

Table 12 continued

Area:	Madison	Madison	Killingworth	Clinton	Madison	Other
Site:	Site II	Site III	Site IV	Aquarius Stables	Mosquito Control	*
Chrysops spp.			1			
Tabanus spp.	3		1			
TOTAL TABANIDAE	3		2			
C. Larvae						
Ae. abserratus	1025					
Ae. canadensis	147					
Ae. cinereus	55					
Ae. excrucians	342					
Cs. melanura	2					
Cs. morsitans	15					
TOTAL CULICIDAE	1586					

*Includes Farmington and Winsted Connecticut

Table 13

Results of virus isolation attempts in suckling mice of arthropods and vertebrates collected during 1978

	Number Tested				Results		
	Larvae	Nymphs	Adults		Negative		Isolates
			Males	Females	Incomplete		
A. Arthropods							
Dermacentor variabilis			18 (2)*	17 (2)	4		
Ixodes cookei	23 (1)	268 (72)	1 (1)	45 (26)	99		1
Ixodes dammini	4 (1)	6 (1)	27 (3)	36 (4)	9		
Ixodes uriae		4 (3)		2 (2)	5		
Anopheles punctipennis				1 (1)	1		
Aedes abserratus	176 (18)		37 (2)	3149 (121)	135		6
Aedes canadensis				4 (2)	2		
Aedes cinereus				2 (1)	1		
Aedes triseriatus				2211 (50)	1		
Aedes excrucians				3 (1)	28	22	
Culex pipiens			1 (1)	262 (8)	6	2	1
Culex restuans			2 (1)	1259 (34)	21	6	8
Culex salinarius				1331 (26)	8	18	
Culiseta melanura			13 (1)	3564 (89)	80	4	6
Culiseta morsitans			24 (1)	229 (4)	5		
Coquillettidia perturbans			2 (1)	2 (1)	2		
Chrysops carbonarius				1 (1)	1		

Table 13 continued

	Number tested				Results		
	Larvae	Nymphs	Adults		Negative		Isolates
			Males	Females	Incomplete		
A. Arthropods, continued							
Hybomitra lasiophthalma				1 (1)	1		
TOTAL TICKS	27 (2)	278 (76)	46 (6)	100 (34)	117		1
TOTAL MOSQUITOES	176 (18)	-	79 (7)	12017 (338)	290	52	21
TOTAL HORSE/DEER FLIES	0	-	0	2 (2)	2		
TOTAL ARTHROPODS	203 (20)	278 (76)	125 (13)	12119 (374)	409	52	22

B. Vertebrates	Brain	Heart	Lung	Liver	Spleen	Kidney	Blood	Negative	Incomplete	Isolates
Peromyscus leucopus	7	7	7	7	7	7	25	67		
Clethrionomys gapperi	2	2	2	2	2	2	3	15		
Microtus pennsylvanicus							1	1		
Glaucomys volans							1	1		
Sorex cinereus	1	1	1	1	1	1		6		
Mustela frenata							1			1
TOTAL MAMMALS	10	10	10	10	10	10	31	90		1

*Number of individuals tested (number of pools)

Table 14

Virus isolations from arthropods and vertebrates collected in Connecticut during 1978

Strain	Species	No.	Age	Date	Area	Site	Re-isolated	Virus
M-1341-78	Mustela frenata	(blood)		10/7	Old Lyme	7		Powassan
Ar-218-78	Ixodes cookei	9	nymphs	10/7	Old Lyme	7	+	Powassan
Ar-208-78	Aedes abserratus	25	adult	6/8-9	Madison	II	+	Jamestown Canyon
Ar-213-78	Aedes abserratus	32	adult	6/14-15	Madison	II	+	Jamestown Canyon
Ar-315-78	Aedes abserratus	25	adult	6/5-6	Madison	II	+	Jamestown Canyon
Ar-317-78	Aedes abserratus	25	adult	6/5-6	Madison	II	+	Jamestown Canyon
Ar-321-78	Aedes abserratus	25	adult	6/5-6	Madison	II	+	Jamestown Canyon
Ar-326-78	Aedes abserratus	25	adult	6/5-6	Madison	II		Jamestown Canyon
Ar-345-78	Culiseta melanura	45	adult	8/21-25	Madison	II		Flanders
Ar-448-78	Culiseta melanura	34	adult	8/7-9	Madison	II	+	Flanders
Ar-452-78	Culiseta melanura	57	adult	8/15-16	Madison	II		Flanders
Ar-461-78	Culiseta melanura	50	adult	8/31-9/1	Madison	II		Flanders
Ar-489-78	Culex pipiens	33	adult	8/31-10/6	Madison	III		Flanders
Ar-490-78	Culex restuans	39	adult	5/30-6/27	Madison	III		Flanders
Ar-494-78	Culex restuans	50	adult	7/19-20	Madison	III		Flanders
Ar-495-78	Culex restuans	44	adult	7/19-20	Madison	III		Flanders
Ar-496-78	Culex restuans	47	adult	7/20-21	Madison	III		Flanders
Ar-497-78	Culex restuans	43	adult	7/24-28	Madison	III		Flanders
Ar-503-78	Culex restuans	50	adult	8/31-9/1	Madison	III		Flanders
Ar-521-78	Culiseta melanura	46	adult	9/21-22	Madison	III		Flanders
Ar-522-78	Culiseta melanura	37	adult	9/25-10/13	Madison	III	+	WEE (subtype II)
Ar-525-78	Culex restuans	28	adult	5/31-7/13	Killingworth	IV		Flanders
Ar-527-78	Culex restuans	45	adult	7/31-10/5	Killingworth	IV		Flanders

Table 15

CF reactions of Ar 623-75 and Ar 1274-76 viruses with reference California group viruses using single inoculation hamster sera

	SERA													
ANTIGENS	Ar 623	Ar 1274	CE	SA	LAC	SSH	JC	SR	KEY	TVT	MEL	SDN	TAH	INK
Ar 623	8	16	0	0	16	0	0	0	64	0	0	0	16	16
Ar 1274	16	16	0	0	16	0	0	8	64	0	0	16	8	16
CE	16	8	64	64	32	64	8	0	16	0	0	8	16	16
SA	16	16	16	>256	32	128	16	16	64	4	0	8	64	16
LAC	16	0	0	16	128	64	8	16	32	0	16	8		
SSH	8	0	0	0	32	64	0	0	0	0	0	8		
JC	8	8	8	16	32	64	64	64	32	0	8	8	16	64
SR	16	8	0	0	16	0	8	32	0	0	0	0	8	64
KEY	16	8	0	0	64	32	16	16	64	8	8	8	32	64
TVT	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MEL	16	0	0	0	32	16	8	16	32	8	16	0	16	32
SDN	0	0	0	0	0	0	0	32	16	0	0	8	8	16
TAH	8	8	0	8	32	16	8	8	0	0	0	0	128	16
INK	16	8	0	0	32	16	32	64	8	0	0	0	16	64

Table 16

50% plaque reduction neutralization titers of Ar 623-75 and Ar 1274-76 viruses with reference California group sera

	Sera											
Virus	CE	SA	LAC	SSH	JC	SR	KEY	TVT	MEL	SDN	TAH	INK
Ar 623-75	<8	128	<8	<8	8	<8	64	<8	8	<8	16	8
Ar 1274-76	<8	128	<8	<8	<8	<8	>64	<8	8	<8	≥32	8
Homologous	≥64	≥256	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥128	≥64

Table 17

Complement-fixation results of Bijou Bridge and other VEE complex viruses[a]

	Antibody			
Antigen	Bijou Bridge	Mucambo	Pixuna	VEE
Bijou Bridge	256[b]	32	<8	<16
Mucambo	256	32	<8	<16
Pixuna	128	16	64	64
VEE	64	<8	<8	128

[a]Bijou Bridge reagents were supplied by Dr. N. Karabatsos, CDC, Fort Collins, Colo.; Mucambo, Pixuna, and VEE ascitic fluids were from the Research Resources Branch, NIH.

[b]Reciprocal of serum titer with optimal antigen in grid titration.

Table 18

Hemagglutination-inhibition results of Bijou Bridge and other VEE complex viruses[a]

	Antibody				
Antigen	Bijou Bridge	Mucambo	Pixuna	VEE	Everglades
Bijou Bridge	2560[b]	320	20	640	160
Mucambo	2560	640	20	640	160
Pixuna	640	160	640	5120	160
VEE	640	160	80	10240	320

[a]Bijou Bridge reagents were supplied by Dr. N. Karabatsos, CDC, Fort Collins, Colo.; Mucambo, Pixuna, VEE, and Everglades ascitic fluids were from the Research Resources Branch, NIH.

[b]Reciprocal of serum titer with 4 units of antigen.

Table 19

Complement-fixation results of Fort Morgan and other WEE complex viruses[a]

	Antibody					
Antigen	Ft. Morgan	WEE subtype II	WEE subtype I	WEE HJ	Aura	Sindbis
Ft. Morgan	64	16	0	0	0	0
WEE, subtype II	128	64	16	-	-	-
WEE, subtype I	16	16	128	-	-	-
WEE, HJ	128	-	-	256	-	-
Aura	8	-	-	-	8	-
Sindbis	0	-	-	-	-	16
Normal	0	0	0	0	0	0

[a] Fort Morgan reagents were supplied by Dr. N. Karabatsos, CDC, Fort Collins, Colo.; WEE HJ and Sindbis ascitic fluids were from the Research Reference Branch, NIH.

Identification of viruses from field material from Ethiopia (O.L. Wood). The Naval Medical Research Unit #5 submitted for identification, viruses isolated from arthropods and animals as part of a broad ecologic study of disease in Ethiopia. Many of the hyperimmune mouse sera and antigens were prepared in Ethiopia prior to the termination there of laboratory activities in April, 1977.

After closure of NAMRU-5 materials remaining in the freezers were inventoried by Ethiopian government officials. Negotiations by the American embassy allowed selected portions of the material to be shipped to Yale in December 1977. All insect pools were recovered. At least a few infected mice or mouse brains from most virus isolates were recovered. Also recovered was the avian sera collection. However, no unprocessed bird or mammalian tissues were recovered due it was said to an intervening freezer failure. Likewise no human sera were recovered.

At this time all virus isolates from bird and animal tissues made initially in Ethiopia have been characterized in respect to virus group and most have been specifically identified.

Antigens of twenty-one strains reacted with the group B grouping fluid by CF:

Eth An 1334	Bird	Eth An 4771	Bird
Eth An 3307	Bird	Eth An 4772	Bird
Eth An 3662	Bird	Eth An 4773	Bird
Eth An 4152	Bird	Eth An 4808	Bird
Eth Ar 4698	Mosquitoes	Eth An 4809	Bird
Eth An 4733	Bird	Eth An 4810	Bird
Eth An 4766	Bird	Eth An 4811	Bird
Eth An 4767	Bird	Eth An 4812	Bird
Eth An 4768	Bird	Eth An 4813	Bird
Eth An 4769	Bird	Eth An 4814	Bird
Eth An 4770			

Sera of seven of these viruses were further reacted by CF with 19 antigens of group B viruses including those known to be in Africa. In each case the highest titer was to West Nile antigen with lesser reaction to Usutu, Japanese encephalitis, Zika and Israel turkey meningoencephalitis viruses. Table 20 shows reciprocal CF test results of grid titration with West Nile antigen and antibody. In addition, Eth An 4733, Eth An 4767, Eth An 4768, Eth An 4808, Eth An 4810, Eth An 4812, Eth An 4713, Eth An 4814, and Eth Ar 4698 reacted by CF to titer with West Nile serum.

Specific West Nile antisera were available at YARU. The sera had been collected from guinea pigs one month after they had received a single injection of West Nile virus. Although antigen titers were low, the sera did not react with either Usutu or Japanese encephalitis at a dilution of >1:8. Eth An 4808, Eth An 4810, Eth An 4812,

Eth An 4813, Eth An 4814, and Eth An 4698 reacted with the West Nile guinea pig sera.

In plaque reduction neutralization tests with the Egypt 101 strain of West Nile virus, sera of Eth An 3307, Eth An 3662, Eth An 4152, Eth An 4811, Eth An 4766, and Eth An 4769 gave 50% or greater reduction at >1:512. In addition the titers of Eth An 4733, Eth An 4767, and Eth An 4768 were reduced 2 or more logs by a West Nile reference serum, thus confirming the identification of these strains of West Nile virus.

Two isolates, Eth Ar 3102 and Eth An 3530 gave good homologous reactions by complement fixation but the antigen did not react with reference antisera pools. Eth Ar 3554 has not yet produced usable antiserum in a mouse system after 3 attempts using both brain and liver preparations. Attempts to adapt the agents to Vero cell cultures were successful and specimens were taken at a point just prior to peak cell destruction for electron microscopy. All three viruses were seen and photographed thus narrowing the group sera needed for further testing.

Eth Ar 3102 from Amblyomma cohaerens ticks collected at the Iara mission near Dembi Dolo had the morphology of a flavivirus. Cells showed cytoplasmic membrane proliferation similar to that reported in flavivirus infections. However, antiserum to 3102 did not inhibit hemagglutination with the antigens Usutu, YF, MVE, and Dengue 2. Neither did 3102 antiserum react with the tick-borne flaviviruses Langat, Powassan, Kadam or Louping ill. The antigen of 3102 did not hemagglutinate so a homologous test was not possible, but the 3102 antiserum had a CF titer of >1:512. The agent is currently being serially passed in cell culture to raise the virus production to a level at which virus protein can be analysed on acrylamide gels to see if a typical flavivirus pattern emerges or if an atypical pattern requires the virus to be placed in pestivirus group.

Eth An 3530 came from bird tissue collected at Bulcha and shows bunyavirus morphology. Further serologic testing is not yet done.

Eth Ar 3554 came from Rhipicephalus pulchellus ticks collected at Kelam on the lower Omo River and shows the morphology of an orbivirus. Neutralization testing with the Kemerovo, Palyam, and NIH #8 polyvalent serum were negative. It is planned to incorporate P^{32} into the virus nucleic acid and compare it with other orbiviruses in acrylamide gel analysis.

Experiments involving mice were in effect suspended from the onset of an epizootic of a murine coronavirus (LIVIM) in December 1977 until the completion of renovation of the animal holding facility to conform to federal standards in late September 1978. Attempts to hold mice in other parts of the building ultimately failed because of LIVIM outbreaks. All mosquito and tick isolates at passage levels

initial through second passage which were recovered from Ethiopia as frozen baby mice were processed in this time period. Large numbers of mice dying of LIVIM make these results uncertain and it has seemed inadvisable to inoculate potentially LIVIM contaminated materials into mice in the new holding area. Therefore only those specimens which had remaining original mouse brain suspension or whole mice from Ethiopia were retested in the new holding area. Of 53 isolates retested 10 have been recovered and 7 of these have been carried through 2 or more passages. Ten specimens could not be retested because all material had been used in the initial passage attempt which was invalidated by the LIVIM outbreaks. An attempt to recover virus in BHK and Vero cells is planned since LIVIM is not known to grow in these systems.

At this writing, mouse based research is again suspended because LIVIM reappeared in the new holding area in late December. Depopulation and general decontamination are planned for mid-January with resumption of research to follow. Meanwhile work on the remaining Ethiopian field material consisting entirely of arthropod pools is suspended.

During much of the time NAMRU-5 was in Ethiopia, work on rabies virus was being carried out at the Ethiopian Central Laboratory. NAMRU supplied mice and occasionally other laboratory facilities but did not carry out any rabies studies of its own. Dr. Makonnen Fekadu was directing the Ethiopian program and in the course of viral isolation studies found three dogs which shed rabies viruses in the saliva over periods of more than a year without clinical signs. Mouse passages of these strains were lyophilized and stored at NAMRU. When freezer contents were shipped out in December, 1977 these virus strains were included. Dr. Makonnen was notified at his present position in Stockholm that the viruses were at YARU and CF testing confirmed that they were rabies and not rabies-related viruses. He was able to visit YARU in May, 1978 and establish that all three strains of the virus were viable using a rabies FA system in CER cells.

Table 20

CF reactions between West Nile and viruses from Ethiopian birds

	WN	1334	3307	3662	4152	4766	4769	4811
WN	2048*	256	512	512	256	512	64	64
1334	1024	128						
3307	1024		512					
3662	1024			256				
4152	1024				256			
4766	1024					256		
4769	1024						64	
4811	1024							32
Normal	0	0	0	0	0	0	0	0

*Reciprocal of serum titer

II. Development of arbovirus techniques and virus models

Attachment of arboviruses to neural and non-neural cells in vitro* (A.L. Smith and G.H. Tignor)

Enumeration of cellular receptors for two strains of Sindbis virus.

As described in the YARU 1977 Annual Report (pp.48-56), neural cells in culture are being exploited in an investigation of viral replicative factors responsible for neurovirulence. Cell surface receptor saturation studies have been performed with the intent of determining whether neuronal cells have more receptors for a neurovirulent virus strain than for a serologically indistinguishable avirulent strain of the same virus. Under standardized conditions of virus growth and attachment, neuroblastoma cells (clone N18) were found to have approximately 25 times more receptors for Sindbis strain SaAr 86 (adult mouse neurovirulent) than for Sindbis strain EgAr 339 (adult mouse avirulent) (Table 21). Bombyx mori cells, which were refractory to infection with SaAr 86, had 1×10^5 surface receptors for this strain, while the average number on five permissive cell types was 1.5×10^6. This might indicate that attachment is one of the restrictive events in this cell type. Estimates for EgAr 339 (average of 2×10^5 receptors per cell on 5 cell types) compare favorably with that of Birdwell and Strauss (1974) for the HR (heat-resistant mutant derived from EgAr 339) strain attached to chick embryo fibroblasts.

Attachment interference assays have also been performed using neural (rat glioma, clone C6) cells. Reciprocal attachment interference between SaAr 86 and eastern equine encephalitis virus (both alphaviruses) was observed (Figure 1). In contrast, attachment of SaAr 86 did not affect attachment of EgAr 339. Thus, there appears to be an interrelationship among binding sites for antigenically related neurotropic viruses, while binding sites for two strains of Sindbis virus differing in their pathogenicity for adult mice are distinct.

Enzymatic inactivation of cellular receptors for Sindbis virus.

Cleavage of receptors from cells which then remain viable indicates that the receptor is not vital to the constitutive functioning of the cell. The lack of inhibition of known cellular functions by inactivation of cellular receptors for viruses is an important consideration in exploring drugs with selective virus inhibitory activity at the cell surface. Enzyme doses which resulted in 100% viability compared to diluent-treated controls for N18, CER, C6 and A. albopictus cells are shown in Table 22 and represent the doses used in receptor inactivation studies (Table 23). Three aspects of these latter experiments are noteworthy: 1) substantial reduction of attachment occurred only after protease treatment, 2) EgAr 339 attachment was affected by enzymes which did not alter SaAr 86 attachment, and 3) none of the enzymes reduced attachment of either strain to

*This project was supported in part by NIH grant AI12541.

A. albopictus cells, the only invertebrate cell line included in these experiments. The last point is of interest because the mosquito cells and CER cells were almost equally tolerant to enzyme treatment and may indicate that the virus receptors on mosquito cells are necessary for cell viability.

As mentioned in a previous YARU Annual Report (1977), regeneration of CER receptors for EgAr 339 is complete after 3 hr in nutritionally supplemented medium at both 37°C and 4°C. Experiments have now been performed to determine whether suppression of cellular synthesis, in turn, suppresses expression of receptors on protease-treated cells (Table 24). Doses of metabolic inhibitors which shut down host protein, RNA or DNA synthesis without affecting cell viability were used. It appears that no new cellular synthesis is required for the expression of new Sindbis virus receptors on the surface of CER cells. The most likely interpretation of this finding, which is in contrast to that with enteroviruses (Levitt and Crowell, 1967), is lateral migration of previously masked receptors or outward migration of internal receptors to positions which allow virus binding.

Determination of the degree of antigenic relatedness between two strains of Sindbis virus. Samples of the two Sindbis strains which had been used in attachment assays were periodically tested by hemagglutination-inhibition (HI), complement-fixation (CF) and serum-dilution neutralization tests to ensure that cross-contamination with other viruses used in the laboratory had not occurred. During the course of these tests, it became clear that the two strains could not be distinguished by standard serologic techniques. For this reason, kinetic HI tests (Casals, 1967) were performed. A typical result, which shows slight differences at 30 min, is given in Table 25.

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Table 21

Infectivity Titers and Numbers of Receptors on Several Cell Types for Sindbis Virus Strains EgAr 339 (adult mouse avirulent) and SaAr 86 (adult mouse neurovirulent)[a]

Cell line	EgAr 339 mean number of receptors per cell (and range) X 10^{-5}[b]	SaAr 86 mean number of receptors per cell (and range) X 10^{-5}[b]
C6	5 (4-6)	6 (4-9)
	5[c]	
N18	0.5 (0.4-0.8)	13 (13-14)
ER	1 (0.8-1.7)	39 (33-46)
L929	2 (1-3)	8 (6-11)
A. albopictus	1 (1-1)	11 (5-20)
B. mori	not done	1 (0.8-1.7)

(a) Numbers of receptors were calculated from Lineweaver-Burk transformations; means and ranges were derived from a minimum of two experiments; virus grown in CER cells; attachment was performed with Sorensen's phosphate buffer (pH 8.0, 0.15 M NaCl) for 3 hr at 4°C.

(b) Calculations are based on the mean number and range of saturating PFU and the mean particle to PFU ratios.

(c) Observed saturation value.

Figure 1a. Hyperbolic Plots for Attachment Competition between ^{3}H-labelled SaAr 86 Strain of Sindbis Virus and EEE Virus in C6 Cells.

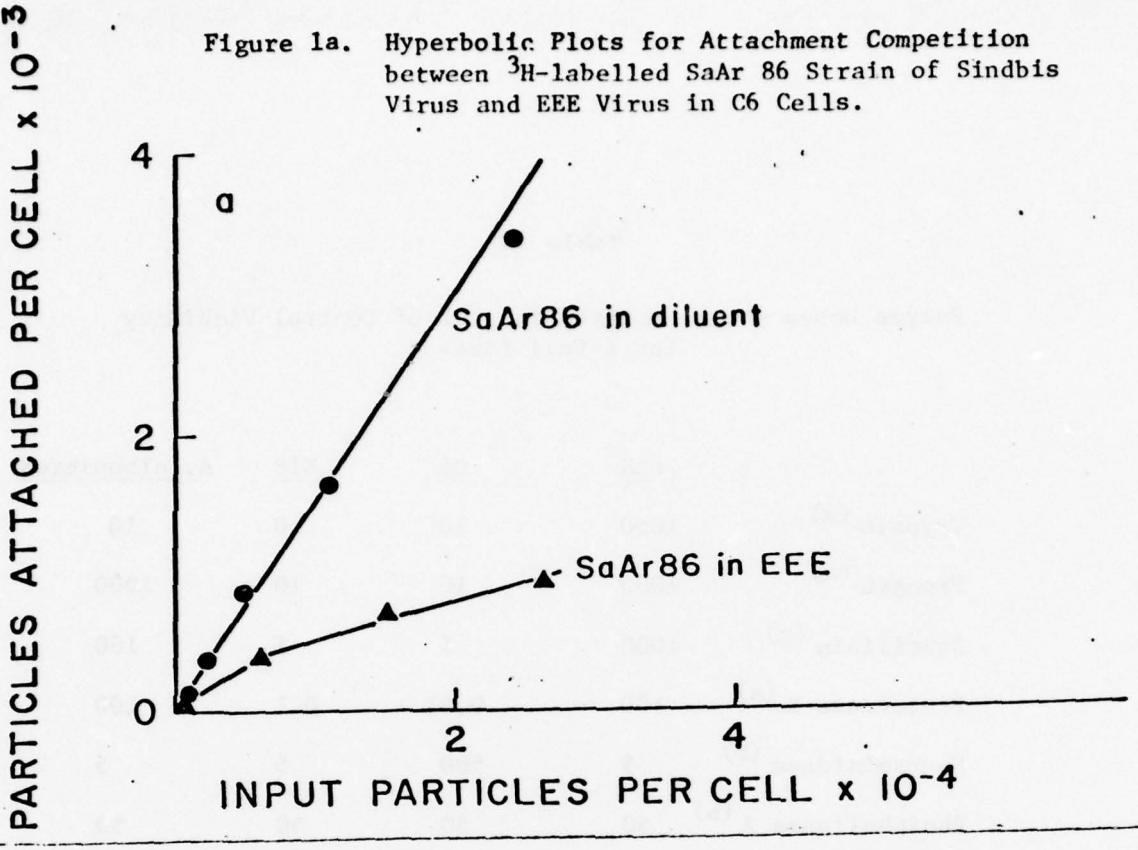

Figure 1b. Hyperbolic Plots for EEE Virus Attachment to C6 Cells after Cellular Pretreatment with Diluent or SaAr 86 Strain of Sindbis Virus.

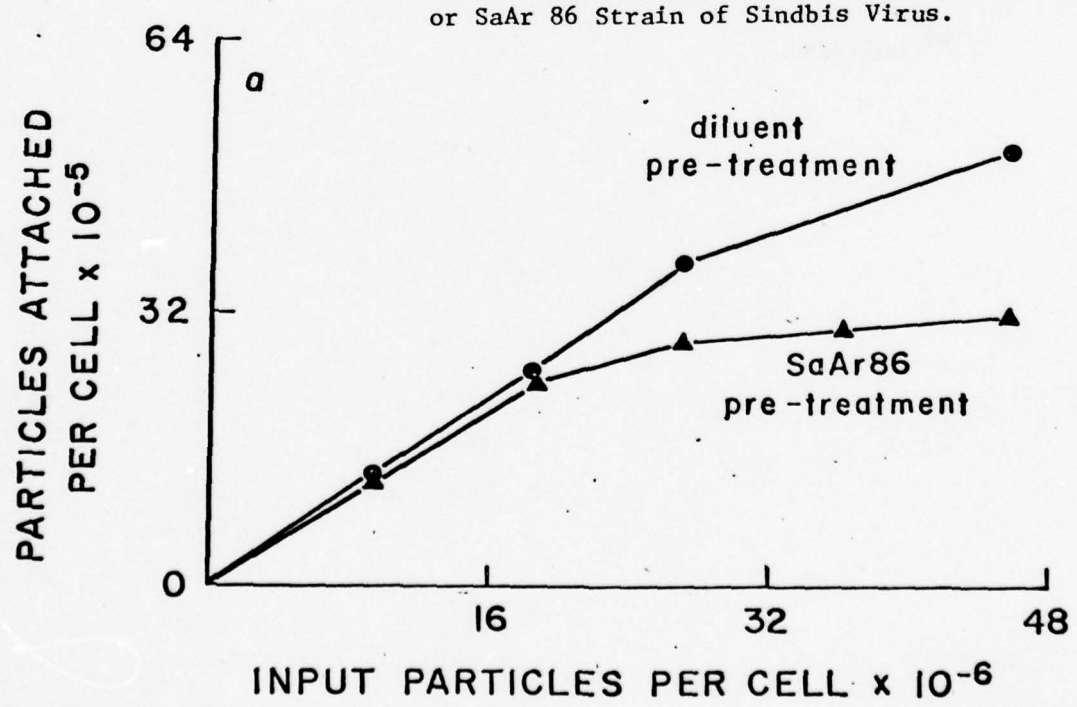

Table 22

Enzyme Doses which Resulted in 100% of Control Viability for 4 Cell Lines

	CER	C6	N18	A. albopictus
Trypsin [a]	1000	10	10	10
Pronase [a]	1000	10	10	1000
Subtilisin [a]	1000	1	1	100
Proteinase K [a]	100	0.01	0.1	100
Neuraminidase [b]	5	500	5	5
Phospholipase A [b]	50	50	50	50
Phospholipase C [b]	5	5	5	5

(a) µg/ml

(b) units/ml

Table 23

Attachment[(a)] of Two Sindbis Virus Strains to Four Cell Lines After Enzyme Treatment of Cells[(b)]

			EgAr 339				SaAr 86	
Enzyme	N18	C6	A.albopictus	CER	N18	C6	A.albopictus	CER
trypsin	64	100	100	100	83	93	100	93
pronase	*15*	*1*	100	*0*	52	*29*	100	57
subtilisin	90	100	100	*42*	94	100	100	72
proteinase K	93	100	100	*11*	100	100	74	67
phospholipase C	N.D.	99	100	60	100	N.D.	100	59
phospholipase A	84	96	96	94	92	97	98	95
neuraminidase	N.D.	100	100	54	100	N.D.	100	80

(a) expressed as % of control; values shown are means of 2 or 3 experiments; variation among experiments was found to be approximately 50%; therefore, only values <50% are considered to be significant reduction of attachment.

(b) enzyme doses are those shown in Table 22.

N.D. = not done

Table 24

Effect of Protein, DNA and RNA Synthesis Inhibitors on Regeneration of EgAr 339 (Sindbis) Virus Receptors on Pronase-treated CER Cells[a]

	Regeneration Time in Hours			
	0	1	2	3
No Inhibitor	0 ± 4	66 ± 5	82 ± 9	100 ± 4
Puromycin (10µg/ml)[b]	9 ± 5	75 ± 8	94 ± 6	100 ± 6
Cytosine arabinoside[b] (50µg/ml)	13 ± 6	70 ± 6	82 ± 5	99 ± 3
Actinomycin D[b] (10µg/ml)	18 ± 10	80 ± 9	97 ± 4	100 ± 5

[a] Expressed as % of labelled virus attached to untreated cells (1×10^{10} particles attached to 10^7 cells); mean values and standard deviations from 3 independent determinations; virus grown in CER cells.

[b] The cells were treated with metabolic inhibitors for 18 hr prior to the experiment and during enzyme treatment and regeneration periods.

Table 25

Comparison of Sindbis Virus Strains EgAr 339 and SaAr 86 by HI Test Using Simultaneous Dilutions of Serum and Antigen and Rate of Reaction

Serum		Antigen, Reaction Time in Hours, Units Inhibited					
	dilution	0.5 hr		2.0 hr		18.0 hr	
strain	1:	EgAr 339[a]	SaAr 86[a]	EgAr339	SaAr86	EgAr339	SaAr86
EgAr339	100	64	64	64	64	128	128
	200	32	32	32	32	64	64
	400	16	16	16	16	16	16
	800	8	8	16	8	16	8
SaAr86	100	64	128	64	128	64	128
	200	16	64	32	64	32	64
	400	8	32	16	32	16	32
	800	8	8	8	16	8	16

(a) antigens were clarified, PEG - concentrated infected culture fluid from CER cells; red blood cell mixture at pH 6.2 .

Early diagnosis by early detection of virus (J. Casals). This laboratory has an active program of studies to develop techniques and methods for early diagnosis of diseases caused by arboviruses and arenaviruses. The approach is two-directional: early detection of virus (or antigen) and early detection of antibody.

The fastest diagnosis of an arbovirus or arenavirus infection would be accomplished by observing the virus directly in clinical specimens, blood, tissues, excretions or secretions. If this is not successful, the diagnosis can be accelerated by inoculation of suspect material into laboratory host systems, preferably cell cultures, followed by daily examination of the inoculated cells for presence of virus.

Exploratory experiments have been carried out in which known infectious virus stock suspensions have been inoculated to cell cultures, in attempts to determine the correlation between amount of virus inoculated and time of appearance of a positive immunofluorescence (IF) reaction. The IF method was chosen at the out-set owing to its sensitivity, simplicity and rapidity of execution; given the necessary reagents, the indirect IF can be completed in 2 to 3 hours. The application of the radioimmunoassay (RIA) to this problem is discussed in another section.

Junin virus. Vero cell monolayers under fluid medium in Lab-Tek 4-chambered slides were inoculated with increasing ten-fold dilutions of a virus stock suspension, the $ICLD_{50}$ of which in newborn mice was $10^{6.5}/0.02$ ml. At daily intervals after inoculation, slides were processed for indirect IF testing with a Junin virus mouse antiserum at dilution 1:8. The result of the experiment is shown in Table 26. Twenty-four hours after inoculation of as little as 1.2×10^2 $ICLD_{50}$ of virus (dilution 10^{-5}) a weak but definite positive reaction was observed; at 3 days, a positive reaction was discernible even after inoculation of 1 $ICLD_{50}$. In contrast, CPE was not noticeable until the 4th day and then at a minimal degree. The procedure allowed identification of a small amount of virus 3 or 4 days before CPE was visible.

Dengue type 2 virus. An experiment similar to that described in the preceeding paragraph was done with dengue type 2 virus, NGB strain, in LLC-MK_2 cells. Lab-Tek slides were inoculated with increasing ten-fold dilutions of a mouse brain tissue stock having an $ICLD_{50}$ of $10^{7.0}$ in newborn mice. Slides for IF were processed on days 1, 2, 3, 4 and 7; the results are shown in Table 27. A weak positive IF reaction was seen on day 2 with the slides inoculated with dilutions 10^{-2} and 10^{-3}; the reaction was fully developed on day 3, with a 4 plus intensity in dilutions 10^{-2}, 10^{-3} and 10^{-4}, weaker with dilution 10^{-5}. There was no positive IF reaction at dilution 10^{-6} or higher. CPE was not present during the first 4 days, was complete or nearly so on the 7th day,

but only in dilutions 10^{-2}, 10^{-3} and 10^{-4}. In this experiment the IF procedure allowed a definite positive diagnosis on day 3 after inoculation, 4 days before CPE was observed.

Banzi virus. Intraperitoneal inoculation of Banzi virus into adult mice results in disease with an incubation period of 7-8 days, followed by death 1 or 2 days later. The titer of the virus by this route of inoculation is of the order of 10^8 $SMICLD_{50}$ and the titration is regularly smooth; inoculation of dilution 10^{-7} usually results in 100% mortality. This disease model is being used as a means to determine whether virus can be detected directly in the blood of the host by IF test and, eventually, by RIA.

Mice 60 days old have been IP inoculated with 150 newborn mouse $ICLD_{50}$ of virus; beginning 24 hours after inoculation and daily afterwards pooled blood samples from 2 exsanguinated mice were titrated for virulence, and thick smears made; the slides and the hemolyzed bloods were stored at -60C, for later study. The part of this experiment so far completed is the determination of the viremia curve. To this end dilutions were made of the hemolyzed, whole blood which were IP inoculated to adult mice; the result of the titrations is given in Table 28.

No virus was demonstrated on the first day after inoculation; beginning with the 2nd day until the 5th, viremia with a titer between $10^{2.9}$ and $10^{3.5}$/ml was observed, during which period the mice appeared normal. The virus titer dropped sharply on the 6th day; on day 7, when 2 of 6 mice were questionably sick, and on day 8 when the left over mice were moribund, no virus was detected. Investigations are continuing to determine whether Banzi virus at these concentrations can be detected directly in the blood, either by IF or RIA.

Rapid diagnosis of virus in mosquito pools by IF. (I. Mattos, S.M. Buckley, and J. Casals). Twenty-five pools of wild-caught mosquitoes captured in southern California were sent to YARU for experimental studies in invertebrate cells, by Drs. T. Work and M. Jozan Work. Isolations had been done in California by inoculation to newborn mice and identification by HI or CF tests on first or second mouse passage; the information given was that 24 of the pools had yielded SLE virus and the 25th pool, WEE virus.

Tests were done at YARU to isolate and identify viruses in the pools employing inoculation of Aedes albopictus cell cultures in chamber-slides (Lab-Tek). The slides were tested by IF with SLE and WEE immune mouse sera on the 4th day after inoculation. Pools negative at that time were tested again and stained on the 6th or 8th day after inoculation. In addition, 21 pools that had been positive in California, and at YARU by IF, were plaqued on Vero cells.

Table 26

Relationship between amount of Junin virus inoculated and time of appearance of a positive IF reaction in Vero cell monolayers

Inoculum, 0.1 ml		Days after inoculation											
Dilution of stock	Newborn mouse $ICLD_{50}$	1		2		3		4		5		7	
		IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE
10^{-2}	1.2×10^{5}	1	0	1	0	2	±	4	3	4	3		
10^{-3}	1.2×10^{4}	1	0	1	0	2	±	4	1	4	1		
10^{-4}	1.2×10^{3}	1	0	1	0	1	±	4	1	4	1		
10^{-5}	1.2×10^{2}	1	0	1	0	1	0	4	1	4	1	4	2-3
10^{-6}	1.2×10^{1}	±	0	±	0	1	0	4	1	4	1	4	2-3
10^{-7}	1.2	0	0	0	0	1	0	3	0	4	±	4	2-3

IF: in a scale of 4, maximum, to 0, none.

CPE: in a scale of 4, complete destruction of monolayer, to 0, none.

Table 27

Relationship between amount of dengue type 2 virus inoculated and time of appearance of a positive IF reaction in LLC-MK_2 cell monolayers

Inoculum, 0.1 ml		Days after inoculation									
Dilution of stock	Newborn mouse $ICLD_{50}$	1		2		3		4		7	
		IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE
10^{-2}	5×10^5	?	0	1	0	4	0	4	0		4
10^{-3}	5×10^4	0	0	1	0	4	0	4	0		4
10^{-4}	5×10^3	0	0	0	0	4	0	4	0		3
10^{-5}	5×10^2	0	0	0	0	1	0	1	0		0
10^{-6}	5×10^1	0	0	0	0	0	0	0	0	0	0
10^{-7}	5	0	0	0	0	0	0	0	0	0	0
10^{-8}	0.5	0	0	0	0	0	0	0	0	0	0

Footnote, see Table 26.

Table 28

Banzi virus: viremia determinations in mice IP inoculated with 150 $ICLD_{50}$ of virus, bled on successive days after inoculation

Bled, day after inoculation	Result of inoculation						Titer LD_{50}/ml
	Dilution of blood inoculated						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
1	0*	0					$10^{0.5}$ or less
2		6	5	1	0	0	$10^{3.5}$
3		6	2	1	0	0	$10^{2.9}$
4		6	3	1	0	0	$10^{3.1}$
5		5	3	0	0	0	$10^{2.9}$
6	2	0	0				$10^{0.9}$
7	0	0					$10^{0.5}$ or less
8	0	0					$10^{0.5}$ or less

*Mice dead of 6 inoculated

The following results were obtained:

	Reported by California laboratory	IF result, YARU
Positive SLE	24/25	19/25
Positive WEE	1/25	1/25
Positive SLE + WEE	0/25	2/25
Negative	0/25	3/25

By Vero cell plaquing 16 pools gave plaques of 21 tested which had been positive by mouse inoculation and IF in A. albopictus cells; the other 5 gave no plaques.

Of the 22 positive results by IF in A. albopictus cells, 15 were positive and identified in 4 days; an additional 6 in 6 days, and one in 8 days.

Rapid and early detection of arbovirus antigen and antibody by RIA and by ELISA. (J. Converse).

Antiserum production to serum proteins, virus and virus antigens. Antiserum to human gamma-globulin and mouse gamma-globulin has been prepared in four rabbits, i.e., two antihuman-IgG and two antimouse-IgG. Using pooled sera of either human and/or mouse the concentrated effluents of selected portions of major protein peaks from Sephadex G-200 gel chromatography (Figure 2), and the serum gamma-globulins prepared by precipitation with saturated ammonium sulfate, were further purified by preparative zone electrophoresis on Pevikon C-870 (Figure 3). All eluent fractions were identified and characterized by immunoelectrophoresis (IEP) and Ouchterlony immunodiffusion gel analysis (IDA) using specific reference antisera.

Animals were inoculated in the four foot pads, the initial inoculation of purified immunoglobulin (Ig) mixed with complete Freunds adjuvant (CFA), followed by bi-weekly injections of purified Ig in incomplete Freunds adjuvant (IFA) for 3-4 months, until appropriate antiserum reactivity was obtained. At that time, animals were bled-out, the serum portion was collected and analyzed by IEP and IDA for specificity using normal whole serum and $(NH_4)_2SO_4$ precipitated gamma-globulin from either mouse or human serum (Figure 4 through 7). Over 155 ml specific anti-mouse -IgG and 200 ml antihuman-IgG serum was prepared. Antisera were stored at -20°C. Prior to being used in serological tests, i.e., $[^{125}I]$-iodination for radioimmunoassay (RIA) and/or alkaline phosphatase conjugation for enzyme-linked immunosorbent assay (ELISA), rabbit anti-mouse IgG and antihuman IgG serum was precipitated with $(NH_4)_2SO_4$ according to the methods described by Heide and Schwick (1978).

Antibodies to selected arboviruses by either single inoculation of live virus, or multiple injections of sucrose-acetone treated virus antigen, into either rabbits or infant and adult mice were prepared following standard procedures described by Hammo: and Sather (1969). In some studies, serial bleedings were taken at daily intervals, the serum separated and stored frozen at -20°C or -70°C until tested.

Preparation of arbovirus antigens. Purified virus antigens were prepared in duplicate batches for the following eight representative arboviruses which are included in three major arbovirus serological groups. Bunyamwera group-Germiston virus, EthAn 4872; group A arbovirus - chikungunya, o'nyong nyong; group B arbovirus - Japanese encephalitis, Banzi, Kunjin, Murray Valley encephalitis, West Nile. All the virus antigens were prepared by growing the virus in Vero cell culture, in either roller bottles (Corning 850 cm^2, #25140) or 150 cm^2 plastic flasks (Corning, #25120), with serum free media. When cytopathic effect (CPE) was + 2 or greater, the fluid was harvested, centrifuged at 10,000 rpm for 30 minutes to remove cellular debris followed by filtering through 0.45 micron filter. Next the virus suspension was precipitated with 2.3% NaCl and 10% polyethylene glycol 6000 added directly to the fluid. After overnight incubation at 4°C the suspension was recentrifuged at 10,000 rpm for 1 hour. The resulting pellet was resuspended in sodium chloride-ethylenediamine tetraacetic acid-tris (hydroxymethyl) amino-methane buffer, (TRIS), pH 7.5, for 1-2 hours at 4°C. The eluted suspension was then further purified by layering it upon a discontinuous sucrose gradient, i.e., 5%, 25%, 60% (w/v) sucrose in STE buffer, and centrifuged at 95,500 x g for 2 hours at 4°C. The virus band, approximately 0.5 - 1.0 ml in volume, distinct but diffuse, was recovered using a J-needle and syringe. All virus antigen suspensions were stored frozen at -20°C.

Sixteen separate virus antigen preparations have been completed with the average recovery of 8.0 ml per run representing an approximate 100 - 200 fold concentration of virus antigen. In RIA and ELISA testing the antigen suspensions were diluted in carbonate-bicarbonate buffer, pH 9.8, usually two-fold dilutions beginning at 1:50 through 1:400. Unused portions were refrozen at -20°C for later use.

Radioiodination of proteins. The radioiodination of rabbit anti-mouse-IgG gamma-globulin and protein-A from Staphylococcus aureus was handled in a similar manner using a modification of the chloramine-T method as described by Greenwood, et al. (1963). Separation of iodinated proteins from unreacted Na[^{125}I] was carried out using gel filtration (Sephadex G-25) and eluting with 0.15 M PBS, pH 7.2 containing 0.02% sodium azide and 10% normal rabbit serum. In the case of the protein-A no rabbit serum was added because of the strong affinity of protein-A for the Fc region of most classes of immunoglobulins in mammals (Kronvall, et al., 1970).

The ammonium sulfate purified rabbit antimouse-IgG gamma-globulin (or protein-A) was labeled at a concentration of 1 mCi/mg of protein. Precipitation with 20% trichloracetic acid indicated that over 93% of the radioactivity in the pooled protein peak fractions was bound to the purified gamma-globulin, or protein-A. Assuming 100% recovery of labeled protein, the specific radio-activity was approximately 0.08-0.5 µCi/µg rabbit gamma-globulin and 0.06-0.1 µCi/µg protein-A. To protect against loss of [^{125}I] labeled protein in storage, normal rabbit serum was added as a carrier protein (but excluded for protein-A). Dilutions of [^{125}I]-rabbit antimouse-IgG were made in 10% normal rabbit serum and 0.15 M PBS, pH 7.2, [^{125}I]-protein-A in PBS only. Protein determinations after removal of free [^{125}I] were not done.

On the basis of preliminary experiments, the labeled proteins were diluted accordingly to contain approximately 2.0 - 6.0 x 10^4 counts per minute (cpm) in a volume of 50 µl for use in the indirect RIA procedures.

Both iodinated proteins were examined by 10 - 40% sucrose-density gradient (SDG) ultracentrifugation for possible aggregation of protein during labeling procedures and storage. A 4.0 ml linear 10-40% sucrose gradient made up in 0.15 M PBS, pH 7.2, and 0.02% sodium azide was used. Labeled protein was layered onto the top of the gradient and centrifuged at 100,000 x g for 18 hour in a model L-2 Beckman Ultracentrifuge using the SW 39 rotor. Fractions of the gradient were obtained by puncturing the bottom of the gradient and collecting of effluent with the aid of a positive pressure-controlling syringe. Estimates of the sedimentation coefficients of serum proteins were obtained by the method of McEven (1967).

Following centrifugation of [^{125}I]-rabbit antimouse-IgG gamma-globulin and [^{125}I]-protein-A, approximately 87-93% and 96% of the applied radioactivity, respectively, was recovered in a peak sedimenting between fractions #14 and #23, coinciding with a sedimentation coefficient of approximately 7S. Also, sample fractions tested from the reference marker protein tube (immune mouse serum) formed precipitin lines with specific goat antimouse-IgG and-IgM antiserum in IDA. Again, the 7S mouse IgG marker protein and the iodinated proteins were located in similar fractions (Figure 8).

Radioimmunoassay testing (RIA). Binding efficiency of [^{125}I] rabbit antimouse-IgG gamma-globulin. The binding activity of the [^{125}I]-iodinated protein was determined by the reaction of the labeled protein with either purified mouse-IgG and/or human-IgG in polystyrene tubes (Lancer) or in hemagglutination plates (Linbro MRC-96) as the carrier surface. Purified gamma-globulin at a stock concentration of 1 mg/ml was diluted in serial two-fold dilutions through 1:40,960 with either PBS-Tween or carbonate-bicarbonate buffer, pH 9.8. Initial volume of 50 µl antigen per tube or well was used. These were incubated overnight or for 48 hour at 4°C followed by washing with PBS-Tween. In one test no wash followed incubation and the antigen was allowed to air dry in the tubes and wells. Incubation of the radio-labeled protein with the

antigen was for 2-3 hour at 37°C. The plates and tubes were washed three times with PBS-Tween to separate bound and free radioactivity (decanting the free counts while bound remained adsorbed). The wells of the microtiter plates were cut out and/or tubes were then placed in 16 x 125 mm polystrene gamma counting tubes (Kimble #59100) and the radioactivity was determined directly in a Nuclear-Chicago Gamma Counting System Model #1085 with Biospan T.M. Automatic analyzer scaler model #8725.

Preliminary results of four separate studies, using two separate iodinated antibody and two separate iodinated protein-A preparations, with either/or mouse-IgG and human-IgG antigens (dilution concentration 20 µg/ml through 0.004 µg/ml), have been negative or equivocal for binding of the radioiodinated protein. Binding efficiency was calculated as equal to the counts per minute (cpm) bound radioactivity in any of the first five dilutions divided by the cpm in 50 µl of the labeled protein. Suitable preparations must bind >10%, however values of <10% were found in all test runs to date.

Indirect solid-phase radioimmunoassay. For the indirect solid-phase radioimmunoassay concentrated purified virus antigen suspensions were diluted 1:50, 1:100, 1:200 and 1:400 with carbonate-bicarbonate buffer, pH 9.8. Two-hundred µl portions of each dilution of virus antigen were added to either the wells of flexible microtiter hemagglutation plates or disposable polystrene tubes, and then held at 4°C for 24-48 hours. The wells and tubes were washed twice with PBS-Tween (200 µl) followed by the addition of 200 µl of anti-virus mouse immune ascites fluid (MIAF) in two-fold dilutions made up previously in 0.1 M PBS, pH 7.2. The microtiter plates and tubes were covered and placed at 37°C for 2-3 hours and afterwards washed twice with PBS-Tween. Fifty-microliter of the second reactent, either [^{125}I] protein-A or [^{125}I] rabbit antimouse-IgG was then added to each well or tube, followed by a further incubation at 37°C for 1-2 hours. Determination of the bound racioactivity remaining was the same as described above.

The initial RIA studies using Germiston sucrose-acetone mouse brain antigen (IMB-SA) and gradient purified antigen; Yellow Fever virus (IMB-SA) and purified antigen (provided by C. Frazier); and purified antigen of chikungunya, o'nyong nyong, Banzi, Japanese B encephalitis, Murray Valley encephalitis and West Nile viruses, at dilutions of 1:50 through 1:400, showed extremely low binding activity, i.e., <10%, using either radiolabeled protein preparation. No differentiation could be made between the virus MIAF dilutions and similar normal mouse serum control dilutions.

The above experiments and those relating to the binding efficiency of the labeled proteins with purified IgG antigens were conducted concurrently, therefore, the finding of very low binding activity in both sets of experiments is not surprising, low binding ratios were noted for all experiments. Disappointing, however, is that no differentiation could be made in either the virus-MIAF dilutions, and the apparent non-

specific reaction with normal mouse serum dilutions. Repeat experiments are of course planned, and additional attention to parameters such as the influence of pH, buffer diluent, plastic vessel used, and incubation times on antigen binding to carrier surfaces will be followed in order to optimize conditions for attachment of the antigen and radiolabeled proteins in these tests.

Surface analysis by [^{125}I]-labeled protein-A binding to virus-infected cell cultures. A microassay for antibody binding to virus infected Vero and Aedes pseudoscutellaris (MOS-61) cell cultures using [^{125}I] labeled protein-A from Staphylococcus aureus was as follows (below). Virus antigen(s) were demonstrated in the cell culture by measuring the binding of MIAF antibodies to infected cells. The [^{125}I] protein-A assay was compared with [^{125}I] rabbit antimouse-IgG assay for measurement of virus antigen(s) present in two cell culture lines.

Cells were tested as monolayers in flat-bottom wells (Costar 24-well Cluster Dish #3524). Monolayers were prepared by adding cells to each well and incubating them at 27°C (A. pseudoscutellaris) or 37°C (Vero) in humid incubator with 5% CO_2 in air. When cells had formed a confluent monolayer, the cells were inoculated with virus and incubated until CPE was +2 or greater. Cell viability, demonstrated by trypan blue exclusion was at least 95%, on the average 2.85×10^5 Vero cells/well and 2.43×10^5 MOS-61 cells/well.

At the beginning of the assay all wells were washed twice with PBS, the cell culture fluid and washes removed from the cells by vacuum aspiration, and 200 µl of immune ascites fluid, 1:25 dilution, or control normal mouse serum, 1:25 dilution, and PBS control was tested in triplicate. An uninoculated plate of the respective cell cultures was treated identically. After incubation for 1-2 hours at 37°C the immune fluid and controls were removed, the cells washed twice with PBS. In the case of MOS-61 the cell monolayers were first fixed for 10 minutes with 1% gluteraldehyde and washed twice with PBS. Next 100 or 200 µl of either [^{125}I] protein-A or [^{125}I] rabbit anti-mouse-IgG was added, incubation for 2 hours at 37°C followed by two washings with PBS. The cell sheets were then scraped and dissolved into 1.0 ml sodium hydroxide (2M) and the contents of each well transferred to polystyrene tubes for [^{125}I] radioactive determination. Appropriate uninoculated control cell cultures were handled in a similar manner.

In the [^{125}I] protein-A assay it was shown that very little [^{125}I] protein-A (2 to 5 times background) bound to cells which had been incubated with normal serum or PBS control alone. Subtracting the amount of [^{125}I] protein-A bound to cells in these controls from that bound after incubation with MIAF corrects for the small amount of [^{125}I] protein-A bound directly to the cells, the remaining [^{125}I] protein-A being that bound to IgG on the cells. Binding ratios of 1.0 or less were found for all controls. Binding ratios equal the

cpm obtained in triplicate tests (averaged) obtained with immune ascites fluid in tests against virus antigen by that obtained against normal serum, or PBS control. A binding ratio of 2 or greater was considered indicative of the presence of antibody bound to cells.

In the preliminary experiments, [^{125}I] protein-A (≅4500 cpm/200 µl) with Japanese B encephalitis virus infected Vero and MOS-61 cells, the [^{125}I] protein-A assay was negative with no significant bound [^{125}I] protein-A. Subsequent titration (in Vero cell culture) of the contents of several individual wells from both infected cell lines indicated $TCID_{50}$/ml of $<10^{2.0}$ for Japanese B encephalitis virus, therefore, indicating the possibility of very low amounts of virus antigen below the detection level of this particular test. Also, these cell monolayers had not been fixed with 1% glutaraldehyde and most of the cell sheet had been removed by the washing procedure.

The [^{125}I] protein-A assay for West Nile virus in West Nile infected Vero and MOS-61 cell culture was more promising. Binding ratios showed significantly more bound [^{125}I] protein-A, i.e., 4 to 10 times more, in both cell lines used. Control wells showed binding ratios of 1.0 or less. West Nile virus titrations of Vero and MOS-61 cells showed $TCID_{50}$/ml $>10^{7.0}$/well. Immunological specificity using reciprocal cross titrations still remain to be done.

Comparison of [^{125}I] protein-A assay with [^{125}I] rabbit antimouse-IgG assay (80-90,000 cpm/200 µl), using identical procedures, showed the [^{125}I] protein-A assay with higher binding than the [^{125}I] rabbit antimouse-IgG, a binding ratio of 1.0 or less was found with MIAF for both Japanese B encephalitis and West Nile virus, in both cell cultures used. In fact, the binding ratios did not differ from those for the normal mouse serum and PBS controls. Again, the labeled antiserum was once used in the previous section regarding binding capacity studies for mouse-IgG, which was later shown to be inadequate for these studies. The above experiments are being repeated, and are presently in progress with new iodinated preparations.

Enzyme-linked immunoassay testing (ELISA): Alkaline phosphatase enzyme was conjugated to rabbit antimouse-IgG gamma globulin by use of glutaraldehyde according to the method of Avrameas, as described by Engvall and Perlmann (1971), and Voller, et al., (1976). Following extensive dialysis in 0.05M tris (hydroxymethyl) aminomethane (TRIS) buffer, pH 7.8, the enzyme conjugated antiserum (ENZ-rabbit antimouse-IgG) was diluted to 5.0 ml with TRIS buffer and stored in the dark at 4°C.

ELISA: Binding capacity of alkaline phosphatase immune serum conjugate. In the assessment of the working strength of the conjugate (mouse-IgG binding capacity) Cooke polystyrene disposable MICROELISA substrate plates (Dynatech Laboratories) were used as the carrier surface. Purified mouse gamma-globulin (IgG) was diluted to 100 µg/ml and 100 ng/ml working stock solutions in carbonate-bicarbonate buffer, pH 9.8. Two-

hundred microliter volume was added to respective plate wells and incubated overnight at 4°C in a humid chamber. Contents of the plates were aspirated, and washed twice with PBS-Tween. Dilutions of the stock conjugate were made in PBS-Tween, i.e., 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. Two-hundred microliter of each dilution were added in duplicate or quadruplicate IgG coated wells, as well as in uncoated wells. Plates were incubated for 2 or 3 hours at room temperature (RT) in a humid chamber. The washing procedure was repeated. Appropriate control wells in all plates were handled identically. Next 200 µl of p-nitrophenyl phosphate substrate solution, made up fresh in 10% diethanolamine buffer, pH 9.8, was added to each well. The color development was observed for 30-60 minutes and then held for 18 hour at RT before the reaction in all wells was stopped by the addition of 3M NaOH (50 µl). The hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase produces p-nitrophenol and inorganic phosphate. At alkaline pH this reaction mixture is converted to a yellow complex readily measured at 410 nm. In these tests all plates were read visually; the highest dilution of the mouse-IgG antigen and dilution of conjugated serum in the last well showing detectable color was determined as the end point.

Utilizing 10 µg of purified mouse-IgG antigen/well, reaction with ENZ-rabbit antimouse-IgG could be read to a dilution of 1:400, however, for working solutions 1:50 and 1:100 dilutions were used routinely. Normal rabbit serum, undiluted or at varying dilutions, in antigen coated and uncoated wells, did not show reaction, therefore, no cross-reactions within this double antibody system were observed. Reactions in all uncoated and control wells remained celar.

Immune conjugate at dilutions of 1:50 and 1:100 could detect purified mouse-IgG antigen through 1:320 dilution in a two-fold dilution series from a stock solution containing 100 µg/ml. This represents detection of approximately 32 ng of mouse-IgG/well, or 160 ng/ml, i.e., assuming all the antigen attached to the wells, in actuality it was probably much less. A dilution of 1:200 conjugate showed reaction through an antigen dilution of 1:160 in the same test. Conjugate dilutions of 1:400 to 1:1600 showed no reaction in any test.

ELISA technique (mouse system) in assay for Bunyaviruses. The sensitivity of ELISA for detecting Bunyaviruses was investigated using the procedures above and dilutions of purified preparations of the virus Germiston. Using ENZ-rabbit antimouse-IgG at a dilution of 1:50, Germiston virus was readily detected at all antigen concentrations, i.e., 1:50, 1:100, 1:200 dilutions with MIAF dilution to 16,384 and 1:400 with MIAF dilution to 1024. Normal rabbit serum, normal mouse serum and non-antigen well controls remained clear throughout the test, and no nonspecific activity reaction was noted.

ELISA technique (mouse system) in assay for Group B arboviruses. Preliminary ELISA tests have been run for detecting Group B arboviruses, following the above procedures and using purified preparations of the

following viruses; West Nile, Banzi, Murray Valley encephalitis, and Kunjin virus. Appropriate serum and non-antigen well controls were incorporated into each plate in test.

With the exception of Murray Valley encephalitis virus, where no enzyme-substrate reaction occurred, all other antigens showed reaction with the 1:50 dilution of ENZ-rabbit antimouse-IgG, at antigen dilutions 1:50 and 1:100. Normal rabbit serum control and non-antigen wells remained clear in all tests, however, serial dilutions of normal mouse serum showed some non-specific reaction, i.e., equal color development, with West Nile and Banzi virus antigen dilutions, but not with Kunjin virus antigen. Kunjin virus at 1:50 and 1:100 dilution could be detected with MIAF dilutions through 1:1024 and 1:16, respectively, without difficulty. Further tests for the sensitivity and immunospecificity of the ELISA test for arboviruses is still in progress with the objective of application in rapid diagnosis and early detection of these viruses.

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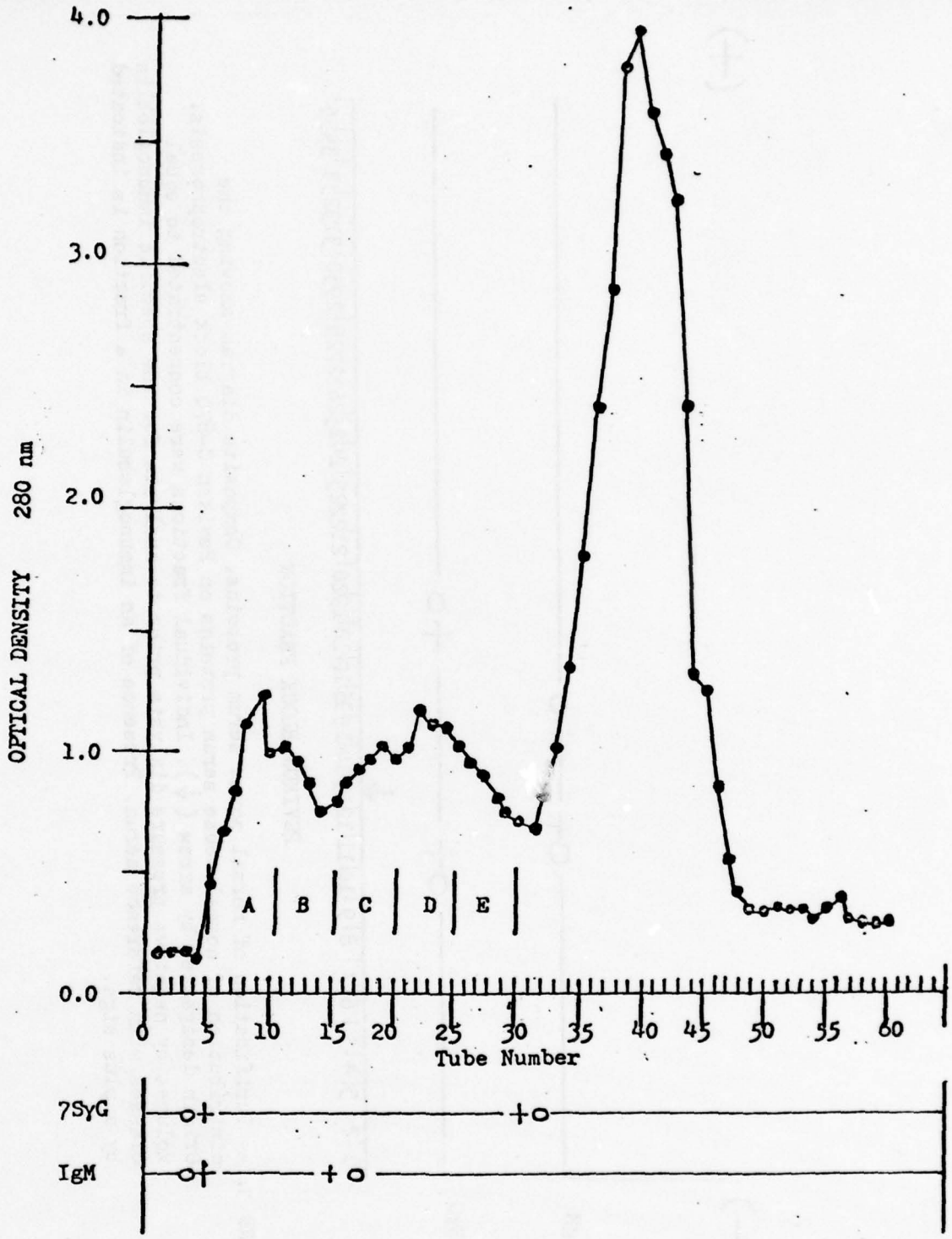


FIGURE 2. -- Distribution of mouse serum proteins after gel chromatography of 7.0 ml normal mouse serum through Sephadex G-200 column with 1 M NaCl-Tris buffer, pH 8.0. Individual fractions were concentrated to equal volumes by negative pressure dialysis prior to immunodiffusion analysis with reference sera. Presence of an immunoglobulin in a fraction is indicated by a plus sign. Subsequent fraction pools are represented by A through E.

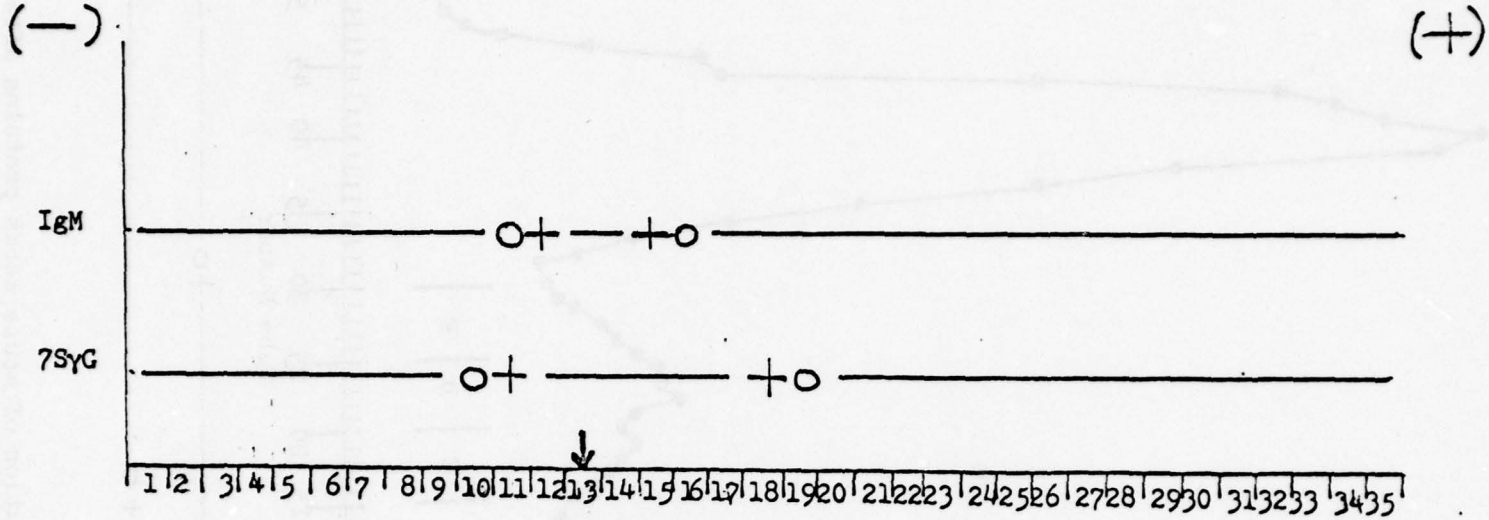
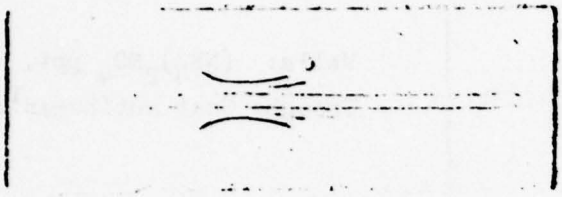


FIGURE 3. -- Purification of normal mouse serum proteins. Composite diagram showing the distribution of normal mouse serum proteins on Pevikon C-870 block electrophoresis. Origin designated by arrow (↓). Individual fractions were concentrated to equal volumes by negative pressure dialysis prior to analysis for the specific immunogloblin classes with reference serum. Presence of an immunoglobulin in a fraction is indicated by a plus sign.

Slide #1

Well: Normal mouse serum
Trough: Goat antimouse serum (whole)

Slide #2

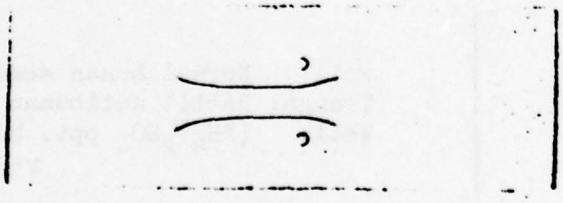


Wells: Mouse serum fractions
Trough: Goat antimouse serum (whole)

FIGURE 4. -- Immunoelectrophoretic pattern of normal mouse serum (slide #1, top well) before Sephadex G-200 gel chromatography.

Slide #2, same mouse serum after Sephadex G-200 gel chromatography, pooled and concentrated fractions C through E (see FIGURE 2). Both slides #1 and #2 developed with goat antimouse serum, whole (BIONETICS Laboratory Products). Cathode is on the left, anode is on the right.

Slide #3



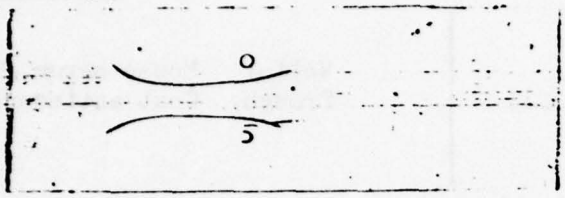
Well: Normal mouse serum
Trough: Rabbit antimouse IgG serum
Well: $(NH_4)_2SO_4$ ppt. mouse γ-globulin

FIGURE 5. -- Immunoelectrophoretic pattern of normal mouse serum (top well) and $(NH_4)_2SO_4$ precipitated mouse gamma globulin (bottom well). Slide developed with rabbit antimouse IgG serum prepared with purified Pevikon block electrophoresis fractions of mouse IgG (see FIGURE 3). Cathode is on the left, anode is on the right.

Slide #1

Outer Troughs: not used
Wells: Normal human serum
Inner Trough: Goat antihuman serum (whole)

Slide #2

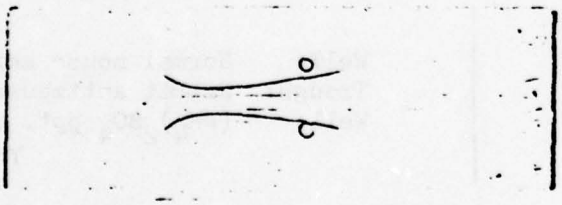


Wells: $(NH_4)_2SO_4$ ppt. human-γ-globulin
Trough: Goat antihuman serum (whole)

FIGURE 6. -- Immunoelectrophoretic pattern of normal human serum (slide #1, both upper and lower wells) before $(NH_4)_2SO_4$ precipitation. Outer troughs not used.

Slide #2, immunoelectrophoretic pattern of $(NH_4)_2SO_4$ precipitated human gamma globulin after fractionation on DEAE-Sephacel column. Both slides #1 and #2 developed with goat antihuman serum, whole (BIONETICS Laboratory Products). Cathode is on the left, anode is on the right.

Slide #3



Well: Normal human serum
Trough: Rabbit antihuman IgG serum
Well: $(NH_4)_2SO_4$ ppt. human-γ-globulin

FIGURE 7. -- Immunoelectrophoretic pattern of normal human serum (top well) and $(NH_4)_2SO_4$ precipitated human gamma globulin (bottom well). Slide developed with rabbit antihuman IgG serum prepared with DEAE-Sephacel purified fractions. Cathode is on the left, anode is on the right.

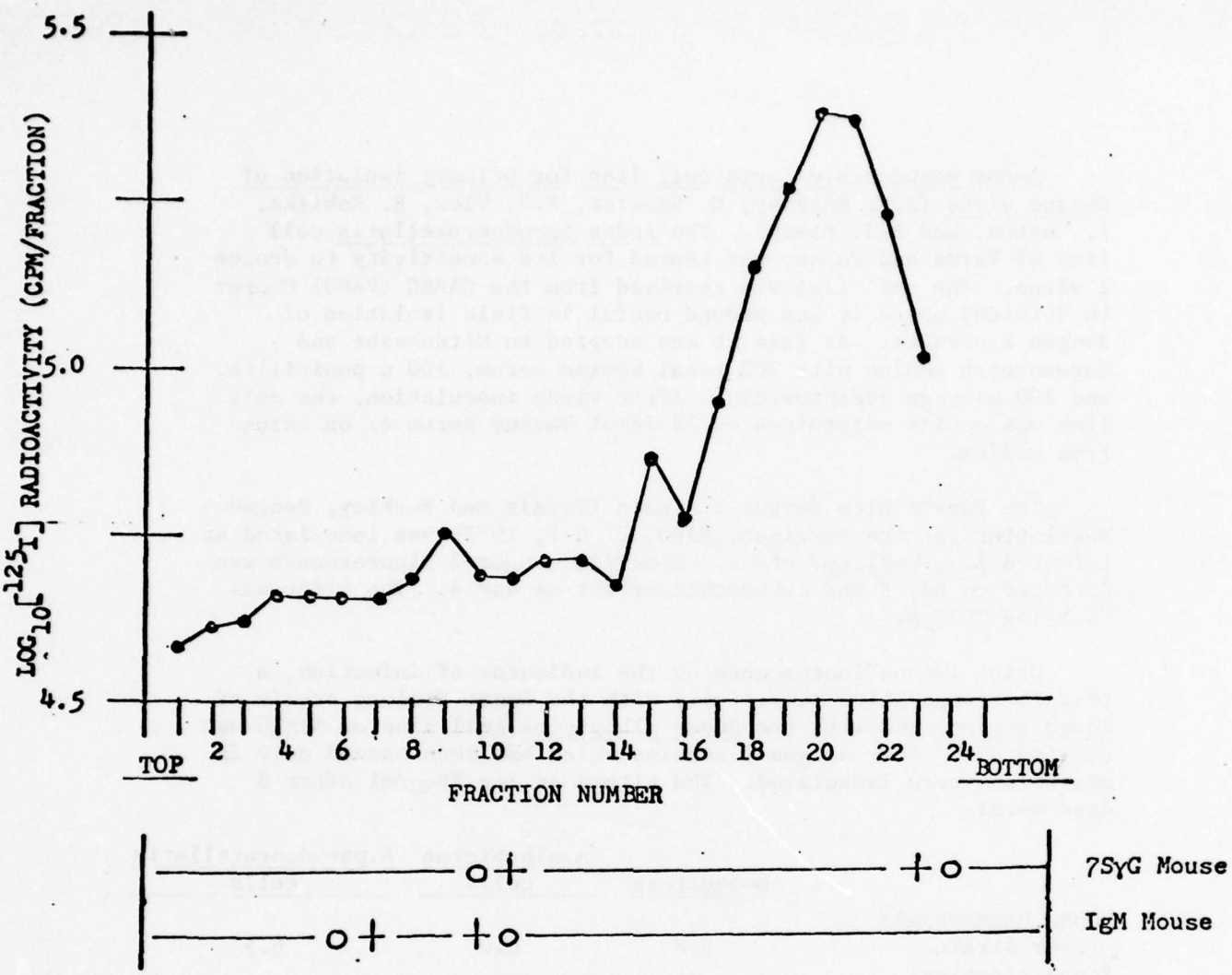


FIGURE 8. -- Sucrose density gradient analysis of $[^{125}I]$-labeled rabbit antimouse IgG gamma-globulin. Gradient fractions were analyzed for $[^{125}I]$ radioactivity (cpm/fraction) and also a second reference marker protein gradient tube was analyzed using specific goat antimouse IgG and antimouse IgM serum. Presence of an immunoglobulin in a fraction is indicated by a plus sign.

Aedes pseudoscutellaris cell line for primary isolation of dengue virus (S.M. Buckley, M. Berwick, E.J. Vier, B. Kobilka, I. Mattos, and B.J. Beaty). The Aedes pseudoscutellaris cell line of Varma and Pudney was tested for its sensitivity to dengue 2 virus. The cell line was received from the CAREC (PAHO) Center in Trinidad where it had proved useful in field isolation of dengue 1 strains. At Yale it was adapted to Mitsuhashi and Maramorosch medium with 20% fetal bovine serum, 100 u penicillin, and 100 microgm streptomycin. After virus inoculation, the cell line was easily maintained on 2% fetal bovine serum or on serum-free medium.

The Puerto Rico dengue 2 strain (Casals and Buckley, Dengue Newsletter for the Americas, PAHO, 2: 6-7, 1973) was inoculated as infected A. albopictus stock. Specific dengue-2 fluorescence was detected on day 3 and cytopathic effect on day 4. The titer was >4.5 log $TCID_{50}$.

Using immunofluorescence as the indicator of infection, a test for comparative sensitivity with the Santo Domingo strain of Aedes aegypti and with the Aedes albopictus cell line of Singh was carried out. Four dengue-2 strains which had been passed only in mosquitoes were inoculated. The titers in log ID_{50}/ml after 8 days were:

	mosquitoes	A. albopictus cells	A. pseudoscutellaris cells
Rosen hemorrhagic fever strain	7.8	6.0	6.5
Rosen classical dengue strain	7.5	6.5	6.5
Malaysian hemorrhagic fever strain	7.1	6.5	6.5
Malaysian classical dengue strain	7.8	6.5	6.5

The mosquitoes were inoculated intrathoracically. The titers in Aedes pseudoscutellaris cell culture were slightly less than the mosquito; the titers in Aedes albopictus cell culture were again slightly less than the Aedes pseudoscutellaris cell culture. In all cases the Aedes pseudoscutellaris cell culture developed easily recognizable cytopathic effect.

The Aedes pseudoscutellaris cell line is simple to maintain under field conditions. The line was transported to Ibadan, Nigeria and to Kuala Lumpur, Malaysia where students of the Yale School of Medicine used it to attempt primary isolation from human blood, under supervision of Dr. Akinyele Fabiyi and Dr. Wil Neill respectively. The isolates are not yet identified; the technique, however appears practical and represents a significant advance for the field laboratory.

The Enzyme linked immunosorbent assay. (O.L.Wood, C.L. Frazier, and A. Smith).

The adaptation of the Enzyme Linked Immunosorbent Assay (ELISA) for work with arboviruses was undertaken with the intent of developing a test that could be easily performed under field conditions. Therefore, whenever possible the use of electronic equipment was avoided. For example, all tests were read by eye instead of with a spectrophotometer.

Initial experiments with ELISA were performed under conditions established in Dr. C. Patton's laboratory at Yale for work with malarial parasites. Although the test when performed with arboviruses is done in the same manner (see below), preliminary results indicated that a number of parameters needed to be altered to make the test applicable.

Random, nonspecific background was noted with several types of polysterene plates when they were tested using several antigen preparations. Plates especially treated for use in ELISA are available from Cooke Engineering Company and proved to be satisfactory for all viruses tested.

Sucrose acetone extracted infected baby mouse brain was used as the first coating antigen and reactions were observed with both immune and nonimmune human sera. Since these nonspecific reactions occurred with several antigen preparations, it was suspected that the sera were reacting with the mouse brain. Tests with normal mouse brain confirmed this. Sera were extracted with acetone, and with acetone and ether with no detectable decrease in nonspecificity. When sera were absorbed with normal mouse brain a slight loss of nonspecific reaction was noted. Post coating of the plates with bovine albumin, fetal calf serum and normal rabbit serum resulted in no appreciable change. Use of an IgG-specific conjugate yielded the same results as the use of a conjugate prepared against human immunoglobulins.

Since serum treatment and post coating had proven futile, a series of experiments were undertaken to determine if antigen prepared from tissue culture might be a better coating antigen. Several methods of preparation in CER cells were tested using Sindbis as a model system. Initially the background reactivity of the negative sera was so high that it was impossible to read the test by eye. As the antigen was purified further, the background reactions diminished. A technique for antigen purification was finally developed that resulted in tests where the eye could be used to distinguish positive from negative sera. Once the antigen was prepared, it was titrated with a reference positive and negative serum to determine the dilution that gave the best resolution between the two sera. This dilution has not changed despite repeated freeze-thaw cycle. Neither lyophilization nor storage at -70° for up to 4 months affected the titer. Treatment of the final antigen with 0.3% BPL to inactivate infectivity did not detectably alter its properties in ELISA.

The Protocol for antigen preparation was as follows:

1. Grow virus in appropriate cell system in serum free medium.
2. Harvest fluid at early CPE and centrifuge at 11,000XG for 30 minutes.
3. Filter supernate through a .045 micron filter.
4. Precipitate virions using polyethylene glycol (PEG) 6000 (10 gm PEG and 2.3 gm of NaCl per 100 ml of supernate).
5. Hold mixture overnight at 4°C to allow precipitation to occur and then pellet precipitate at 11,000 XG for 1 hour.
6. Resuspend the pellet in STE buffer (NaCl 5.84 gm/ℓ EDTA 0.37/gm/ℓ and Tris HCl 1.21 gm/ℓ pH 7.2).
7. Rate zonal ultracentrifugation to yield a virus band; 10 ml of resuspended pellet layered on a discontinous sucrose STE buffer gradient consisting of 1.0 ml of a 5% W/V sucrose solution over 2 ml of a 25% W/V sucrose solution over a bottom 3 ml cushion of 60% W/V sucrose.
8. The visible band in the 60% sucrose region is harvested and stored at -70°C for use as antigen.

The test was performed as follows:

1. Dilute antigen in coating buffer (Na_2CO_3, 1.59 gm/ℓ, $NaHCO_3$, 2.93 gm/ℓ, pH 9.6) and add .21 ml to each well of an ELISA plate. Allow to stand overnight at 4°C.
2. Wash plates 3 times with pH 7.2 phospate buffered saline-Tween (PBST) (0.5 ml/ℓ of tween 20).
3. Dilute sera in PBST and add .21 ml to the appropriate wells.
4. Incubate at room temperature for 2 hr, then wash plates 3 times with PBST.
5. Dilute conjugate in PBST and add .21 ml to each well. Allow to react for 2 hours at room temperature.
6. Wash plates three times with PBST.
7. Dilute substrate in appropriate buffer and add .21 ml to all wells.
8. Allow color to develop until reference positive serum is the desired color and then add .05 ml of 3M NaOH to stop the reaction.
9. In these tests when an alkaline phosphatase conjugate was used the substrate was Sigma 104 (p-nitrophenyl phosphate) 1 mg/ml in a diethanolamine (97 ml/1, pH 9.8) buffer. NaN_3, 0.02%, was added to all solutions as an antimicrobial agent.

A Sindbis antigen was used to test 24 human sera from the Senegal collection at YARU. The test was done with coded sera and read by two individuals. The data presented in Table 29 show that there was 100% correlation with the HI test as far as presence or absence of antibody was concerned, but ELISA was generally more sensitive.

A Venezuelan equine encephalomyelitis (VEE) (TC80 strain) coating was prepared using the same protocol. When it was tested with pre and post vaccination sera (Table 30), there was good correlation between vaccination status and ELISA titer, and among HI, ELISA and neutralization results. One vaccine failure, MG, was detected with all three tests. The positive prevaccination reactions of RS (4/9/62) could be

caused by a cross reaction with Mayaro antibodies. TA had received an EEE vaccine prior to 1971 and this could account for the low titered reaction in both of his sera in the ELISA. The initial post vaccine sera for EG and KC were negative in both HI and neutralization tests. These sera were not available for testing by ELISA, but subsequent sera from both individuals did show the presence of antibodies. There is an inexplicable lack of diagnostic rise in the pre and post sera of RK although a slight rise is noted. Results of neutralization tests done on a prior post sera show that antibodies were present.

A coating antigen was also prepared for Germiston in CER cells. Since known positive specific Germiston human sera were not available, human sera from Liberia that were HI positive to a Bunyamwera antigen were used. Bunyamwera and Germiston cross react with both HI and CF tests. All sera were tested by HI against both a Bunyamwera and Germiston antigen and in the ELISA; the results are presented in Table 31 . Nine of the fifteen sera were either positive or negative in all three tests while four were positive in ELISA only and one in the HI test only. One sera that was positive in HI with both antigens was negative in ELISA. It is not certain whether the positive reactions observed were homologous or caused by heterologous cross reactions. Further testing of the antigen with Germiston specific rabbit sera and of the human sera with a Bunyamwera antigen is planned.

An experiment was done to determine the amount of coating virus that attached to the plate as a function of time and virus concentration. Sindbis virus was labeled with H^3 uridine and thus only the amount of intact virus that had attached was recorded. Although surface antigen not associated with RNA might attach to the plate and not be measured, it was thought that other labeling methods might lead to the inclusion of labeled cell products in the attachment data. The results are depicted in Figure 9 . All points above the solid line give a positive ELISA with a reference sera. As can be seen the amount of attachment increases appreciably with time in the 1:100 to 1:400 antigen dilution range. This is the range that most coating antigens are used at in ELISA. If concentrated antigen is used to coat the plates, short coating periods are acceptable, but if high dilutions of antigen are used the overnight incubation period is preferable. It should be noted that plates coated with high concentration of antigen for short periods of time do give a higher reading with negative sera and thus this makes the test less reliable. When the intensity of the ELISA reaction was correlated with the number of counts, PFU, and particles attached for two separate Sindbis preparations it was observed that there was a direct correlation between the amount of material attached to the plates and the intensity of the reaction (Table 32) . The amount attached for two preparations was virtually equal but did represent different dilutions of the starting material.

It was decided to extend the ELISA technique to another arbovirus group, the Bunyaviridae and to ascertain whether the technique was as sensitive and specific as the HAI and neutralization test. Hyperimmune sera had been prepared previously in rabbits against some virus of the Bunyamwera group: Bunyamwera, Batai, Cache Valley, Guaroa, Germiston,

Ilesha, Sororoca, and Wyeomyia. A rabbit antiserum was prepared against another closely related African virus, Birao. Normal rabbit serum and an antiserum against a group C virus, Oriboca, served as controls. Antigens were prepared in Vero cell cultures in the manner described above for Sindbis virus for all antisera. Antigens were titrated at 1:50, 1:100, 1:200 and 1:400 against four 2-fold dilutions of homologous antiserum and against normal rabbit serum with starting dilutions of 1:10. On the same plate, one antigen dilution was arbitrarily chosen to coat four rows of 12 wells. Usually antigen was used at 1:100. Each row of these wells received much greater dilutions of antiserum starting at 1:20 and proceeding by 2-fold steps to greater than 1:40,000. Sera tested included homologous, antiserum to a virus closely related, antiserum to a virus distantly related by HAI, and normal rabbit serum. The antigens were specific in that color did not develop in wells containing normal rabbit serum. Also the antisera were very high titered. In the case of Bataai, Ilesha, and Bunyamwera antisera the final titers were 1:10,240. Results of cross-comparison of the Bunyamwera group viruses are shown in Table 33; this test corresponds in specificity to the HI and N tests.

Preliminary experiments have been performed with the sandwich technique of antigen detection. When untreated sera were used to coat the plates all the wells showed a positive reaction. When $(NH_4)_2SO_4$ precipitated mouse ascitic fluid globulin was used as a coat, only wells that contained coat, the human serum used to detect the antigen, and the conjugate showed a positive reaction. Although part of the nonspecific background has been removed, further work must be done before the technique is usable.

Table 29

Comparison of anti-Sindbis ELISA and HI titers in Senegalese human sera

Sera	ELISA	HI
16	0[a]	0
40	0	0
47	0	0
51	10	10
120	0	0
141	0	0
184	0	0
385	160	20
398	80	40
417	0	0
547	0	0
563	0	0
576	0	0
585	160	40
664	80	40
701	80	20
775	40	40
866	40	40
913	0	0
488	0	0
958	0	0
997	80	>80
JK[b]	0	0
385[c]	160	20

[a] reciprocal of titer; 0 = <10

[b] New Haven human serum

[c] internal repeat

Table 30

Anti-VEE ELISA titers following VEE Vaccination

Vaccinee	Date	Vaccination Status	ELISA	HI	NT
JH		pre	0[a]	0	0
		post	>80	640	+
AS	10/11/73	pre	0		
	06/20/75	post	160		
RK	07/05/73	pre	10		
	08/13/73	post			+
	01/16/74	post	20		
WB	10/18/73	pre	0		
	05/30/74	post	40		+
MG	01/21/69	pre	0	0	-
	11/02/72	post	0	0	-
EG	12/10/71	pre	0		-
	02/15/72	post	0	0	-
	03/14/72	post		0	
	11/27/72	post	40		
	02/25/74	post	20		
GK	10/20/72	pre	0		-
	11/27/72	post	>320		+
KC	09/30/68	pre	0	0	
	03/21/72	post		0	
	06/26/72	post	20		
TA	03/08/71	pre	10[b]		
	03/14/72	post		0	-
	01/22/73	post	10		
RS	04/09/62	pre	80[c]		
	12/15/67	post	80	20	-
	01/22/73	post	40		
	06/06/75	post	80		
JF	1978	pre	0		
CR	1978	pre	0		
AM	1976	post	80		
	1978	post	80		

[a] reciprocal of serum titer; 0 = <10.
[b] received EEE vaccine prior to 1971.
[c] infected with Mayaro virus in 1959.

Table 31

Anti-Germiston ELISA titers of Liberian human survey sera

Sera	ELISA Germiston	HI Germiston	HI Bunyamwera
6	20[a]	10	0
8	0[b]	0	0
15	0	0	0
23	0	0	80
24	0	0	160
35	0	0	0
38	40	0	80
49	0	0	0
54	40	10	20
66	0	0	0
88	10	0	20
106	40	10	20
111	20	10	10
139	0	10	80
156	40	10	320

[a] reciprocal of serum titer.

[b] 0 = <10

Figure 9

Counts attached as a function of time and dilution

Sindbis antigen on ELISA plates

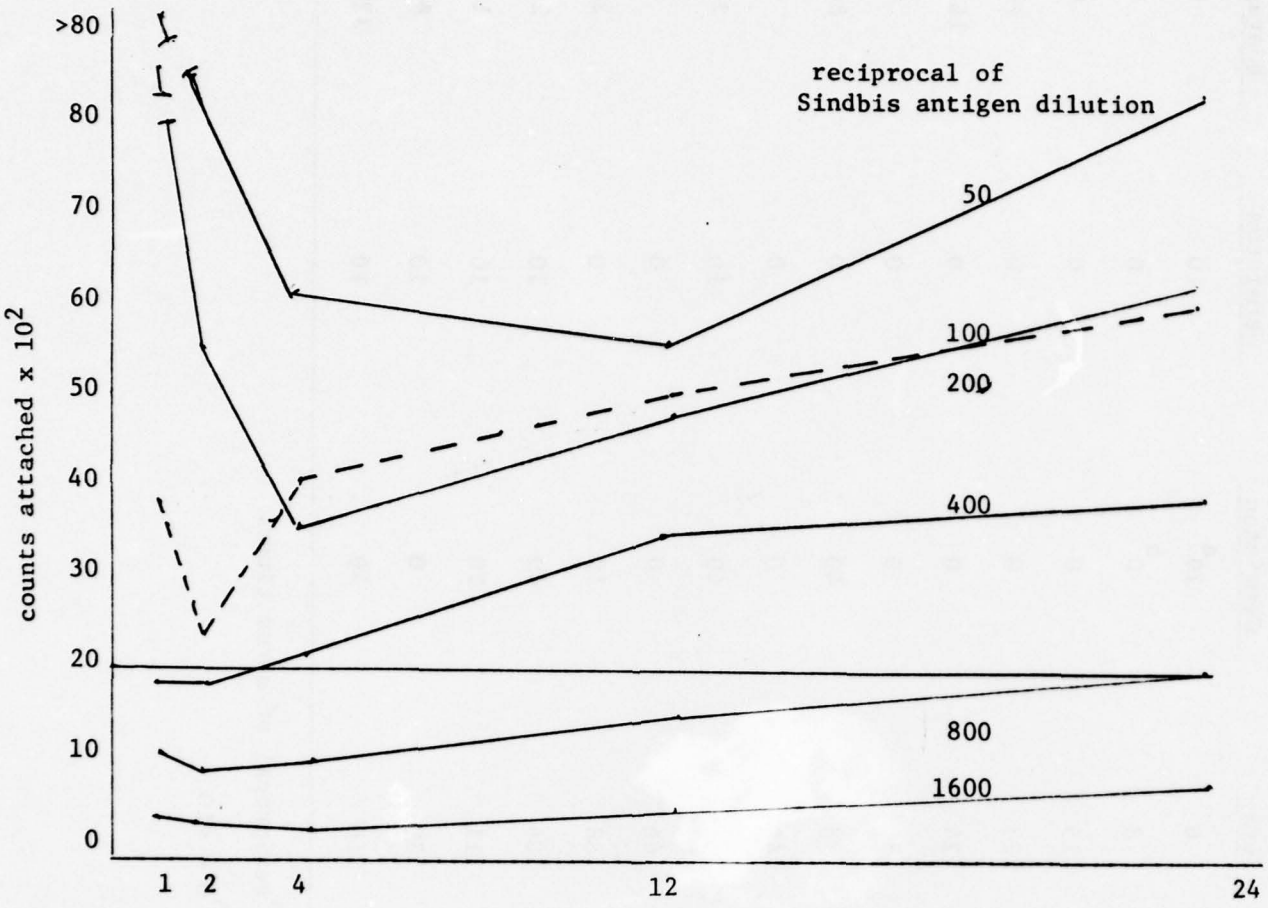


Table 32

ELISA result as a function of Sindbis virus counts, PFU, and particles attached

	Color intensity			
	4	3	2	1
counts	3400	1500	1050	550
	2100	980	580	400
PFU's	58.8×10^4	27.4×10^4	16.2×10^4	11.2×10^4
	55.0×10^4	24.0×10^4	16.8×10^4	8.8×10^4
particles	37.4×10^6	16.5×10^6	11.6×10^6	6.1×10^6
	25.2×10^6	11.8×10^6	7.0×10^6	4.8×10^6

Table 33

ELISA results comparing Bunyamwera group viruses using whole virion antigens

Antigens	Rabbit sera (3 injections) BUN	GER	BIR	BAT	ILE	CV	GRO	Control ORI	Control NORMAL
BUN	10,240	40	320	1280	1280	320	320	40	10
GER	1280	2560	320	1280	2560	320	10	10	0
BIR	320	40	1280	10	320	40	40	40	0
BAT	5120	160	640	10,240	2560	320	160	10	0
ILE	5120	320	1280	640	10,240	640	320	20	0
CV	640	10	160	80	320	1280	80	40	0
GRO	10	0	>320	0	10	0	320	10	0
ORI	10	0	0	0	0	10	10	80	10

Strain characterization of geographically different Kemerovo group viruses by analyses of their RNA segments (D.L. Knudson, A.J. Main, S.M. Buckley, J. Casals, and R.E. Shope).

The orbiviruses contain double stranded RNA in 10 segments which can be labeled with ^{32}P and analyzed by polyacrylamide gel electrophoresis. We have studied 16 strains of Orbiviruses in the Kemerovo serogroup to demonstrate the usefulness of the analysis of the RNA segments for epidemiological studies.

The orbiviruses form a genus of arthropod-borne viruses in the family Reoviridae. There are at least 80 orbiviruses in 10 serogroups and 7 ungrouped serotypes. The genus contains the following groups: Colorado tick fever, bluetongue, African horsesickness, Changuinola, Kemerovo, and several others of human and veterinary disease importance. The Kemerovo group viruses were chosen for this study because 16 closely related strains were available from a variety of hosts and from different geographic regions of Asia, Europe, and Africa, including one strain isolated in 1961 by J.R. Schmidt of NAMRU-3, from a migrating Redstart, Phoenicurus phoenicurus, which was suspected on ecologic grounds of transporting Kemerovo virus from the Kemerovo region in Siberia. It was in Siberia that the prototype strain was isolated the following year from Ixodes persulcatus ticks and from man.

In addition the strains had been studied by neutralization and complement-fixation tests extensively by Libikova, Buckley, and Casals and formed two serologic subtypes, Kemerovo and Tribec. The Tribec subtype however, derived from 3 different geographic foci in Czechoslovakia and might be expected to have evolved into different variants genetically.

Thus the RNA segment analysis might tell whether the Kemerovo virus from the migrating bird was the same as the viruses from Siberia; whether the Siberian viruses which came from 2 neighboring villages were identical or different; whether the human isolate differed from the tick isolates in Siberia; and whether the Slovakian Tribec and Lipovnik isolates from the 3 different geographic areas were genetically different.

The strains were passed once in BHK-21 cells from mouse brain stock, then labeled on second passage with ^{32}P and harvested at 48 hours. The infected cells were sedimented, separated from the medium, extracted with a non-ionic detergent, and the RNA was precipitated overnight with 2 volumes ethanol. The precipitates were dissolved in buffer and layered on 10% polyacrylamide slab gels. The 16 strains were processed simultaneously. The gels were dried, and the autoradiographic RNA patterns were analyzed.

Because of the properties of the 10% gel, purification of the RNA is not required, nor is high titered virus needed. These inocula had titers ranging between $10^{-2.3}$ and $10^{-5.5}$ $TCID_{50}$/ml.

Figure 10 shows the origin of the 16 strains. There were 6 strains from 2 villages in the Kemerovo region of Siberia. One of these was isolated from man, the others from ticks. Nine strains originated in Slovakia, all from Ixodes ricinus ticks. One of these, Tribec, came from the Tribec mountains in Southwest Slovakia; another 4 strains came from Lipovnik in eastern Slovakia; and 4 strains of virus came from Koliba near Bratislava in western Slovakia. Figure 11 shows graphically the estimated summer nesting range of the European Redstart, from Siberia to Western Europe, and the winter sub-Saharan range. These ranges are based on sightings and on recaptures of banded birds. The arrow shows a hypothetical migratory route of the Redstart from which Kemerovo virus was isolated.

The complement-fixation test distinguished, but barely distinquished, the Kemerovo isolates from the Slovakian isolates, however it did not distinguish among isolates from Slovakia or among isolates from Siberia (Table 34). The Egyptian isolate appeared to be serologically the same as the Siberian serotype.

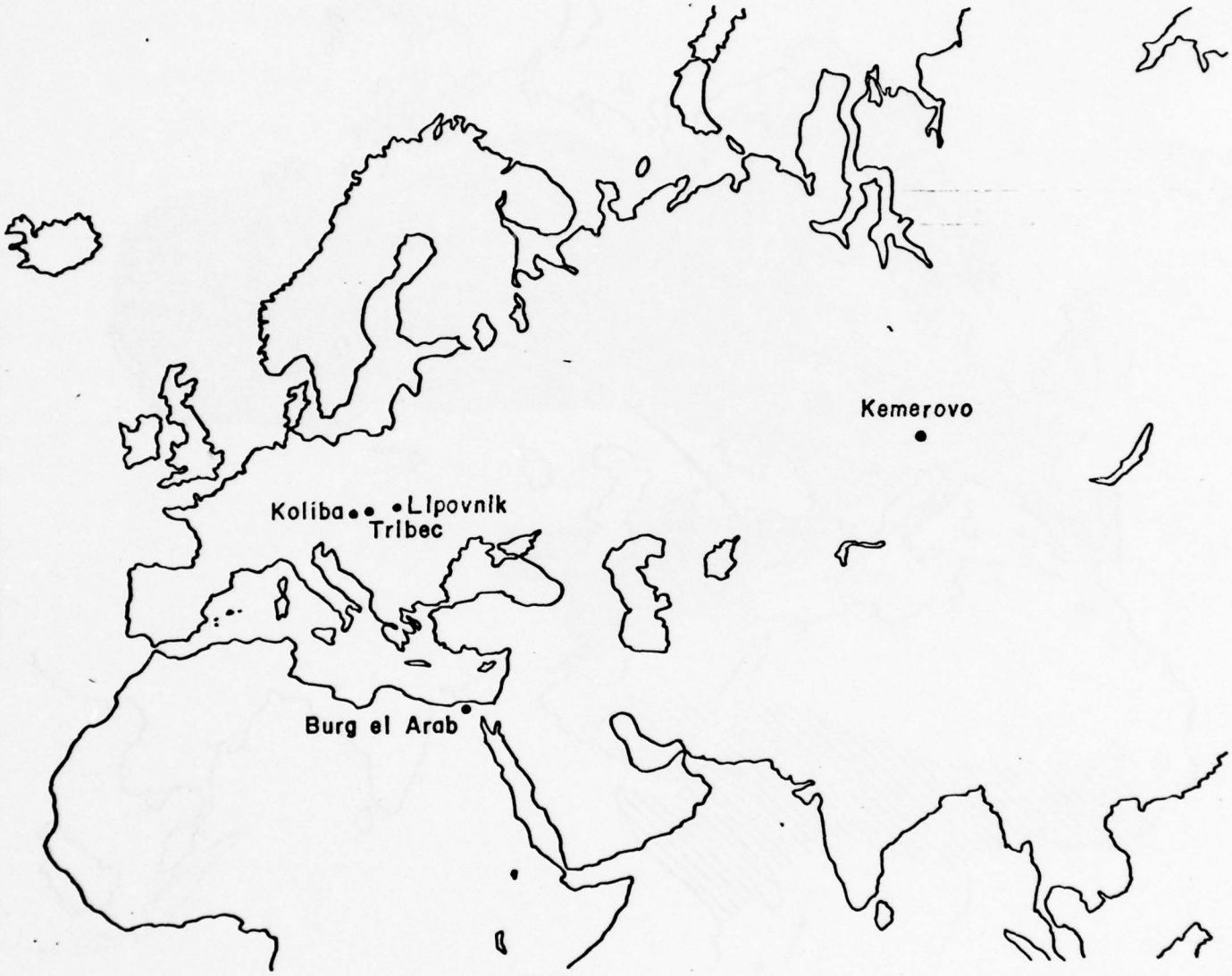
The neutralization test (Table 35), like the CF, showed uniformity among the Siberian isolates which differed from the Slovakian isolates. Some Slovakian sera were more broadly cross-reactive than others, indicating a possible genetic diversity. In one study, there appeared to be a slight difference between Tribec and Lipovnik prototype strain by neutralization test. Again the bird isolate was not distinguished from the Siberian viruses by neutralization test.

The polyacrylamide gel RNA segment patterns are shown in Figure 12. All viruses had 10 segments, indicating that none were mixtures of orbiviruses. There was remarkable uniformity of the Siberian isolates. The L-75 strain from man was the same as the tick isolates. One tick isolate, KM-3, differed in 2 segments from the others. The Redstart virus RNA was nearly identical also, providing strong evidence that the origin of the bird isolate was the same as those from Siberia.

None of the Slovakian isolates were identical (although they all had 10 segments with the general pattern of orbiviruses). Each of the 3 Slovakian foci had distinctive patterns and there was even diversity among isolates from the same focus, possibly explaining why some of the sera were more broadly cross-reactive by neutralization tests than others.

It is not possible without further study to determine which segment codes for the protein or proteins responsible for the CF

Figure 10



Origins of 16 strains of Kemerovo virus in USSR, Czechoslovakia and Egypt.

Figure 11

Summer nesting range (stippled) and winter range (sub-Saharan) of the European Redstart (Phoenicurus phoenicurus). Arrow shows possible migratory route of the Redstart from which Kemerovo virus was isolated.

and neutralization reactions, however, the RNA segment analysis should facilitate such a determination in future genetic recombination experiments. In addition the technique allows accurate rapid identification of orbiviruses without need to produce an immune serum and can be applied to determination of markers for vaccines, and subtle genetic changes which may occur in persistently infected animals or when orbiviruses change in virulence or host range during epidemics, or by passage from host to vector.

Table 34

Complement-fixation relationships of Kemerovo and Tribec viruses[a]

	Sera		
Antigen	Kemerovo L-75	Kemerovo KM-3	Tribec prototype
Kemerovo L-75	256[b]	32	128
Kemerovo KM-3	256	32	128
Tribec prototype	32	16	256

[a] Libikova and Casals, Acta virol. 15: 65-78, 1971.

[b] reciprocal of CF antibody titer with optimal antigen dilution.

Table 35

Neutralization relationships of Kemerovo and Tribec viruses[a]

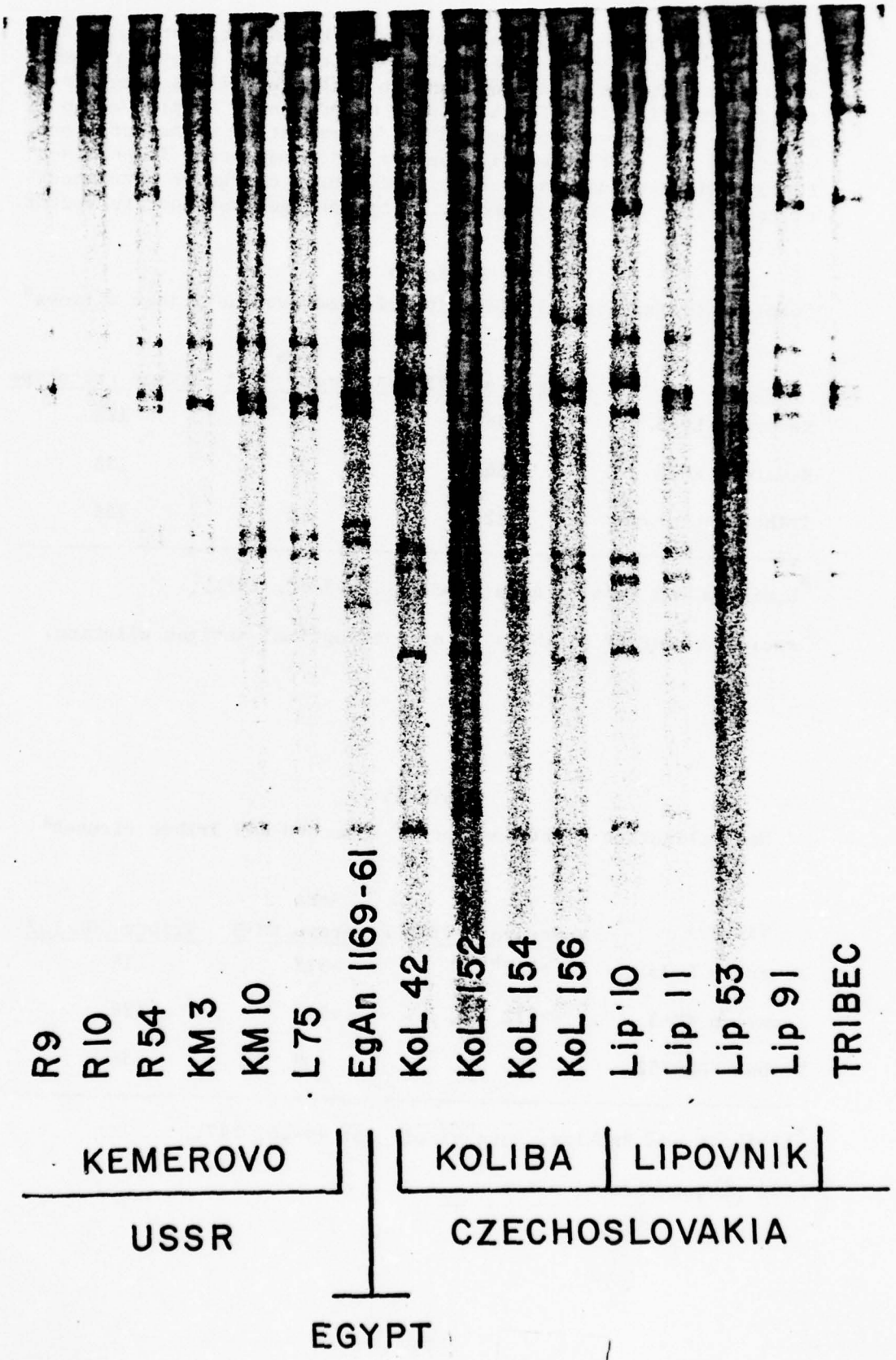
	Sera		
Virus	Kemerovo L-75	Kemerovo KM-3	Tribec KOL-152
Kemerovo L-75	>512[b]	>512	16
Kemerovo KM-3	>512	>512	128
Tribec KOL-152	<16	<8	256

[a] Libikova and Buckley, Acta virol. 15: 79-86, 1971.

[b] 50% plaque reduction titer.

Figure 12

RNA segment analyses of Kemerovo strain orbiviruses



RNA segment analyses of Kemerovo serogroup orbiviruses (D.L. Knudson, A.J. Main, and R.E. Shope)

Analyses of the RNA segment profiles of the sixteen Kemerovo strains provided the impetus to examine the dsRNA profiles of twenty-three other members of the Kemerovo serogroup. The majority of these serogroup viruses were isolated from the tick, Ixodes uriae, about 40° north latitude. One isolate, Nugget, was isolated from I. uriae taken from Macquarie Island (Lat. 54°30'S, Long. 159°E) in the South Pacific.

The mouse brain virus stocks were passaged once in the BHK-21 cell line, and they were prepared for dsRNA analyses as described for the Kemerovo strains (see above). The data describing the isolate, strain number, suckling mouse brain passage history, area of isolation, and autoradiogram of the slab gels are presented in Figures 13 and 14.

These data suggest that the majority of the isolates exhibit distinct RNA profiles of ten segments which is consistent with their classification as orbiviruses. There are instances where segments appear to co-run, and as a result, some isolates seem to have less than 10 segments. This point, however, will be clarified when the autoradiograms are analyzed by scanning densitometry. The RNA profiles of strains isolated from a specific geographic region were often indistinguishable. In a few instances, variation in a single segment between two strains was also seen. For example, the two isolates of Yaquina Head were essentially identical except for their third segment from the bottom of the gel (Figure 13). Nugget virus was the only isolate examined that appeared to possess more than ten segments. This virus is clearly a candidate for additional studies, such as virus cloning experiments. In general, viruses which originated from specific areas were similar, but extensive variability in the dsRNA profiles was observed for isolates from different regions.

RNA segment analyses of American and Australian Bluetongue serotypes (D.L. Knudson and R.E. Shope - This work was completed at the U.S.D.A., Plum Island Animal Disease Center in collaboration with Drs. C. Campbell and W.K. Butterfield)

In 1977, YARU identified an unknown virus from Australia as indistinguishable by complement-fixation tests from bluetongue type 10, the bluetongue virus vaccine strain. The new virus, known as bluetongue type 20, was isolated in 1974 from a pool of mixed Culicoides species from the Northern Territory of Australia. Type 20 also exhibited a low level cross-reaction by CF with the epizootic hemorrhagic disease of deer (EHD) virus. This account represents the first isolation of bluetongue virus from Australia, and as a result, these findings provided the rationale for the analyses of the dsRNA segment profiles of bluetongue viruses and other related viruses.

Figure 13

RNA segment analyses of Kemerovo serogroup orbiviruses

	Virus	Strain	SMB Passage	Area
1.	Great Island	CanAr 41	4	North Atlantic (Canada)
2.	Great Island	CanAr 176	2	North Atlantic (Canada)
3.	Bauline	CanAr 14	3	North Atlantic (Canada)
4.	Bauline	CanAr 128	1	North Atlantic (Canada)
5.	Bauline	CanAr 133	1	North Atlantic (Canada)
6.	Cape Wrath	ScotAr 20	3	North Atlantic (Scotland)
7.	Tindholmur	DenAr 3	2	North Atlantic (Faeroe Islands)
8.	Mykines	DenAr 12	1	North Atlantic (Faeroe Islands)
9.	Mykines	DenAr 8	3	North Atlantic (Faeroe Islands)
10.		Fin V808	4	North Atlantic (Norway)
11.		Fin V873	3	North Atlantic (Norway)
12.		Fin V962	4	North Atlantic (Norway)
13.	Yaquina Head	USA 15	7	North Pacific (Oregon)
14.	Yaquina Head	USA 62	8	North Pacific (Oregon)

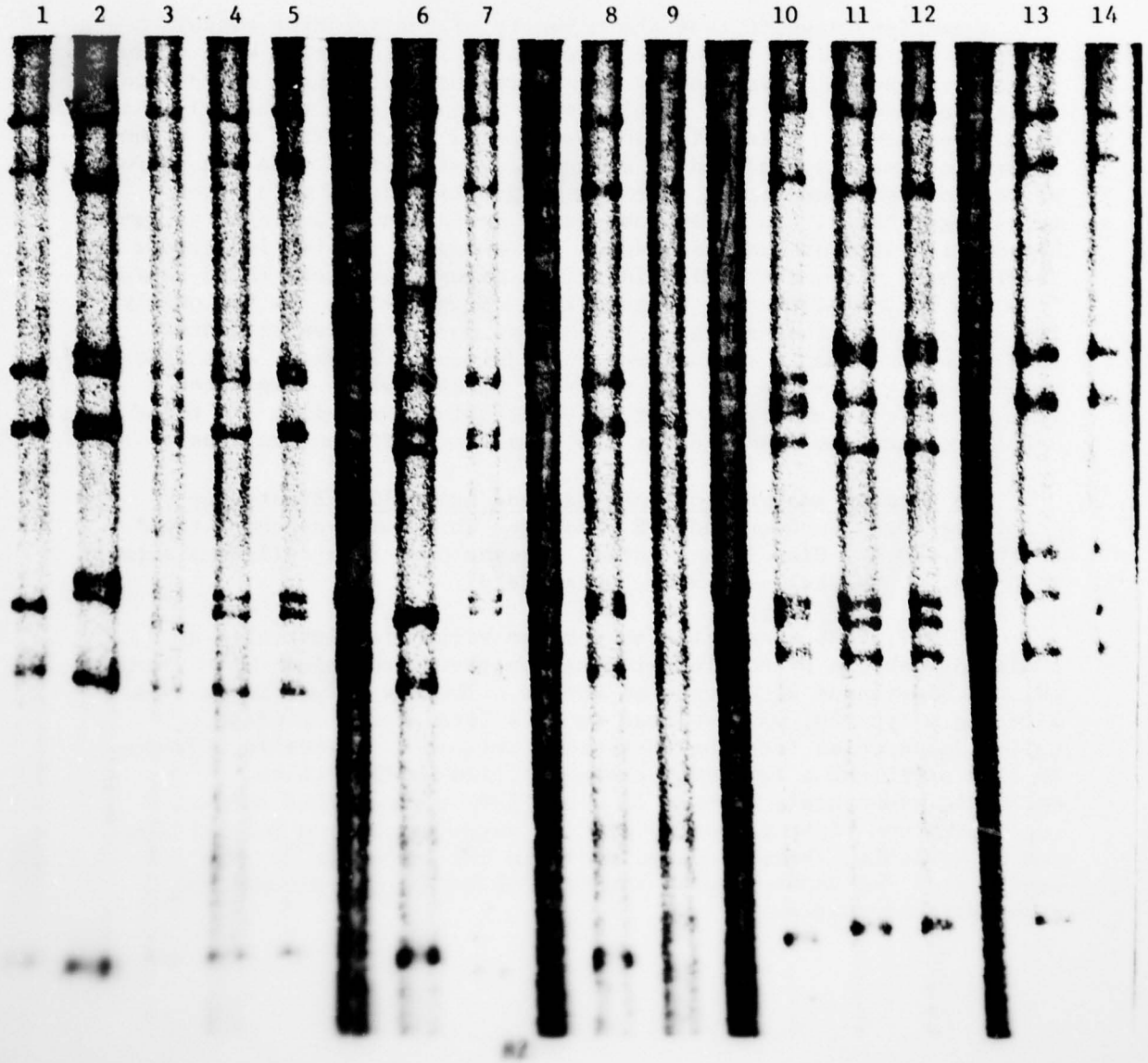
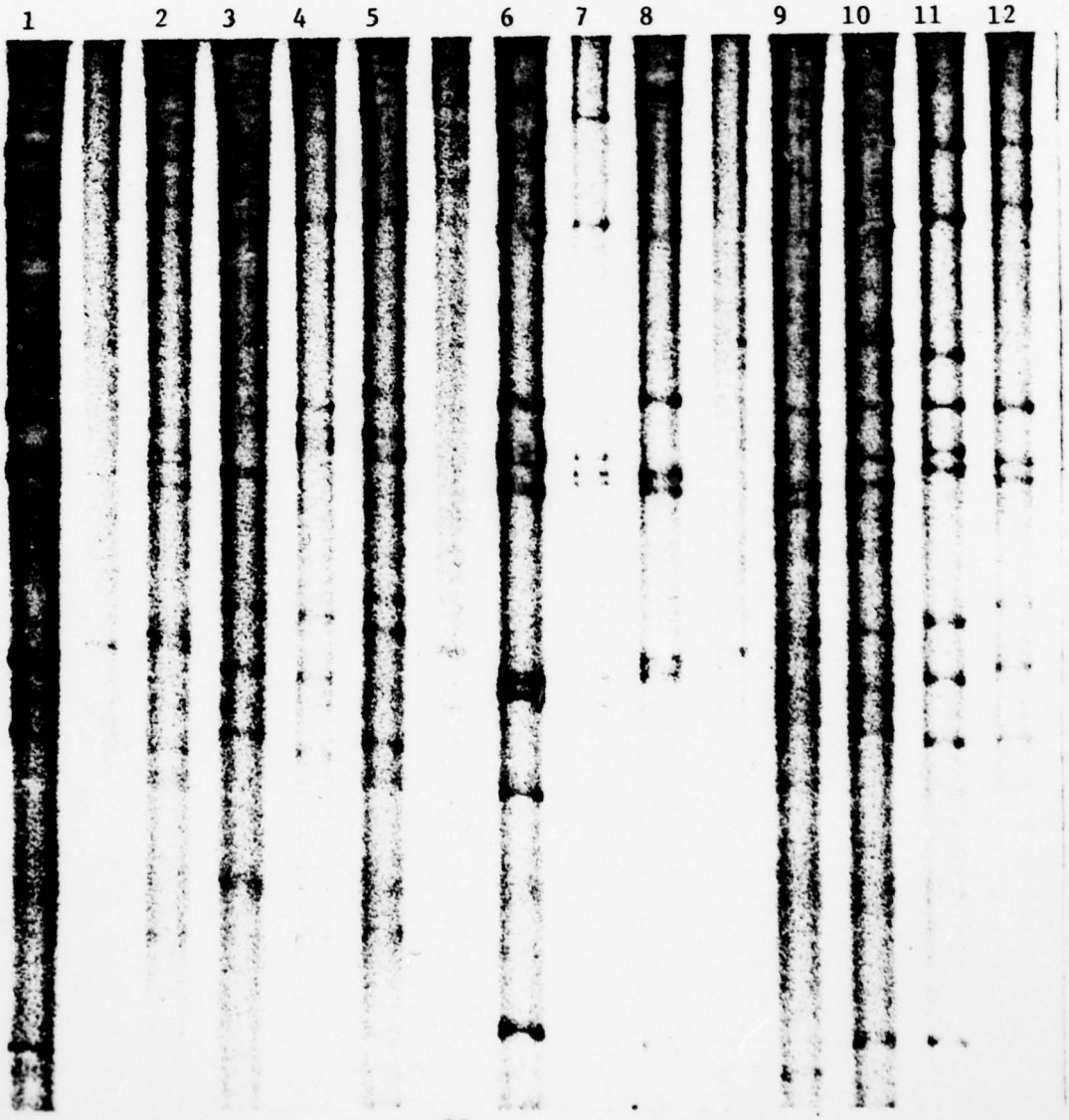


Figure 14

RNA segment analyses of Kemerovo serogroup orbiviruses

Virus	Strain	SMB Passage	Area
1. Great Island	CanAr 45	3	North Atlantic (Canada)
2. Baku	LEIV 46A	7	USSR
3. Huacho	CalAr 883	6	South America
4. Mono Lake	CalAr 861	7	North America
5. Chenuda	EgAr 1152	25	North Africa
6. Lipovnik	Lip 91	2	Europe
7. Tribec	original	14	Europe
8. Kemerovo	R-10	8	USSR
9. Nugget	AusMI-14847	6	South Pacific (Australia)
10. Okhotskiy	LEIV 287ka	6	North Pacific (USSR)
11. Poovoot	RML 71	6	North Pacific (Alaska)
12. Kenai	71-1629	4	North Pacific (Alaska)

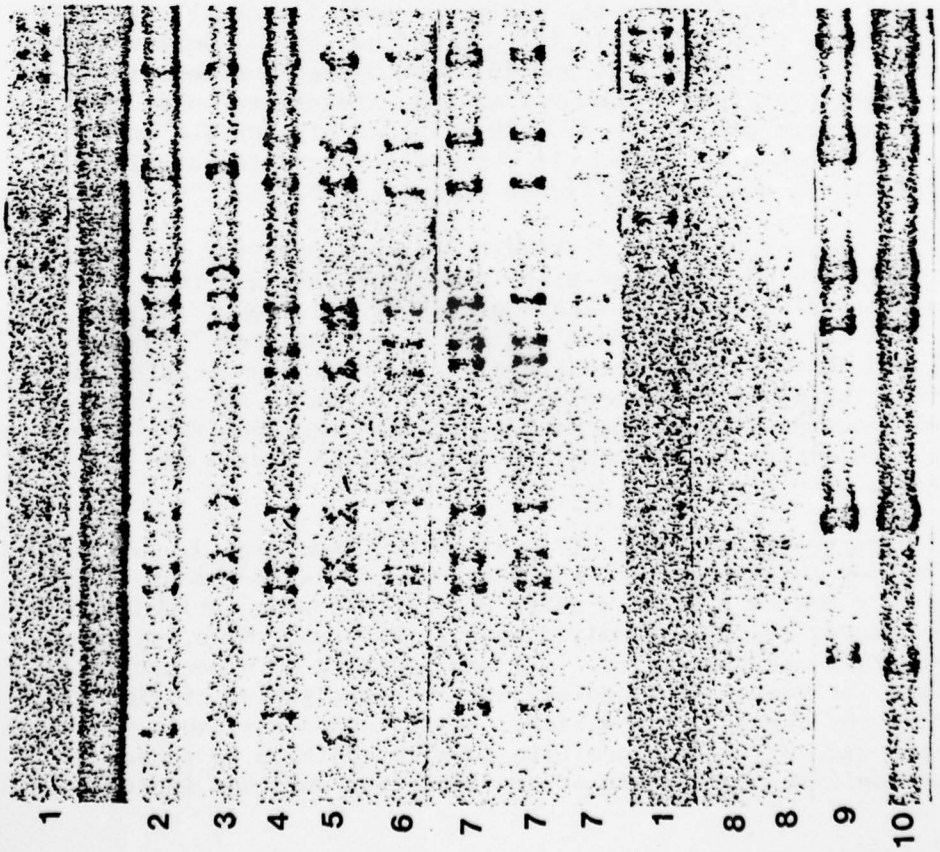


The virus stocks were prepared for RNA analyses as described previously with the exception that the MVPK (porcine kidney) cell line was used instead of the BHK-21 cell line. The data are presented in Figure 15, and the RNA profiles for four bluetongue serotypes found in the U.S.A. are compared with the profiles of type 20, EHD, and Ibaraki. The RNA profiles for bluetongue viruses 10, 11, 13, and 17 were similar with only minor variations in the mobility of a few RNA segments, and thus, each serotype exhibited a unique RNA profile. The two EHD strains were also distinguishable, but more similar to Ibaraki than to the bluetongue serotypes. In general, the RNA profile for type 20 was similar to the other bluetongue types, but it was not the typical bluetongue-like RNA pattern. Whether the type 20 virus represents a reassortant species from existing Australian orbiviruses remains to be demonstrated.

Figure 15

RNA segment analyses of American and Australian bluetongue serotypes

Virus	Type or Strain	Passage history	Area
1. Reovirus	3	L929 cell line	-
2. Bluetongue	20	MVPK cell line	Australia
3. Bluetongue	20	SMB	Australia
4. Bluetongue	17	sheep-MVPK	U.S.A.
5. Bluetongue	10	sheep-MVPK	U.S.A.
6. Bluetongue	13	sheep-VERO-BHK	U.S.A.
7. Bluetongue	11	sheep	U.S.A.
8. Ibaraki		VERO-BHK	Japan
9. Epizootic hemorrhagic disease		VERO	Alberta
10. Epizootic hemorrhagic disease		VERO	New Jersey



III. Seroepidemiological surveys.

Sera from Venezuela (G. Calderon and J. Casals). Sixty-nine sera collected in the State of Zulia were tested by HI test for antibodies against 18 different arbovirus antigens. Due to the small number of sera and to the fact that they represented 11 different animal species, this study can hardly be called a survey, it is rather an exploration; however, its results indicate that certain arboviruses--or related ones not included in the study--were present in the area.

The distribution of sera by species was: Didelphis, 27; hamster, sentinel, 14; man, 12; Tupinambis, 5. The remaining 11 sera were from 7 species, none of which contributed more than 3 samples. The sera, after treatment with kaolin and goose red cell adsorption, were tested against 4 or 8 units of the following antigens: VEE, EEE, WEE, Una, Aura, Mayaro, Aroa, dengue 2, yellow fever, Bussuquara, Ilheus, SLE, Powassan, Oriboca, Itaqui, Maguari, Guaroa and Bunyamwera. The result of the tests is given in Table 36. Only 19 of the 69 sera had antibodies for any of the antigens employed; 6 sera were specific for VEE, with titers up to 1:80, and 15 had antibodies against group B viruses (flavivirus). A specific diagnosis of the group B positives (considering the antigens used) is possible in only a few cases, perhaps no. 15 for SLE and no. 60 for yellow fever; 5 sera, nos. 3, 33, 50, 64 and 66, gave a reaction associated with superinfection, although with moderate titers only. The remaining sera had titers of 1:10 only, mainly for SLE and/or Ilheus; although these low titer reactions were technically satisfactory, they are difficult to interpret.

In view of the fact that with the sera positive for group B, the titers were highest with Ilheus and SLE, and that a few sera reacted with either Ilheus or SLE at dilution 1:10, one can make the assumption that one of these 2 viruses (of those included in the study) were responsible for the antibodies; and considering that, in general, the SLE antigen is apt to be more sensitive than Ilheus, one can tentatively conclude that Ilheus virus was active in the area. Reactions with Guaroa and Maguari, were minimal.

Sera from Senegal (J. Pilaski, W.G. Downs, and J. Casals). The survey initiated last year has been continued through 1978. Sera were tested against 8 units of the following antigens: chikungunya, Sindbis, Banzi, dengue 2, MVE, West Nile, yellow fever, Zika and Bunyamwera. MVE was included as a sensitive antigen for group B antibodies, not in anticipation of finding activity by the virus in Africa. As it turned out, this antigen did not uncover positive sera in addition to those found positive against the other flavivirus antigens included in

Table 36

Hemagglutination-inhibition test sera from Venezuela (Zulia State)

Serum		Antigen									
No.	Animal	VEE	Aroa	D2	YF	Bussu	Ilheus	SLE	POW	Guaroa	Maguari
3	Cerdocyon	10	20		40	20	40	40	20		
15	Didelphis							20			
16	Didelphis	40									
30	Didelphis	10									
33	Didelphis		40	10	20	40	160	160	20		
42	Tupinambis						10				
50	Didelphis		80	80	80	80	160	80	40		
51	Hamster						10	10			
52	Hamster		10				10	10			
53	Hamster						10	10			
54	Hamster						10				
60	Man	10			20						
62	Man						10				
63	Man	80									
64	Man		40	80	20	40	80	40	20		
65	Man				10						10
66	Man		20		10	20	40	40			
67	Man	80								10	10
69	Man						10				

Only positive sera of 69 tested have been tabulated; all blank spaces indicate negative reaction at dilution 1:10, lowest used.

Reciprocal of serum titer.

the survey; however, in a considerable number of cases the titers of the sera were as high or 1 or 2 dilutions higher with MVE than with the other antigens.

The interpretation of the results within group B was made as follows: a serum was considered positive for an antigen when its titer for this antigen was 2 or more dilutions higher than for any other antigen, and possibly positive when only 1 dilution higher; a serum was labeled as giving a superinfection-type reaction when the titers with 2 or more antigens were alike and high, for example 1:320 or higher; and as superinfection-type low titer, when the titers were no higher than 1:40 or 1:80 with several antigens. Results with MVE antigen were disregarded when interpreting the results.

A summary of the survey's results is given in Table 37. It is to be understood that the interpretation of the results applies only to the antigens used; what would have happened if additional antigens had been used is a moot question. Antibodies for chikungunya and Sindbis were not infrequent, about 13% and 15%, respectively, of the sera were positive. A large proportion of sera, 76% were positive for group B antigens; nearly half of the sera had across the board reactions with all the antigens; tentative diagnoses could be made for West Nile, yellow fever, Banzi and Zika, but not for dengue 2. The logical conclusion is that several group B viruses have been active in the area of Senegal from which the sera came. Nine sera reacted with Bunyamwera antigen, but whether they represent infections with this virus or with other agents of the Bunyamwera group--Ilesha, Germiston--cannot be stated.

Table 38 gives examples of results on which the interpretation of the tests was based.

West African sera for Lassa fever (J. Casals). Lassa fever virus. The survey to detect immune persons among hospital staff personnel in West Africa, particularly Liberia, conducted in association with Dr. John D. Frame, College of Physicians and Surgeons, Columbia University, New York, N.Y. has continued through 1978. Spot-slides bearing Lassa fever virus infected cells were generously supplied by Dr. Karl M. Johnson, CDC, Atlanta, Ga.

Between 15 April and 15 December, 1978, 278 sera from residents in Liberia, Niger and Rwanda were screened at dilution 1:4 only; 6 sera were positive, 3 gave a $\pm$ reaction, i.e., positive but borderline; 8 were questionable, probably negative; and 261 were negative. All the positive sera were from Liberia

Yugoslavian sera for Congo-Crimean hemorrhagic fever (O. Imami, and J. Casals). CCHF virus. A small survey for IF antibodies against this virus was done in association with Dr. O. Imami, Pristina, Yugoslavia; 70 sera from lifetime residents of Serbia

Table 37

Hemagglutination-inhibition test survey with human sera from Senegal

Number of sera tested	85		
Positives for any antigen		68	
Group A, positives			24
chikungunya			9
possible chikungunya			2
Sindbis			4
possible Sindbis			9
Group B, positives			65
superinfection			13
superinfection, low titers			17
West Nile			2
possible West Nile			13
yellow fever			2
possible yellow fever			5
Banzi			4
possible Banzi			4
Zika			1
possible Zika			4
Group Bunyamwera			9

Table 38

Hemagglutination-inhibition test sera from Senegal: illustrative examples of antigenic group B diagnoses

Diagnosed as	Serum No.	Antigens					
		WN	YF	ZIKA	BANZI	D2	MVE
Superinfection	2	320	320	320	320	320	1280
	14	320	640	640	640	640	2560
Superinfection, low titer	24	40	20	40	10	0	40
	30	80	40	80	40	10	80
West Nile	74	40	0	0	0	0	10
Possible West Nile	65	20	10	10	10	0	10
Yellow fever	85	40	160	40	40	20	40
Possible yellow fever	38	0	10	0	0	0	0
Zika	32	320	160	1280+	160	80	320
Possible Zika	19	20	80	160	20	10	80
Banzi	55	20	10	20	80	0	40
Possible Banzi	60	20	10	10	40	0	20

Reciprocal of titers; 0, negative at 1:10

were screened at dilution 1:4. The sera were from currently healthy persons, who had no recollection of a severe hemorrhagic disease; 3 were positive.

Laboratory workers sera for Pichinde virus. (J. Casals). At the request of Dr. D.H.L. Bishop, University of Alabama, 57 sera from 45 persons associated with a laboratory in which work with Pichinde virus was being conducted, were tested for antibodies against Pichinde and other arenaviruses.

The sera were at dilution 1:4 by immunofluorescence (IF) test against Pichinde antigen; a number were also tested against Tacaribe and Junin viruses, but positives were found only against Pichinde virus, with one exception (see section: Diagnosis of human disease, Tacaribe virus infection). All positive sera at dilution 1:4 were titrated to determine their end-points; sera that at dilution 1:4 gave a questionable or weak reaction with readings of $\pm$, 1 or 2 plus, were retested beginning at dilution 1:2 or undiluted. All sera were also tested by CF in increasing two-fold dilutions beginning at 1:4, extending to 1:32, against Pichinde, Tacaribe, two other viruses not arenaviruses and a normal tissue antigen. With the exception of one serum (see above) positive specific reactions were found only for Pichinde virus. The results of the tests are shown in Table 39.

In the IF test, 17 sera were positive with titers between 1:8 and 1:128; 14 of these same sera and none negative by IF, were also positive by CF with titers from 1:4 to 1:32 or higher. These results illustrate the considerable opportunity of acquiring laboratory infections when working with Pichinde virus; as far as we know, no clinical manifestations were associated with these infections.

Multipurpose serological survey: Guam, United States, Colombia, West Irian, Papua and New Guinea (D. Asher, A. Diwan, R. Benfantes, D.C. Gajdusek, C.J. Gibbs, and J. Casals). During the summer of 1977, a survey was carried out comprising a total of 1748 sera; the outcome and general purpose were described in the 1977 annual report. In brief, the purpose of the survey is to determine whether a relationship exists between presence of antibodies for arboviruses and certain neurological diseases of as yet undetermined etiology. In addition, the survey includes sera from isolated and primitive populations in a continuing effort to map distribution of arbovirus antibodies.

The first part of the survey consisted of screening the sera at dilutions 1:10 and 1:20 by HI against 25 arbovirus antigens; the second part of the study, conducted during October, 1978, had as an aim to titrate a number of selected sera that had been positive in the screening test. In addition, a number of sera not assayed before were tested for antibodies against Ross River antigen.

1. Four hundred seventy-three sera from normal individuals of native populations from West Irian, Papua, New Guinea and Guam, were

Table 39

IF and CF test results with sera from individuals exposed to Pichinde virus in the laboratory

Serum No.	Test and reciprocal of titer		Serum No.	Test and reciprocal of titer	
	IF	CF		IF	CF
1	0	NS	30	0	0
2	16	NS	31	64	8
3	128	32+	32	128	32
4	128	32+	33	0	0
5	0	NS	34	0	0
6	0	NS	35	16	8
7	0	0	36	0	0
8	128	32+	37	0	0
9	0	NS	38	0	0
10	128	32+	39	8	0
11	0	0	40	0	0
12	0	0	41	0	0
13	0	0	42	8	4
14	0	0	43	0	0
15	16	8	44	0	0
16	0	0	45	0	NS
17	0	0	46	32	8
18	0	0	47	0	0
19	64	32+	48	0	0
20	0	0	49	0	0
21	0	0	50	0	0
22	0	0	51	0	0
23	8	4	52	0	0
24	0	0	53	0	0
25	0	0	54	64	4
26	0	0	55	0	0
27	128	32+	56	16	0
28	0	0	57	0	0
29	0	0	58	0	0

0, negative reaction at dilution 1:2 or 1:4.
NS, non-specific

tested for antibodies against Ross River antigen. The following numbers were positive: 10 at dilution 1:10; 20, at dilution 1:20; 5 at dilution 1:40; and 3 at dilution 1:80.

2. Two hundred four sera from U.S. residents with chronic forms of CNS disease were tested for antibodies against EEE, WEE, SLE, Powassan, yellow fever and California encephalitis. Nineteen were positive for SLE, at dilution 1:40, and 3 at dilution 1:80. One had a titer of 1:10 for EEE, and 2 for Powassan, at the same dilution; 4 were positive for yellow fever at dilutions 1:10 or 1:20.

3. Two hundred forty-seven sera from persons in Guam, West Irian and Papua were tested against dengue 2, JE and Murray Valley encephalitis viruses. One hundred seventy were positive for MVE with titers up to 1:1280. One hundred forty-eight were positive for JE, most of which were also reactors with MVE; the titers with JE were, in general, 1 or 2 dilutions lower than with MVE. Dengue 2 antibodies were found in 38 of 109 sera tested, but at lower dilutions, the highest titers being 1:80.

4. Seventy-eight sera from South American natives were tested as follows: 23 against Mayaro and 55 against SLE, Ilheus, Bussuquara, yellow fever and dengue 2; the first dilution was 1:40. 11 were positive with Mayaro, with titers as high as 1:640 (6 sera) and 1:1280 (one serum). Nine sera were positive for SLE, 13 with Ilheus, 9 for yellow fever and 3 for Bussuquara. From the distribution of titers it appeared as though SLE and Ilheus may have been responsible for the antibodies.

IV. Diagnosis of human disease

Tacaribe virus infection (J. Casals). A laboratory investigator, G.G., engaged at various times on work with Pichinde and Tacaribe viruses was taken ill some time in 1976, with fever, malaise, and systemic generalized and uncomfortable symptoms; circumstantial evidence pointed to a possible infection with Tacaribe virus. In the course of a serological survey done in the personnel where G.G. worked (see section: Seroepidemiological surveys) it was found that many persons, including G.G. had antibodies against Pichinde virus. In order to settle the etiological question, serial serum samples from G.G. were tested for antibodies against Tacaribe, Pichinde and other related and control antigens, by immunofluorescence (IF) and CF tests; the result is shown in Table 40.

The sera from G.G. reacted only with antigens as shown in Table 40. The results indicate that G.G. suffered two infections in succession; the first one with Tacaribe (or a closely related virus) between April and November, 1976; and the second with Pichinde (or a similar agent) between December 1976 and October 1977.

Korean hemorrhagic fever (J. Casals). Dr. J. Schachter, University of California, San Francisco, requested assistance to investigate the validity of a claim made to him suggesting that there may exist an etiological relationshp between Korean hemorrhagic fever and infection with a Chlamydia. Dr. Schachter supplied antigens: a yolk sac Chlamydia antigen 1-7-77, and a normal yolk sac control antigen; and a reference serum "Men. 3-9-73, LGV", with an anticipated CF titer of 1:128 or 1:256. A CF test was performed in our laboratory with sera from 4 persons who had recovered from hemorrhagic fever with renal syndrome (HFRS), another name for Korean hemorrhagic fever; these sera had been generously supplied by Dr. A. Butenko, Moscow. The sera had been found positive by Dr. H.W. Lee, Seoul, Korea, against a virus recently isolated by him, which is now considered on valid evidence to be the causal agent of Korean hemorrhagic fever. As shown in Table 41, the 4 sera known to have antibodies against Dr. Lee's agent reacted negatively with the Chlamydia antigen; serum #298 and one of the controls were anticomplementary and gave nonspecific reactions. A serological relationship between HFRS and Chlamydia infection was not confirmed by this test.

Table 40

Results of immunofluorescence and complement-fixation tests with serial sera from an individual exposed to Pichinde and Tacaribe viruses in the laboratory

Serum, bleeding date	Test, antigen, reciprocal of titer			
	IF		CF	
	Pichinde	Tacaribe	Pichinde	Tacaribe
6/4/75	0	0	Ac	Ac
10/27/75	0	0	0	0
4/8/76	0	0	0	0
11/19/76	0	16	0	4
12/7/76	0	16	0	4
10/12/77	16	4	8	2-4
6/20/78	8	4	8	2-4

0, negative reaction at dilution 1:2.
Ac, anticomplementary

Table 41

Complement-fixation test
Absence of relationship between hemorrhagic fever with renal syndrome (HFRS) and Chlamydia infection

Antigen	Serum, titer						
	Chla.	HFRS 285	HFRS 290	HFRS 294	HFRS 298	Control/US resident	Control/US resident
Chlamydia	1:256	0	0	0	1:16	0	1:8
Normal yolk sac	0	0	0	0	1:16	0	1:8
None, diluent	0	0	0	0	1:8	0	1:8

Antigens used in dilutions 1:32, 1:64 and 1:128, which for Chlamydia were equivalent to 8, 4 and 2 units, respectively.

Sera, first dilution 1:16 for Chla., 1:8 for the others.

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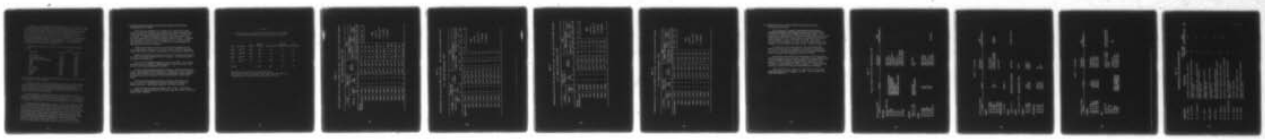

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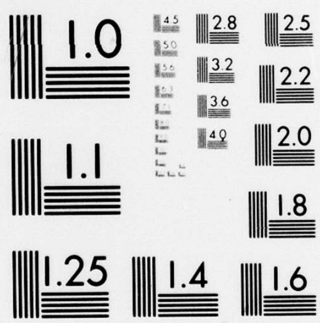

MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS-1963-A

La Crosse encephalitis in Westchester County, N.Y. (R.E. Shope, B.J. Beaty, and J. Casals). The Arbovirus Unit, as a service to the staff of Yale-New Haven Hospital, carries out diagnostic serology of encephalitis cases. Human cases of arbovirus encephalitis acquired in Connecticut have never been recognized. This year we came close; a 13 year old child from Pound Ridge, N.Y. (near Stamford, Connecticut) was hospitalized at Yale-New Haven Hospital on July 30, 1978 with fever and convulsions. He continued with convulsions, became comatose and his life was maintained by a respirator until August 30 when he died.

Complement fixation test of paired sera gave the following results:

Antigens	Sera	
	July 30	August 12
La Crosse	<1:4	1:16
Jamestown Canyon	<1:4	<1:4
California encephalitis	<1:4	<1:4
WEE	<1:4	<1:4
EEE	<1:4	<1:4
VEE	<1:4	<1:4
Powassan	<1:4	<1:4
St. Louis	<1:4	<1:4
Rabies	<1:4	<1:4
Herpes	<1:4	<1:4
LCM	<1:4	<1:4

Attempts to isolate La Crosse virus from post mortem brain in mice and mosquitoes were negative.

Epidemiological studies have been initiated in collaboration with Dr. Margaret Grayson of the New York State Health Department to survey for La Crosse antibody in family and neighbors, to survey small mammals, and to locate a possible focus of infected Aedes mosquitoes in Pound Ridge.

CF antibody to Jamestown Canyon virus was found in another patient from Meriden, Connecticut with encephalitis; however titers of 1:16 were present in both acute and convalescent sera, making it impossible to link the disease to Jamestown Canyon virus infection.

Arthrogryposis in man (R.E. Shope). Blood specimens were sent by Dr. Leslie Spence, Univeristy of Toronto, Ontario, Canada from a 5 month old child born with arthrogryposis (multiple rigid joint deformities), and from his mother and father. Because of the known occurrence of arthrogryposis in sheep and calves born of animals suffering Akabane virus infection during gestation, the sera were tested by HI with Simbu group antigens. Tests were negative with Aino, Akabane, Buttonwillow, Mermet, Nola, Oropouche, Sango, Sathuperi, Shamonda, Simbu, and Thimiri. Thus, no link in man between arthrogryposis and Simbu group virus infection was found.

V. Duration of yellow fever neutralizing antibody following 17D vaccination (W.G. Downs).

Sera from 134 World War II veterans were received from the Center for Disease Control, Fort Collins for yellow fever neutralization testing in a collaborative project with Drs. T. Monath and J. Poland. Serologic testing was done in both laboratories with the Yale laboratory not knowing the Fort Collins results. Although the vaccination records are not available on all of the veterans, they served in theaters of war in which all U.S. servicemen were expected to have been vaccinated.

Tests in mice were carried out in 6-week-old females by the i.c. route without addition of accessory factor; 61.6% of 125 sera showed protection or partial protection in the mouse test (Table 42).

Plaque reduction neutralization tests in Vero cells were carried out with virus doses between 21 and 77 plaques. Results are shown in Tables 43 through 46.

In ten of the negatives tested in Vero cell PRNT, 7 are clearly negative, 3 show a possible slight degree of plaque reduction, only in one (12142) approaching 50% reduction.

In the 20 sera tested which were scored as "partial protection" in mice the following 5 show evidence of significant plaque reduction: 12132, 12140, 12145, 12222, 12293; and an additional 5 show evidence of plaque reduction in region of 50%: 12138, 12144, 12178, 12190, 12247.

In the 10 sera tested which were scored as "protection" in mice, all can be considered as showing a significant degree of plaque reduction. Only #12148 might be considered marginal.

There was good correlation between the 2 tests. Over 50% of the veterans, vaccinated more than 30 years ago, maintained detectable yellow fever antibody.

Table 42

Results of mouse neutralization tests of U.S. Army W.W.II veterans with French neurotropic yellow fever virus

Date	Dilution	MLD_{50}	Number of Sera	Protection*	Partial protection	No Protection
1/16	$10^{-4.4}$	360	30	12	5	13
1/23	$10^{-4.4}$	630	30	8	9	13
1/30	$10^{-4.4}$	63	30	13	8	9
2/6	$10^{-4.5}$	31	35	19	3	13
Totals			125	52	25	48

*Protection = mortality ratio 0/6, 1/6, 1/5.
Partial protection = mortality ratio 2/6, 3/6, 4/6, 2/5, 3/5.
No protection = mortality ratio 5/6, 6/6, 4/5, 5/5.

Table 43

Neutralization Test in Mice and PRNT in Vero Cells. Sera from Fort Collins-WWII Veterans

(Y.F. immunized approximately, 1943)

In mice		Female mice 6 week old ♀ 6/box		Virus dilution		VERO						
						Number of Plaques						
						Serum dilution			Pre-control		Post-control	
Serum No.	Mouse Card No.	D	S	←	→	1:8	1:16	1:32	1:8	1:32	1:8	1:32
(Partial positives) 10657	12190	2	4	$10^{-4.4}$	$10^{-4.3}$	26	35	50	74	conf.	16	34
10661	12194	3	3	$10^{-4.4}$	$10^{-4.3}$	47	63	68				
10677	12198	2	4	$10^{-4.4}$	$10^{-4.3}$	40	60	69				
10678	12199	3	3	$10^{-4.4}$	$10^{-4.3}$	46	69	71	PRNT			
10679	12200	4	2	$10^{-4.4}$	$10^{-4.3}$	conf.	conf.	conf.	6-21-78			
10683	12222	2	4	$10^{-4.4}$	$10^{-4.3}$	18	61	79	Virus $10^{-4.3}$			
10692	12230	3	3	$10^{-4.4}$	$10^{-4.3}$	conf.	conf.	73	+ .75% BPA			
10721	12293	2	4	$10^{-4.5}$	$10^{-4.3}$	11	11	conf.	77 plaques			
10742	12314	2	4	$10^{-4.5}$	$10^{-4.3}$	42	69	conf.				
10743	12315	3	3	$10^{-4.5}$	$10^{-4.3}$	conf.	conf.	conf.				
10709	12247	2	4	$10^{-4.4}$	$10^{-4.3}$	29	53	68				
10704	12242	2	4	$10^{-4.4}$	$10^{-4.3}$	cont.	conf.	conf.				

Table 44

Neutralization Test in Mice and PRNT in Vero Cells. Sera from Fort Collins-WWII Veterans

(Y.F. immunized approximately, 1943)

In Mice		Female mice 6 week old 6 ♀ 6/box		Virus dilution		VERO						
						Number of Plaques						
						Serum dilution			Pre-control JL		Post-control HD	
Sera No.	Mouse Card No.	D	S	←	→	1:8	1:16	1:32	1:8	1:64	1:8	1:64
(Negative) 4961	12126	6	0	$10^{-4.4}$	$10^{-4.4}$	16	16	15	15	17	4	10
4963	12129	6	0	$10^{-4.4}$	$10^{-4.4}$	9	9	7				
4965	12130	6	0	$10^{-4.4}$	$10^{-4.4}$	9	11	12	PRNT			
4969	12134	6	0	$10^{-4.4}$	$10^{-4.4}$	11	18	12	5-24-78			
4970	12135	6	0	$10^{-4.4}$	$10^{-4.4}$	14	14	12	Virus $10^{-4.4}$			
4971	12136	6	0	$10^{-4.4}$	$10^{-4.4}$	14	10	12	+ .75% BPA			
4972	12137	6	0	$10^{-4.4}$	$10^{-4.4}$	11	11	8	21 plaques			
4977	12142	5	1	$10^{-4.4}$	$10^{-4.4}$	8	8	8				
4982	12146	6	0	$10^{-4.4}$	$10^{-4.4}$	9	10	9				
10635	12150	6	0	$10^{-4.4}$	$10^{-4.4}$	15	17	13				

Table 45

Neutralization Test in Mice and PRNT in Vero Cells. Sera from Fort Collins-WWII Veterans

(Y.F. immunized approximately, 1943)

In mice		Female mice 6 week old 6♀ 6/box		Virus dilution		VERO						
						Number of Plaques						
						Serum dilution			Pre-control		Post-control	
Serum No.	Mouse Card No.	D	S	←	→	1:8	1:16	1:32	1:8	1:32	1:8	1:32
(Partial positives) 4967	12132	3	3	$10^{-4.4}$	$10^{-4.3}$	12	14	24	61	59	9	26
4973	12138	2	4	$10^{-4.4}$	$10^{-4.3}$	23	33	44				
4975	12140	2	4	$10^{-4.4}$	$10^{-4.3}$	9	15	22				
4979	12144	2	4	$10^{-4.4}$	$10^{-4.3}$	22	32	49				
4980	12145	3	3	$10^{-4.4}$	$10^{-4.3}$	4	8	28				
10645	12178	4	2	$10^{-4.4}$	$10^{-4.3}$	33	46	44				
10652	12185	4	2	$10^{-4.4}$	$10^{-4.3}$	69	67	64				
10555	12188	4	2	$10^{-4.4}$	$10^{-4.3}$	62	60	62				

PRNT
6-14-78
Virus $10^{-4.3}$
+ .75% BPA
56 plaques

Table 46

Neutralization Test in Mice and PRNT in Vero Cells. Sera from Fort Collins-WWII Veterans

(Y.F. immunized approximately, 1943)

In mice		Female mice 6 week old 6 ♀ 6/box		Virus dilution		VERO Number of plaques						
						Serum dilution			Pre-control		Post-control	
Serum No.	Mouse Card No.	D	S	←	→	1:8	1:16	1:32	1:8	1:32	1:8	1:32
(Positives)4976	12141	1	5	$10^{-4.4}$	$10^{-4.3}$	2	3	8	70	conf.	24	53
4978	12143	1	5	$10^{-4.4}$	$10^{-4.3}$	20	51	57				
10632	12147	0	6	$10^{-4.4}$	$10^{-4.3}$	4	24	41				
10633	12148	0	6	$10^{-4.4}$	$10^{-4.3}$	33	62	67				
10634	12149	0	6	$10^{-4.4}$	$10^{-4.3}$	11	25	43				
10639	12154	1	5	$10^{-4.4}$	$10^{-4.3}$	14	20	51				
10640	12155	1	5	$10^{-4.4}$	$10^{-4.3}$	25	44	49				
10690	12228	1	5	$10^{-4.4}$	$10^{-4.3}$	16	32	35				
10715	12287	1	5	$10^{-4.5}$	$10^{-4.3}$	26	39	49				
10718	12290	0	6	$10^{-4.5}$	$10^{-4.3}$	5	11	13				

PRNT
6-29-78
Virus $10^{-4.3}$
+ .75% BPA
62 plaques

VI. Distribution of reagents, WHO Collaborating Centre for Reference and Research

Distribution of reagents, World Health Organization-Collaborating Centre for Reference and Research. (R.E. Shope, J. Casals, S. Buckley, A.J. Main, Jr., and O.L. Wood). The equivalent of 528 ampoules of arbovirus reagents were distributed from the WHO Centre to laboratories in 24 countries during the period January 1, 1978 to December 31, 1978. This total consisted of 231 ampoules of virus stock, 56 ampoules of virus antigen, and 241 ampoules of mouse ascitic fluid or immune sera.

Of the virus stocks distributed, this represented 149 different arboviruses; of antigens, 27 different arboviruses; and of sera, 73 different arboviruses (not counting individual viruses represented in polyvalent ascitic fluids).

During this same period, the equivalent of 183 ampoules of arbovirus reagents were referred to this Centre from laboratories in 10 different countries. The referrals consisted of 118 ampoules of virus specimens (Table 47), 22 ampoules of virus antigens, and 43 ampoules of immune reagents. In addition, 738 sera were received for arbovirus antibody survey testing.

Three different cell lines were distributed during 1977: Aedes albopictus, Aedes aegypti, and CER. The recipients are listed in Table 48.

Table 47

Viruses referred to YARU for identification, 1978

Country of origin; strain	Source	Information from donor	YARU identification
Australia			
Ngaingan MRM 14556		Ungrouped	
CS 110	*Culicoides brevitarsis*	Simbu group	
Ch 19520	*Culex annulirostris*	Ungrouped	
Ch 19546	*Culex annulirostris*	Turlock group ?	
CS 79	*Lasiohelea* sp.	Ungrouped	
CS 122	*Ixodes uriae*	Group B	
CS 132	*Culicoides brevitarsis*	Rhabdovirus	
CS 58		D'Aguilar-related	
CS 147		D'Aguilar-related	
CH 16287		Same as Ch 16313	
Belgium			
Dak An D 5314	*Tatera kempi*	Keuraliba	
Dak Ar B 2181	*Mansonia uniformia*	Yata	
Brazil			
Br H 4129	man	Simbu group	
Br H 4186	man	Simbu group	
Br H 2011	man	Simbu group	
Br H 10329	man	Guaroa?	
Br H 4185	man	Simbu group	
Br H 4327	man	Simbu group	Oropouche
Br Ar 263191		Palyam group?	

Table 47 continued

Country of origin; strain	Source	Information from donor	YARU identification
Brazil			
Be Ar 295042		Palyam group?	
Be Ar 327600		New group B	
Br H 10344	man	non-mouse pathogen	Candiru
Br H 9556	man	non-mouse pathogen	Candiru
Be An 238758			
Be Ar 293022		Arenavirus?	
Czechoslovakia			
Cz Arg 265	*Argas* sp.		
France			
Brest Ar T 13	*Ornithodoros maritimus*	ungrouped	Soldado-related
Greece			
Aedes virus, AP 82	*Rhipicephalus bursa*	Congo?	
Nigeria			
Ib H 75649	man	dengue-1	
Ib Ar 92039	mosquito	dengue?	
Ib Ar 92040	mosquito	dengue?	
Philippines			
Ph Ar 281	mosquito	JE	
Ph Ar 384	mosquito	JE	
Ph Ar 814	mosquito	Getah	
Ph Ar 1091	mosquito	JE	

Table 47 continued

Country of origin; strain	Source	Information from donor	YARU identification
Senegal			
Dak Ar 398	tick	Wad Medani?	
Dak Ar B 9005	mosquito	yellow fever	
Dak Ar B 8883	mosquito	yellow fever	
Dak Ar D 25865	mosquito	yellow fever	
Dak Ar D 25225	mosquito	yellow fever	
USA			
Ib An 57245		related to Orungo	Orungo-relationship
YV 177		related to Orungo	not confirmed by CF
Ug MP 359		Orungo	
O.cor. 76			
SV E 3		alphavirus	EEE
74-33702	*Aedes triseriatus*	La Crosse-like	
77-38299	*Aedes canadensis*	La Crosse-like	
76-M443	*Sigmodon hispidus*	California group	

Table 48

Distribution of Cell Cultures during 1978

Investigator	Institution	Singh's Aedes albopictus	Aedes pseudoscutellaris	CER
Dr. A. Fabiyi	University of Ibadan, Ibadan, Nigeria	X	X	
Dr. A.R. Filipe	Institute of Hygiene and Tropical Medicine Lisbon, Portugal	X		
Dr. M. Jozan-Work	c/o Dr. Stanley, Dept. of Microbiology University of Western Australia, Perth, Australia	X	X	
Dr. J. Kern	American Type Culture Collection Bethesda, Maryland	X	X	
David Lundino	W. Alton Jones Cell Science Center Lake Placid, N.Y.	X		
Carol Pacey	Dept. of Entomology, Waters Hall, Kansas State University, Mahattan, Kansas		X	
Dr. F. Pinheiro	Belem Virus Laboratory, Belem, Brazil	X	X	
Dr. A. Rudnick	University of Malaya, Kuala Lumpur, Malaysia		X	
Dr. P. Sinarachatanat	Dept. of Microbiology, Mahidol University Bangkok, Thailand	X		
Dr. O. Imami	Pristina, Yugoslavia			X
Dr. P. Sureau	Institut Pasteur, Teheran, Iran			X
Dr. B. Lachmi	Israel Institute for Biological Research Ness-Ziona, Israel			X

Table 48 continued

Distribution of Cell Cultures during 1978

Investigator	Institution	Singh's Aedes albopictus	Aedes pseudoscutellaris	CER
Dr. A. Fagbami	University of Ibadan, Nigeria			X
Dr. W. Neill	University of California, Kuala Lumpur, Malaysia			X
Dr. L. Hamde	Alexandria, Egypt			X
Dr. G. French	USAMRIID, Fort Detrick, Maryland			X
Dr. C. Hayes	University of Maryland, Lahore, Pakistan			X
Dr. J. Pilaski	Dusseldorf, Germany			X