Progr. med. Virol., vol. 19, pp. 257–323 (Karger, Basel 1975)

Growth Cycle of Arboviruses in Vertebrate and Arthropod Cells[1]

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Contents

1 The early volumes of Progress in Medical Virology are now out of print. Rather than reprinting the entire volume, certain timely articles have been selected for up-dating. The previous review on this subject was written by Dr. MANFRED MUSSGAY and appeared in volume 6.

I. Introduction

This present review is an attempt to bring up to date the article written on the same topic about ten years ago [MUSSGAY, 1964]. The division into the same sections as in the previous report is used, with the exception that a new section on 'Virus Growth in Cell Cultures of Arthropods' is included. Within some sections the subtitles have been changed. Only contributions of the past decade are considered; for references of earlier work the reader is referred to the first review. The great number of publications on the multiplication of arboviruses in different host systems did not allow a complete coverage of existing literature within the frame of this article, and therefore a certain selection of references had to be made.

II. Arbovirus Structure

The criteria for defining a given agent as an arbovirus have been discussed by CASALS [1971]. Only properties relating to the transmission cycle should be considered in answering the question of whether a virus is arthropod-borne or not. There are at present between 250 and 300 serotypes which, with various degreses of legitimacy are assembled in the arbovirus set. About four-fifths of the arboviruses are put together in antigenic groups on the basis of serological overlaps. This serological grouping reflects in some respect the heterogeneity in structure of arboviruses, but it also differentiates serological groups within a virus family with the same architecture. For understanding of the multiplication processes knowledge of the architecture of a virus is important, and therefore this section tries to redistribute arboviruses into a number of established groups and to give averages of information on their properties.

A. Togaviruses

Members of this family of 'classical' arboviruses are enveloped, single-stranded RNA viruses with cubic symmetry of their nucleocapsid. The family contains two genera named alpha- and flaviviruses (formerly group A and B arboviruses, respectively), which can be distinguished serologically. A list of the member viruses has been given by WILDY [1971].

The Eastern, Western and Venezuelan encephalitis viruses are the most prominent members of the genus alphavirus; Sindbis virus constitutes the type species but considerable information has been obtained for Semliki Forest and Chikungunya viruses. The name flavivirus was derived from yellow fever virus, which is the type species of the genus; other members studied are the dengue virus types, the Japanese, Murray Valley, St. Louis and tick-borne encephalitis viruses, and West Nile virus.

Alphaviruses are about 60 nm in diameter, flaviviruses are distinctly smaller (about 45 nm); this difference is also reflected by the sedimentation coefficients which range between 240 and 300S for alphaviruses and 170 and 220S for flaviviruses. The buoyant density depends on the gradient material used and varies between 1.18 (in sucrose) and 1.24 g/ml (in CsCl) for viruses belonging to both genera. In the electron microscope, togavirions appear as spherical particles consisting of an envelope and an isometric core. The envelope carries more or less prominent projections and displays 'unit mem-

brane' characteristics in thin sections. The core constitutes the viral nucleocapsid whose cubic symmetry has been proven for alphaviruses. A single nonglycosylated polypeptide with a molecular weight of 30,000–35,000 (alphaviruses) and 13,000–18,000 (flaviviruses) participates in the architecture of the nucleocapsid. The envelope of alphaviruses consists of probably two glycoprotein species with molecular weights >50,000 daltons. In flaviviruses an envelope polypeptide of a similar size has been found, but in addition a small protein of 7,700–8,700 daltons is present.

The arthropod-borne togaviruses agglutinate erythrocytes of a number of animal species under defined pH conditions. This technique is widely used for antigenic grouping. The structures responsible for hemagglutination in alphaviruses have been identified as the glycoprotein projections on the viral surface.

The RNA of togaviruses has a molecular weight of about 4×10^6 daltons and is essentially single-stranded and linear. It can be obtained in an infectious form, mild detergent treatment being sufficient for extraction. Within the nucleocapsid, the RNA is accessible to the action of ribonuclease.

While most members of the togavirus family known up to now are arthropod-borne, viruses with a similar architecture exist which spread conventionally; these include rubella, equine arteritis, bovine diarrhea, hog cholera and mouse lactic dehydrogenase viruses. Papers reviewing the structure and comparative aspects of arthropod-borne and nonarthropod-borne (nonarbo) togaviruses have been published recently [HORZINEK, 1973a, b].

B. Bunyaviruses

The term 'Bunyaviridae' has been informally proposed by MURPHY *et al.* [1973] to encompass more than 130 viruses, most of which were listed in the Bunyamwera serologic supergroup of the arboviruses. Although serologically unrelated to the supergroup, some additional viruses have been included into the bunyavirus group because of their similarity of morphology and morphogenesis. Members of this new taxon, which will possibly achieve family status [MURPHY, personal communication], are spherical, enveloped, single-stranded RNA viruses with helical symmetry of their nucleocapsid.

Bunyamwera virus has been proposed as the type species of the Bunyavirus genus; 12 definite and 18 presumptive genera have been listed (table I).

Bunyaviruses measure about 100 nm in diameter; sedimentation coefficients of 450S and 518S have been determined for Uukuniemi [PETTERSSON *et al.*, 1971] and Rift Valley fever virus [POLSON and STANNARD, 1970],

respectively. The buoyant density of Uukuniemi virus is 1.18 g/ml in sucrose and 1.20 g/ml in CsCl. Representative viruses of several of the member serogroups have been studied in the electron microscope. Because of the apparent fragility of the envelope, the roughly spherical shape of the virion is preserved only when the harsh treatment of pelleting and resuspension is avoided [POLSON and STANNARD, 1970] or after fixation [SAIKKU and VON BONSDORFF, 1968]. Projections are present on the viral envelope and are arranged in a hexagonal pattern; after spontaneous or detergent-induced disintegration of the envelope the nucleocapsid can be visualized. It consists of a loosely wound helix measuring about 9 nm in width and composed of one strand approximately 2 nm thick and more than 1 μm long [VON BONSDORFF *et al.*, 1969; SAIKKU *et al.*, 1971]. In Uukuniemi virus two polypeptides with molecular weights of 65,000–75,000 and of 25,000 daltons could be identified and correlated to the envelope and nucleocapsid, respectively [PETTERSSON *et al.*, 1971]; others [ROSATO *et al.*, 1973] reported three structural proteins with molecular weights of 83,000–85,000, 30,000–33,000 and 20,000–23,000 for five Bunyamwera supergroup viruses.

For about 80% of the bunyaviruses listed in the catalogue of arthropod-borne viruses [TAYLOR, 1971] pH dependent hemagglutination has been reported, although frequently at very poor titers. In analogy to myxoviruses, togaviruses, etc. the structure responsible for this activity can be expected to be the envelope surface projection.

When extracted with SDS and centrifuged in sucrose gradients, the RNA of Uukuniemi virus sedimented in two peaks at 21 and 27S; these peaks were rendered acid-soluble by pancreatic RNase. In the nucleoprotein strands obtained after detergent treatment of Uukuniemi virus, the RNA is almost completely protected from the action of the enzyme [PETTERSSON *et al,*. 1971].

All viruses of the Bunyamwera serologic supergroup appear to be mosquito-borne; serologically unrelated bunyaviruses have been isolated from *Phlebotomus* flies, ticks and mammals as well [TAYLOR, 1971]. It is anticipated that nonarbo bunyaviruses exist in nature.

C. Rhabdoviruses

The genus rhabdovirus [WILDY, 1971] includes more than 20 viruses of similar structure isolated from plants, arthropods and vertebrates. Rhabdoviruses are bullet-shaped, single-stranded RNA viruses with helical symmetry of their nucleocapsid.

Table I. The *Bunyaviridae* family[1]

Bunyavirus genus (members of Bunyamwera serologic supergroup 1–3; 25 representatives studied by electron microscopy)	Unnamed presumptive genus (not included in Bunyamwera serologic supergroup, but representative viruses of each group studied by electron microscopy)
1. Bunyamwera serogroup/16 members	1. Anopheles A serogroup/6 members
2. Bwamba serogroup/2 members	2. Anopheles B serogroup/2 members
3. C serogroup/11 members	3. Bakau serogroup/2 members
4. Capim serogroup/7 members	4. Bhanja virus/ungrouped
5. California serogroup/11 members	5. Congo serogroup/2 members
6. Guama serogroup/8 members	6. Ganjam serogroup/2 members
7. Koongol serogroup/2 members	7. Kaisodi serogroup/3 members
8. Patois serogroup/4 members	8. Lone Star virus/ungrouped
9. Simbu serogroup/16 members	9. Mapputta serogroup/2 members
10. Tete serogroup/3 members	10. Nairobi sheep disease virus/ungrouped
11. Olifantsvlei serogroup/2 members	11. Pacui virus/ungrouped
12. 6 unassigned viruses	12. Phlebotomus fever serogroup/16 members
	13. Rift Valley fever virus/ungrouped
	14. Tataguine virus/ungrouped
	15. Thogoto serogroup/2 members
	16. Turlock serogroup/4 members
	17. Uukuniemi serogroup/6 members
	18. Witwatersrand virus/ungrouped

1 From MURPHY *et al.* [1973]. The collaboration of Dr. R. E. SHOPE and Dr. J. CASALS of the Yale Arbovirus Research Unit/WHO International Reference Center for Arthropod-borne Viruses, New Haven, Conn., in preparing this listing, is gratefully acknowledged.

Vesicular stomatitis virus is the type species of the genus; according to their serological properties the vertebrate rhabdoviruses have been distributed into seven subgroups by MURPHY and SHOPE [1972].

Vertebrate viruses of this group have a diameter of about 70 nm and range from about 130 to 220 nm in length; infectious virions sediment at >600S. The buoyant density of vesicular stomatitis virus is 1.16–1.18 g/ml in sucrose and 1.20 g/ml in CsCl. The virus particles are bullet-shaped, i.e. cylindrical with one hemispherical and one planar end. The internal helical structure appears as a series of transverse striations in untreated particles. It is surrounded by a membranous envelope studded on its outer surface

with fine projections. It has been suggested that these projections interact with internal virus components. The structural and biological relations of virus components obtained after treatment with solvents and detergents have been reviewed by CARTWRIGHT *et al.* [1970]. Vesicular stomatitis virus contains three major proteins with molecular weights of 75,000, 57,000 and 32,500 daltons, which were shown to be subunits of the surface projections, ribonucleoprotein and a matrix protein, respectively. Different models have been proposed for vesicular stomatitis virus, and localization and interrelation of the structural proteins have been attempted [CARTWRIGHT *et al.*, 1972].

The presence of a hemagglutinin associated with intact virions was demonstrated for several rhabdoviruses. The optimum conditions for demonstration of this activity included low temperature, pH 6.2 and the use of goose erythrocytes.

From sedimentation data and electron microscopic measurements a molecular weight of $3.4–3.8 \times 10^6$ daltons has been estimated for the RNA of the virion; the lack of infectivity of extracted RNA is explained by the finding of a virion-associated polymerase [BALTIMORE *et al.*, 1970].

The arthropod-borne rhabdoviruses have been isolated from mosquitoes, *Phlebotomus* flies and mites [TAYLOR, 1971]. The most prominent non-arbo member of the group is rabies virus. Marburg virus, whose structure is comparable but not identical to that of rhabdoviruses, is conceivably arthropod-borne, since multiplication in mosquitoes has been observed under laboratory conditions. Reviews on vesicular stomatitis and related viruses have been presented by HOWATSON [1970] and on Marburg virus by SIEGERT [1972].

D. Picornaviruses

Presently, Nodamura virus is the only arthropod-borne representative of the family *Picornaviridae* [MURPHY *et al.*, 1970b]; these are small, naked, isometric, single-stranded RNA viruses with icosahedral capsid symmetry. Nodamura virus appears serologically unrelated to other picornaviruses [SCHERER and HURLBUT, 1967].

In thin sections through infected cells and negatively stained preparations the virion had a diameter of 28–29 nm; it sedimented at 135S in sucrose gradients. The buoyant density in CsCl was 1.34 g/ml. Electron microscopically the virus was indistinguishable from picornaviruses. Preliminary evidence suggests that there are three structural polypeptides, the major one

having a molecular weight of 45,000 daltons [MURPHY *et al.,* 1970b; NEWMAN and BROWN, 1973]. The most significant finding is the observation that Nodamura virus contains two species of RNA sedimenting at 22S and 15S, respectively, which may be present in two distinct particle species. Each RNA species had low infectivity in mice but this was enhanced about 100-fold by mixing them.

In these properties Nodamura virus differs from all the small RNA viruses of vertebrates and invertebrates so far described. Its tentative classification as an enterovirus [WILDY, 1971] must be reconsidered when more data become available. In its protein composition the virus appears to resemble members of the genus Calicivirus [NEWMAN and BROWN, 1973]. The comparative virology of the small RNA viruses has been reviewed recently by BROWN and HULL [1973].

E. Diplornaviruses

Certain arboviruses including African horse sickness, bluetongue, Colorado tick fever, epizootic hemorrhagic disease of deer, Kemerovo and about 25 others constitute a new taxonomic group [BORDEN *et al.,* 1971; MURPHY *et al.,* 1971a]; its members share the basic properties of a double-stranded RNA genome in several segments, cubic capsid symmetry and lack of an envelope. In these properties they resemble reoviruses. However, the bluetongue-like viruses (for which the term orbiviruses has been coined) and reoviruses are considered as genera of the diplornavirus taxon, for which the rank of a family has been assumed [MELNICK, 1973]. A common orbivirus antigen is lacking, but ten serologically distinct subgroups could be established on the basis of cross-complement fixation tests [BORDEN *et al.,* 1971]. Within the bluetongue-like viruses 16 and within the African horse sickness viruses 9 antigenic types have been identified [VERWOERD, 1970; HOWELL and VERWOERD, 1971]. Hemagglutination has been observed only incidentally.

Viruses of this group measure between 50 and 80 nm in diameter. For bluetongue virus a sedimentation coefficient of 650S and a buoyant density of 1.38 g/ml in CsCl has been estimated [VERWOERD, 1969]. Using the electron microscope, evidence for a common icosahedral virion construction has been accumulated; 32 capsomeres arranged in a single-layered capsid showing a T = 3 surface lattice have been identified [MURPHY *et al.,* 1971a]. Morphologically, orbiviruses thus differ clearly from reoviruses, for which double-

layered capsids and different numbers of capsomeres have been determined [VERWOERD, 1970]. Seven polypeptides were found to be present in the capsid of bluetongue virus, which on the basis of their respective molecular weights could be correlated to certain genome segments. The double-stranded RNA occurs in 10 discrete pieces which amount to a total of 15×10^6 daltons for the viral nucleic acid [VERWOERD *et al.*, 1970, 1972].

A review on diplornaviruses has been published by VERWOERD [1970] and on bluetongue virus by HOWELL and VERWOERD [1971].

This concludes the series of structural groups of RNA viruses for which arthropod-borne members have been identified. Only recently, DNA viruses have attained the attention of arbovirologists. African swine fever virus, a probable member of cytoplasmic DNA viruses or 'iridoviruses' [WILDY, 1971] has definitely been shown to multiply in an argasid tick species [PLOWRIGHT *et al.*, 1970]. Cotia virus, which was isolated from mosquitoes in Brazil, has been observed to have a virion morphology similar to members of the pox-virus group [BORDEN, SHOPE and MURPHY, cited in CASALS, 1971]; because of the lack of data it will not be considered further.

F. Iridoviruses

African swine fever virus is an enveloped icosahedral DNA virus having a diameter of 175–215 nm. The virion consists of a dense nucleoid surrounded by an electron lucent area and enclosed in a distinct hexagonal outer shell. The DNA nature of the viral genome has been proven by extraction of an infectious nucleic acid and specific enzyme treatment [ADLDINGER *et al.*, 1966]. African swine fever virus up to now is the only arthropod-borne as well as the only mammalian iridovirus; *Ornithodorus moubata* ticks appear to play a predominant role in epizootiology of the disease. The literature on African swine fever virus has been reviewed by HESS [1971].

G. Comments

Summarizing this section, it can be said that since the appearance of the first article progress has been made in the knowledge of the structure of viruses included in the arbovirus group. It has become evident that the term arbovirus must be used to designate an epidemiological behavior which is irrelevant to the criteria on which current general systems of viral classification

are based [CASALS, 1971]. The arbovirus set must be disassembled and individual viruses distributed among the pertinent taxons of the system. However, one should keep in mind that information concerning the essential properties for classification in a general system – type of nucleic acid, capsid symmetry and envelope – is only available for less than 10% of the arboviruses.

III. Virus Growth Cycle in Tissue Cultures of Vertebrate Cells

A. Susceptibility of Cells and Viral Cytopathic Action

The Appendix of the previous review [MUSSGAY, 1964] shows that many of the arboviruses grow in a wide variety of cell systems. Primary cell cultures of green monkey, hamster kidney, chick embryo and human amnion as well as continuous cell lines like Vero, BHK, L, and HeLa support growth of various arboviruses. Several additional reports have been given on this subject.

It was demonstrated that growth of Venezuelan equine encephalitis virus resulted in higher virus yield in roller cultures than in suspension cultures of chick embryo cells [NOVOKHATSKII and ERSHOV, 1970]. The replication of some flaviviruses (dengue, tick-borne encephalitis, yellow fever, St. Louis encephalitis virus) in cultures of primate and porcine origin was described by several authors [PAUL, 1968; STIM and HENDERSON, 1966; BOGOMOLOVA *et al.*, 1971; BRES *et al.*, 1971; VILAS, 1965; EMEL'YANOV and MONASTYREVA, 1969]. Reproduction of St. Louis encephalitis virus was achieved in leukocyte culture [BARINSKY *et al.*, 1968]. Rhabdoviruses seem to have the broadest host spectrum. Cells of human, primate and porcine origin support the growth of vesicular stomatitis and Marburg viruses [HOFMANN and KUNZ, 1968; MAASS *et al.*, 1969; PIRTLE *et al.*, 1970]. Vesicular stomatitis virus was also grown in several white blood cell types [BAUMBERGER, 1970; EUSTATIA and VAN DER VEEN, 1971]. Human lymphoblasts were capable of replicating vesicular stomatitis virus [WALLACE, 1969]. Each major leukocyte type of the peripheral blood of healthy donors was studied *in vitro* for its ability to support vesicular stomatitis virus replication. In these studies, monocytes and macrophages were found to be the principle host cells [EDELMAN and WHEELOCK, 1967].

The application of other cell systems from warm-blooded animals for the propagation of RNA-containing arboviruses has been described. Vene-

zuelan equine encephalitis virus was reported to grow in embryonic cell cultures of wild birds [DICKERMAN and BONACORSA, 1972] and also in the human diploid cell strain WI–38 [REITMAN and GREEN, 1970]. Recently, some reports on propagation of arboviruses in vertebrate cells other than mammalian and avian have appeared. The culture of cells from poikilothermic animals offers a unique opportunity to study the growth of viruses of homeothermic vertebrates (e.g. arboviruses) in cells of hosts far removed phylogenetically from the normal hosts and at temperatures below those of their normal hosts. OFFICER [1964] described the replication of two alphaviruses (Venezuelan and Eastern equine encephalitis) in a fish cell line from rainbow trout, and KROEKER and BIRD [1965] showed that a cell line from the goldfish, *Carassius auratus*, was capable of supporting the growth of the alphavirus Sindbis. Two established cell lines, derived from the embryonic tissues of chinook salmon and from steelhead trout, are able to support the replication of Western equine encephalitis virus with a resulting virus yield at least as high as that found in a culture of chick embryo cells [NIMS *et al.*, 1970]. Two other viruses, Sindbis and vesicular stomatitis, replicated in a cell line derived from fathead minnows [SOLIS and MORA, 1970]. Primary cell cultures obtained from tadpoles of *Rana temporaria* supported the growth of three tick-borne arboviruses, namely Quaranfil, louping ill and Langat [PUDNEY and VARMA, 1971]. CLARK [1972] grew Lagos bat virus, a rhabdovirus, in two cell lines of reptilian origin. Yields of released virus obtained in a cell line from *Vipera russeli* after the first passage were similar to those obtained in BHK cells. First passage yields of Lagos bat virus in a cell line from *Gekko gecko* were slightly less than the yields obtained in BHK cells. The growth of arboviruses in arthropod cells will be discussed in section IV.

As mentioned in the previous report, different arboviruses may vary in the intensity of their cytopathic action; for example, some alphaviruses cause a marked cytopathic effect in chick embryo cells which also support the growth of many tick-borne encephalitis viruses but without an accompanying cytopathic effect. Even related viruses may have a different behavior in this regard. Three viruses of the Phlebotomus fever group – Chagres, Sicilian and '1–81' – multiplied in hamster embryo cells with the appearance of a cytopathic effect, whereas another virus belonging to this group – Naples – replicated without producing a cytopathic effect [SALIM, 1968b]. Also, it is possible that a certain arbovirus strain propagates in a cell species without causing a cytopathic effect, whereas growth of this virus in another cell system with comparable titers results in cell destruction. This was demonstrated by EMEL'YANOV and MONASTYREVA [1969]: St. Louis encephalitis

virus grew to high titers in cultures of human embryo fibroblasts, BHK, chick embryo, and HeLa cells, but a cytopathic effect was manifested only in chick embryo cells. Louping ill virus multiplied in calf kidney cells, human embryonic kidney cells, human amnion cells, elk testis and kidney cells without cytopathic changes, whereas in ERK and HeLa cells virus multiplication caused cell destruction [KÄÄRIÄINEN, 1965]. Freshly isolated virus strains in nature may differ from laboratory strains with respect to cytopathogenic activity when grown in different cell cultures. DUBBS and SCHERER [1969] described serial passages of Japanese B encephalitis virus in L cells which produced a variant virus with enhanced cytopathic potency. The multiplication of Tahyna virus, belonging to the bunyavirus group, was tested in eight different cell lines including heart, kidney, liver and lung. A cell line derived from rabbit lung proved to be most susceptible to Tahyna virus. Pig kidney cells were found the least susceptible [SEFCOVICOVÁ, 1965a]. Reproduction of tick-borne encephalitis virus was studied in pig embryo and Hep-2 cell cultures. It was shown that the maximal number of pig embryo cells participating simultaneously in virus synthesis was approximately 20% more than that of Hep-2 cells. However, production of infectious virus was more intensive in Hep-2 cells than in pig embryo cells [BOGOMOLOVA *et al.*, 1971].

Cytopathic potency of a virus may be enhanced or decreased after several passages not only in a cell system but also in animals. A neuro-adapted strain of Tahyna virus exhibited a marked cytopathic effect in primary hamster kidney cells, whereas an extraneural variant which was passaged by extraneural inoculation of viremic blood multiplied in the same cell cultures without regular cell degeneration in the first passage [SEFCOVICOVÁ, 1965b]. KÄÄRIÄINEN [1965] reported that a freshly isolated strain of tick-borne encephalitis virus produced marked cytopathic alterations during the first ten successive passages in HeLa cells; when further passages were made, the cytopathic effect was diminished.

Environmental conditions prove to be of importance for yield of virus and to some degree for establishing a cytopathic effect. TRIBBLE *et al.* [1971] investigated the replication of Venezuelan equine encephalitis virus in various mammalian cells propagated in serum-free and chemically defined media. Since media containing lactalbumin hydrolysate and serum supported virus growth to higher yields than chemically defined medium, it was suggested that cell nutrition plays a major role in enhancing virus replication. An attempt was made by HEARN *et al.* [1971] to identify nutritional additives that influence the replication of Venezuelan equine encephalitis virus in

suspension cultures of L cells grown in a defined, serum-free medium.

Proline, serine and choline enhanced the titer of a virulent virus strain. ZWARTOUW and ALGAR [1968] used freshly prepared suspensions of chick embryo cells in concentrations up to 10^8 cells/ml to propagate Semliki Forest virus with yields of 10^{11} PFU/ml of medium. The cell suspensions required no nutrient other than glucose and the cells were suspended in Earle's saline. Incubation at 31 °C and maintenance of a pH between 7 and 7.4 was necessary. Tissue cells cultured *in vitro* have a high glycolysis rate compared with cells freshly prepared from tissues; therefore, the latter offers the possibility of cultivating high concentrations of cells [PAUL, 1965]. ZASUKHINA and MARININA [1969] found a prolonged replication period of Chikungunya virus in several-day-old chick embryo cells as compared with young cultures.

The plaque test is frequently performed with arboviruses. Carboxymethylcellulose, starch or agarose are often used instead of agar, which contains sulfated polysaccharides highly inhibitory to certain arboviruses. Others, notably the flaviviruses, do not produce cytopathic alterations when susceptible cells are incubated under fluid media, but produce plaques when the same cells are incubated under solid media. Only the more rapidly cytolytic members appear to produce plaques regularly. Plaque characteristics of 52 arboviruses were described by BERGOLD and MAZZALI [1968]. Recently, plaque assay and plaque neutralization in a stable line of porcine kidney cells were described for several flaviviruses, especially members of the dengue and St. Louis complex [WESTAWAY, 1966]. The conditions for plaque formation with a sandfly fever virus were described by SALIM [1968a]. The Naples strain multiplies in hamster embryo cells without producing a cytopathic effect; plaques appear only when hamster embryo cells are infected in suspension and seeded in Petri dishes to form a monolayer under carboxymethylcellulose. The influence of several known virus plaque-enhancing compounds on plaque development of all dengue virus serotypes in simian kidney cell lines was studied by STIM [1970]. Plaques appeared sooner and/or were increased in size if heparin, protamine sulfate, DEAE-dextran, actinomycin, pancreatin or glutamine were used as additives in the solid overlay. In another report, the addition of protamine sulfate or DEAE-dextran increased the titer and plaque size of Tahyna virus under agar and agarose overlay [MÁLKOVÁ, 1971a]. It had already been demonstrated earlier that the plaque inhibition of agar can be partially overcome by the addition of polycations such as DEAE-dextran or protamine sulfate. However, it has to be kept in mind that the addition of these compounds may cause undesirable effects, since protamine has been reported to inhibit virus-cell interactions

[COLTER *et al.*, 1964], and DEAE-dextran has been shown to enhance interferon production [DIANZINI *et al.*, 1969; VILČEK *et al.*, 1972]. Additional mechanisms may be involved in the action of DEAE-dextran [WALLIS and MELNICK, 1968].

Cytopathic lesions produced by RNA-containing arboviruses are characterized by nuclear and cytoplasmic alterations. In the nucleus, margination of chromatin, vacuolization of the nucleoplasm, disappearance of nucleoli and pycnosis are observed. The cytoplasm is contracted and cystic transformation and accumulation of basophilic substances occur. However, arboviruses do not produce alterations in infected cells distinguishable from those of nonarboviruses of the same family.

B. Synthesis of Virus Particles

Most of the studies dealing with the biochemical events occurring in infected cells have been performed with members of the togavirus family. Therefore, results obtained with these viruses will be discussed in detail and only general aspects will be given for rhabdo- and diplornaviruses.

1. Adsorption and Penetration

Kinetics of adsorption have been described in the previous review [MUSSGAY, 1964]. Little is known about whether receptors on the surface of susceptible cells are responsible for virus attachment. Yet, since arboviruses, and mainly alphaviruses, are capable of infecting and proliferating in a variety of cell cultures derived from phylogenetically unrelated species including mammalian, avian, reptilian, amphibian and piscine, one can speculate that these viruses either have a broad range of receptors, or receptors widely common in nature, or that they do not require receptor sites to adsorb onto and penetrate into cells. HAHON and COOKE [1967] assumed that the requirements for virus attachment are virus-specific and not cell-specific. The adsorption of an alphavirus, Venezuelan equine encephalitis virus, onto cells was highly specific in its salt requirement, in that maximal binding occurred only in the presence of defined concentrations of NaCl. The presence of divalent cations in the medium appeared to inhibit virus attachment. This salt requirement for adsorption of alphaviruses may be a group characteristic because maximal attachment of yellow fever virus (flavivirus group) onto cells occurred in the presence of divalent cations [HAHON and COOKE, 1967]. The salt requirement, in addition to the evidence of pH dependence and tempera-

ture independence of the adsorption of virus onto cells, indicates that the initial union involves electrostatic forces. The nucleocapsid component of Sindbis virus particles is unable to attach to host cells [BOSE and SAGIK, 1970; DOBOS and FAULKNER, 1970]. These results emphasize the role of the envelope protein in the interaction between enveloped arboviruses and cells. Recent experiments have shown that the structural phospholipids of Semliki Forest virus envelope, although necessary for the structural integrity of the virion, are not necessary for virus infectivity [FRIEDMAN and PASTAN, 1969].

After attachment, Venezuelan equine encephalitis virus remains at the cell surface for several minutes, where it is susceptible to antiserum [HAHON and COOKE, 1967]. Virus penetration proceeds as a first order reaction and is completed after approximately 30 min at 35 °C. In contrast to the temperature independence of the attachment reaction, the penetration of virus is temperature-dependent. Virus penetration is optimal at 37 °C and minimal at 4 °C. In addition, penetration of Venezuelan equine encephalitis virus was independent from the ionic content of the medium [HAHON and COOKE, 1967].

Two different modes of action are supposed for penetration of vesicular stomatitis virus. Electron microscopic studies by HEINE and SCHNAITMAN [1971] have indicated that vesicular stomatitis virus enters cells by fusion of the viral and host membranes followed by release of the nucleocapsid directly into the host cell cytoplasm. This fusion occurs within 2–4 min at 37 °C. In contrast, SIMPSON *et al.* [1969] observed vesicular stomatitis virus only in phagocytic vacuoles and suggested that penetration takes place by phagocytosis followed by intracellular uncoating of the virus. Early events observed by electron microscopy during bluetongue virus infection are described by several authors [CROMACK *et al.*, 1971; LECATSAS, 1968]. Adsorbed virus was seen on the cell surface 10 min after inoculation. Phagocytic vesicles containing virus particles were observed during the next 30 min, and finally virus nucleoids or complete particles were seen within lysosomes between 30 min and 2 h postinfection.

2. Replication of RNA

The nucleic acid of alphaviruses serves as both genome and messenger RNA (mRNA). The data which indicate that the viral RNA functions indeed as a messenger depend primarily upon the fact that RNA extracted from virus particles is infectious [for references see MUSSGAY, 1964]. The start of RNA replication requires a step involving the synthesis of protein coded by

the viral genome, since the entry of input RNA into a replicative structure is prevented by inhibition of protein synthesis [FRIEDMAN *et al.*, 1967; FRIEDMAN and SREEVALSAN, 1970; SCHEELE and PFEFFERKORN, 1969]. It seems that RNA polymerase is the product synthesized during these early events in virus replication, since this enzyme is the first virus-coded protein required for initiation of RNA replication. Later in infection, however, RNA synthesis becomes independent of protein synthesis [SCHEELE and PFEFFERKORN, 1969; FRIEDMAN and GRIMLEY, 1969]. The infecting virion RNA serves as a messenger at least in some form early during infection; FRIEDMAN *et al.* [1967] reported that the RNA from the infecting virus was converted from a 42S to a 26S form within 30 min after infection. Therefore, it remains to be decided whether the whole viral genome or only part of it is translated during the early phase of infection.

The infecting RNA rapidly becomes associated with membranous cytoplasmic structures. It seems that synthesis of alphavirus RNA occurs at these sites; during this process, replicative forms (RF) or replicative intermediate (RI) forms can be isolated [for definition of RF and RI the reader is referred to BISHOP and LEVINTOW, 1971]. RF and RI are the first newly synthesized RNAs which can be detected in infected cells [FRIEDMAN, 1968a; SREEVALSAN and YIN, 1969; KÄÄRIÄINEN and GOMATOS, 1969; FRIEDMAN and SREEVALSAN, 1970]. Viral RNA polymerase and nascent viral RNA have been recovered with membrane fractions of infected cells [MARTIN and SONNABEND, 1967; GRIMLEY *et al.*, 1968; SREEVALSAN and YIN, 1969].

Besides RF and RI – which will be discussed below – single-stranded viral-specific RNA components appear in virus-infected cells. LEVIN and FRIEDMAN [1971] identified four forms of single-stranded viral RNA in alphavirus-infected cells: a 42S form, the only form present in the virion, and a 38S, a 33S and a 26S RNA which were assumed to be mRNAs of alphaviruses. It remains to be determined whether the latter form (26S) is identical to the 26S form called interjacent RNA [SONNABEND *et al.*, 1967] which is detected early in infection, possibly as a result of the breakdown of the infecting 42S RNA [FRIEDMAN *et al.*, 1967], and which was recently shown to contain one-third of the base sequences of the RNA of the virus particle [SIMMONS and STRAUSS, 1972a]. The messenger nature of 33S and 26S RNA was confirmed by studies of KENNEDY [1972] and MOWSHOWITZ [1973] who isolated these RNA species from membrane-bound polyribosomes obtained from chick embryo cells infected with Semliki Forest virus. Other authors [ROSEMOND and SREEVALSAN, 1973] isolated a 28S and a 15–18S RNA component associated with ribosomes; these forms of mRNA may be identical to 26S

and 15S RNA containing poly A segments. The presence of adenylate-rich sequences in Sindbis virus RNA was described [JOHNSTON and BOSE, 1972]. KÄÄRIÄINEN and GOMATOS [1969] identified RNA components sedimenting at 42S, 26S and 22S. The same species are synthesized in an *in vitro* system with an enzyme isolated from cells infected with Semliki Forest virus [MICHEL and GOMATOS, 1973].

Besides these single-stranded RNA forms, synthesis of double-stranded or partially double-stranded RNA occurs in alphavirus-infected cells. These components represent forms of RF and RI. Several years ago a 20S RF with double-stranded regions was identified [FRIEDMAN *et al.*, 1966; PFEFFERKORN *et al.*, 1967; MARTIN and SONNABEND, 1967; FRIEDMAN and BEREZESKY, 1967]; this RF was also synthesized *in vitro* [MARTIN and SONNABEND, 1967]. This RF is analogous to the RNase-resistant core produced by RNase treatment of RI, and it was assumed that RF originates as an artefact during the extraction procedure [WEISSMANN *et al.*, 1968]. However, the possibility that RF is a 'dead' end product derived from RI after exhaustion of the RNA polymerase was also discussed [GIRARD, 1969]. RI reveals a polydisperse nature in sucrose density gradient sedimentation [FRIEDMAN, 1968a; SREEVALSAN and YIN, 1969]. By improving the analytical methods, various kinds of RF were described in the meantime and shall be considered in detail in connection with RI.

At least two kinds of RF occur *in vivo*, whereas in an *in vitro* reaction four forms could be identified after ribonuclease treatment [LEVIN and FRIEDMAN, 1971]. STOLLAR *et al.* [1972] described three distinct forms of double-stranded RNA with sedimentation coefficients of 20S, 15S, and 12S, which were all found in infected chick embryo cells; in contrast to this cell system the authors detected only 20S and 12S RNA in infected BHK cells, and 20S RNA and only small amounts of double-stranded RNA smaller than 20S in infected mosquito cells.

In a detailed study SIMMONS and STRAUSS [1972b] reported the existence of three species of RF and determined their sedimentation values to be 23.5S, 20S and 16S. The authors presented a model for the replication of Sindbis virus RNA in which the existence of two different types of RI is postulated. They are designated as RIa and RIb. Both RI forms use complementary (minus) strands of different lengths. No explanation is given as to the mode of origin of the complementary (minus) strands. Virion RNA originates at RIa. Single-stranded RNA forms of smaller size classes grow out of RIb, indicating the existence of a mechanism which prevents the replicase enzyme from reading the minus strand throughout. It may be that there are two

different replicases which differ in their ability either to read the whole template, or to understand a stop signal. Therefore, the two RI forms are convertible according to the enzyme which is reading them. As a mean product, the 26S RNA, known as interjacent RNA, originates from the RIb. Probably this RNA and a somewhat larger one, which arises in much smaller amounts and may be identical with the 33S RNA detected by LEVIN and FRIEDMAN [1971], serve as messenger during infection. This mechanism allows the synthesis of large amounts of 26S RNA, which contains only one-third of the base sequences of the virion RNA, during the middle of the infection cycle and that of virion RNA near the end of the cycle. The control mechanism which activates the synthesis of only a part of the viral genome is unknown. The existence of additional single-stranded RNA species detected by several authors (see above) cannot be explained with this model. Digestion with RNase of RIa and RIb gives rise to the three detected species of RF, because the termination point of the replicase in RIb is susceptible to RNase, therefore leaving behind two double-stranded RNA components unequal in size, which are called RF II and RF III (20S and 16S, respectively). RF I (23.5S) originates from RIa and has the full length corresponding to virion RNA.

According to the concept of WEISSMANN *et al.* [1968], who have stated that in the replication structure the complementary strand is associated with one or more plus strands of varying length but is largely present in single-stranded form inside the cell, the different species of RF in the model of SIMMONS and STRAUSS [1972a, b] derive from corresponding RI after extraction and RNase treatment, whereby fully double-stranded molecules with single-strand breaks are produced. Except for the unknown mode of origin of the complementary minus strand there is no real double-stranded form involved in the replication cycle. In some contrast to this, MICHEL and GOMATOS [1973] found strong evidence that RI and different species of RF were precursors of the single-stranded RNA. Pulse-chase experiments revealed that the amount of pulse-labeled RNA species remaining in RF and RI with the enzyme-template complex decreased during the subsequent chase in an amount equivalent to that appearing in single-stranded RNA components. Moreover, more than 95% of the RNase-resistant RNA were perfect duplexes. The RFs were represented by at least two forms with molecular weights of 4.4×10^6 and 5×10^6. No RF belonging to a larger size class was found. Less than 5% of the RNase-resistant RNA components was present in an RI. The core of the RI resistant to RNase had a molecular weight of 5.6×10^6. Therefore, it was concluded that the detected RF species did not

derive from RI after RNase digestion, and are not 'dead' end products. However, it remains possible that RF species actually exist as RI forms, but with only one nascent RNA chain held in place by a short region of hydrogen bonding. The authors were unsuccessful in finding RF or RI composed of the full length comparable with virion RNA. Provided that the 42S virion RNA is a single polynucleotide chain as reported [ARIF and FAULKNER, 1972; SIMMONS and STRAUSS, 1972a], it must be derived from more than one RI or RF, respectively. In this regard a polynucleotide chain ligase, like that found during bacteriophage replication [SILVER *et al.*, 1972], is necessary. The observation of FRIEDMAN *et al.* [1967] that the RNA from the infecting virus was converted from a 42S to a 26S form within 30 min after infection fits into the consideration that the genome consists of distinct pieces which are covalently linked in the virion. Another possibility would be that there exists more than one initiation site on the virion RNA for the synthesis of several distinct complementary RNA strands containing only part of the genome. It cannot be ruled out that at least part of the structures found by MICHEL and GOMATOS [1973] are involved in the synthesis of the complementary minus strand of RNA. There was no explanation for the origin of this kind of RNA in the study of SIMMONS and STRAUSS [1972a, b].

At present it is not possible to make a final statement on the real function of RF in alphavirus replication.

In the course of replication of flaviviruses a similar pattern of newly synthesized RNA as in alphavirus-infected cells appears. Two equally prominent peaks of RNA were found during replication of dengue 2 virus which represent the virion RNA (45S) and the RF (20S) [STOLLAR *et al.*, 1967]. RNA of the 26 to 28S variety was present only in very small amounts. Both strands of the RF are synthesized prior to 12 h after infection. At later times, however, only the viral or positive strand of the RF appears to be synthesized. Similar results were found in cells infected with Japanese B encephalitis virus [ZEBOVITZ *et al.*, 1972], with the exception that an additional RNA with a sedimentation coefficient of 8S was detected. The RNA synthesized by St. Louis encephalitis virus included, besides the 43S virion RNA and the 20S double-stranded form, a 26S species in greater amounts as determined during replication of dengue 2 virus [TRENT *et al.*, 1969]. The 26S RNA, however, was found to be relatively resistant to ribonuclease. Viral RNA polymerase has been detected in cells infected with St. Louis encephalitis virus [QURESHI and TRENT, 1972]. Yet CARDIFF *et al.* [1973] described a lack of identifiable 40S RNA in the *in vitro* polymerase product, suggesting that the enzyme system has special requirements for the production of virion RNA.

It may be concluded from these data that there exist no fundamental differences in the mechanisms by which alpha- and flaviviruses multiply.

The RNA synthesized after infection with a rhabdovirus, vesicular stomatitis virus, has been described in a recent review [HOWATSON, 1970]. Biochemical studies on the replication of this virus are complicated by the fact that crude virus preparations contain not only bullet-shaped infectious B particles but also defective T particles which interfere specifically with the growth of B particles [HUANG and WAGNER, 1966a]. HOWATSON [1970] mentioned the presence of the following species of single-stranded RNA: 38S (virion B), 31S, 26S (virion T), 21S, 15S, and 13S; and double-stranded RNA: 13–15S, 19–20S and 13–50S which represents the RI. Details, however, vary among different laboratories [SCHAFFER and SOERGEL, 1972; WILD, 1971; NEWMAN and BROWN, 1969; STAMPFER *et al.,* 1969]. The 15S and 13S RNAs seem to be mRNA since these species are found in association with polysomes. MUDD and SUMMERS [1970b] and WILD [1971] fractionated the 15S region on polyacrylamide gels and found multiple species. In addition, a 28S RNA also revealed messenger activity [HUANG *et al.,* 1970; HUANG and MANDERS, 1972]. The lack of infectivity of RNA isolated from the virions [HUANG and WAGNER, 1966a], which contrasts with the readily demonstrable infectivity of RNA from arboviruses of the togavirus family, led to the detection of an RNA polymerase which is inside the vesicular stomatitis virion and which uses the virion RNA as a template for the synthesis of complementary RNA [BALTIMORE *et al.,* 1970]; consequently this enzyme can be described as an RNA transcriptase. This transcriptase is responsible for repetitive transcription of the virion genome. The transcription process is not only complete but also sequential [BISHOP, 1971; ROY and BISHOP, 1972]. Since the mRNA of vesicular stomatitis virus in infected cells is also complementary to the virion genome [HUANG *et al.,* 1970; MUDD and SUMMERS, 1970b], presumably the function of the polymerase is to supply an infected cell with these mRNA species by transcription of the virion RNA. By convention, mRNA is described [SUBAK-SHARPE, 1971] as the plus strand so that the virion RNA is thereby designated as a minus strand. During the process of RNA replication two enzymes, a transcriptase and a replicase, are responsible for supplying all types of RNA needed for rhabdovirus multiplication.

Only a few reports exist on the replication of bluetongue virus RNA. All 10 genome segments of bluetongue virus, a diplornavirus, are transcribed after infection into single-stranded mRNA species but the products are not formed in equimolar amounts. Therefore, specific regulation at the level of

transcription is indicated which is an integrated part of the viral function since the *in vivo* control of transcription is the same as the *in vitro* control [HUISMANS, 1970; HUISMANS and VERWOERD, 1973].

3. Synthesis of Viral Protein

The first product of virus-directed macromolecular synthesis in toga-virus-infected cells seems to be the RNA polymerase. Between 2 and 3 h after infection of BHK 21 and chick embryo cells with Sindbis and Semliki Forest virus, 60–75% of the total protein synthesis is virus-directed, in particular, synthesis of the membrane protein in the virus constitutes 25% of total protein synthesis. Virus protein appears to be produced on polysomes associated with cellular membranes [FRIEDMAN, 1968b; STRAUSS *et al.*, 1969]. Attempts have been made to isolate virus-coded polypeptides from infected cells.

In chick embryo cells infected with Semliki Forest virus five major viral proteins were produced early in infection; three of these proteins correspond to viral structural proteins. Late in infection three major proteins – only the structural proteins – were produced [FRIEDMAN, 1968c]. In contrast to these results, HAY *et al.* [1968] reported that six new proteins were formed in the same virus-cell system. All six proteins were first detectable 3 h after infection and were present in the same proportions thereafter. Two of the proteins were identified as those of the complete virus particle. 12–16 polypeptides induced by Sindbis virus infection have been identified in BHK 21 cells [STRAUSS *et al.*, 1969]. As early as 30 min after infection of chick embryo cells with Sindbis virus, seven virus-specific polypeptides have been detected. The same pattern of viral polypeptides could be seen 2, 3 and 5 h after infection [ENZMANN, unpublished results].

Several authors examined the polypeptide pattern of flaviviruses [WESTAWAY and REEDMAN, 1969; SHAPIRO *et al.*, 1971; TRENT and QURESHI, 1971]. In a recent report the proteins specified during infection by the flaviviruses Kunjin, Japanese B encephalitis, St. Louis encephalitis and dengue 2 are defined and compared in two different cell lines [WESTAWAY, 1973]. Three structural and, in addition, seven nonstructural proteins were specified in Vero and PS cells by the genome of these viruses. All polypeptide profiles are similar but distinguishable from one another. The proteins are synthesized in the same but not in equimolar proportions for long periods. SHAPIRO *et al.* [1972a] have shown that it is possible to distinguish mosquito-borne from tick-borne flaviviruses on the basis of the electrophoretic mobility of a structural protein.

Several species of RNA found in togavirus-infected cells have been postulated to be mRNA, including 33S, 28S, 26S, 15–18S, and 8S RNA (see above). Since 26S RNA is the major RNA species present during the period when virion protein synthesis is maximal, it seems likely that 26S RNA is the messenger for the structural proteins of the virion. The primary gene product after translation of this mRNA was revealed to be the precursor of the structural proteins which arise by proteolytic cleavage of this large polypeptide [SCHLESINGER and SCHLESINGER, 1973; WAITE, 1973]. The other forms of mRNA found in minor amounts in alphavirus-infected cells seem to be responsible for translation of additional polypeptides which are only required in small quantities during virus replication [SIMMONS and STRAUSS, 1972b]. The control mechanism by which the viral genome makes it possible to provide structural proteins in abundance and functional proteins in only small numbers seems to be working at the transcription level in synthesizing restricted amounts of mRNA. However, this process is not yet fully understood.

Aspects of protein synthesis directed by vesicular stomatitis virus have been reviewed by WAGNER and SCHNAITMAN [1970], BALTIMORE *et al.* [1971] and WAGNER *et al.* [1972a].

Five virion polypeptides and nine nonstructural polypeptides are found in vesicular stomatitis virus-infected cells [MUDD and SUMMERS, 1970b; KANG and PREVEC, 1971]. The RNA-dependent RNA polymerase responsible for the transcription of mRNA of vesicular stomatitis virus appears to be a component of the virion [BALTIMORE *et al.*, 1970]. It seems to be associated with the ribonucleoprotein (nucleocapsid) complex. During the process of transcription apparently no protein is released from the complex [BISHOP and ROY, 1972; EMERSON and WAGNER, 1972; SZILÁGYI and URYVAYEV, 1973]. Instead of a monocistronic message coding for a large precursor polypeptide, smaller messages from the intact input RNA strand may be transcribed, each coding for individual polypeptides. It may be assumed that there is a regulation at the transcriptional level of the relative proportions of structural and nonstructural proteins synthesized. The mRNA species synthesized on viral RNA as template presumably attach to either membrane-associated ribosomes or to cytoplasmic ribosomes. It seems possible that some membrane-bound messengers translate proteins which are inserted directly into membranes [WAGNER *et al.*, 1972a]. During this process specific glycosylation of G protein takes place, which is essential for membrane insertion of this polypeptide [PRINTZ and WAGNER, 1971]. It seems likely, therefore, that vesicular stomatitis virus RNA and proteins N, L, and

NS [for nomenclature of proteins see WAGNER *et al.*, 1972b] are assembled into nucleocapsids in the cytoplasm, whereby the RNA is intimately associated with protein which may organize the RNA into loose helical coils. This complex is called the ribonucleoprotein. An additional protein layer apparently serves to tighten the coiling of the ribonucleoprotein into a cylinder which exhibits a helical symmetry. The nucleocapsids later attach to cytoplasmic membranes containing viral G and M proteins [KNUDSON, 1973; COWARD *et al.*, 1971]. Introduction of negatively charged phosphate groups into specific regions of the polypeptide chains before or after assembly of subviral structures may facilitate the mutual recognition of protein components during the sequential steps of virus assembly [SOKOL and CLARK, 1973]. Both plasma and intracytoplasmic vacuolar membranes serve as maturation sites for vesicular stomatitis virus. The principal site of maturation seems to be a host-dependent phenomenon [ZEE *et al.*, 1970]. Possibly the origin of a cultured cell is of importance. KLENK and CHOPPIN [1969] have shown chemical differences among the plasma membranes of primary monkey kidney cells, BHK and hamster kidney cells. This difference in membrane composition and structure may be responsible for determining the maturation site of vesicular stomatitis virus. Possibly the lipid affinity of the viral envelope proteins plays a major role in selection of the maturation site.

A recent report [VERWOERD *et al.*, 1972] deals with viral protein synthesis of a diplornavirus, bluetongue virus. It seems that a coding relationship exists between the genome segments of bluetongue virus and the polypeptides synthesized. Here again regulation of macromolecular synthesis seems to be due to processes during transcription

4. Morphogenesis

In the previous review it was reported that the most striking feature of alphavirus morphogenesis was the appearance of numerous intracytoplasmic vacuoles delimited by a membrane onto which virus precursor particles were attached. This observation supports the above-mentioned studies in which synthesis of both viral RNA and protein was suggested to occur in association with cellular membranes. In the meantime, several electron microscopic studies revealed additional details of alphavirus morphogenesis.

Intracytoplasmic membranous structures of a unique type were the first evidence of subcellular alteration associated with the replication of several alphaviruses [ACHESON and TAMM, 1967; FRIEDMAN and BEREZESKY, 1967; GRIMLEY *et al.*, 1968]. The structures referred to as type 1a cytopathic va-

cuoles (CPV-1) were membrane-limited and characteristically lined by regular membranous spherules attached to the interior surface of the vacuoles; they measured 50 nm in diameter, and contained typically a fine central density, but were neither virus core nor virions. CPV-1 membranes seem to represent a significant element of the alphavirus replication complex, possibly of RNA synthesis [FRIEDMAN *et al.*, 1972; GRIMLEY *et al.*, 1972]. The other type of cytoplasmic vacuoles is the one originally described in cells infected with several alphaviruses [for references see MUSSGAY, 1964] and is numerous, especially in the late phase of the virus replication cycle. These vacuoles, termed CPV-2 [GRIMLEY *et al.*, 1968], are studded with closely spaced particles of uniform size (25–30 nm) believed to represent viral precursors designated by different authors as nucleocapsids, nucleoids or cores. CPV-2 appear to arise from dilated regions of the endoplasmic reticulum and are generally smaller in diameter than CPV-1 (0.6–2 nm), averaging 0.35 nm. In a progressed stage of infection CPV-2 contain mature virions within their lumens. The number of CPV-1 decline when the proportion of CPV-2 increases during the course of infection. It is not clear how virus-specific membranous tubular structures are formed and how the cell contributes to their formation. The outer surface of the CPV-2 appears to be the site of origin for the nucleocapsids. Virus RNA from a preexisting RNA pool is coated with the newly synthesized proteins to form virus cores at a very rapid rate almost as quickly as these proteins can be supplied [FRIEDMAN, 1968b; BEN-ISHAI *et al.*, 1968]. After formation, the core particles acquire their external membrane coat specified by the virus genome in transit through modified membranes, either the plasma membrane or the membranes of cytoplasmic vacuoles. There is disagreement as to the relative importance of these two sites of viral maturation [ACHESON and TAMM, 1967; BROWN *et al.*, 1972; WAITE *et al.*, 1972; GIL-FERNÁNDEZ *et al.*, 1973; LASCANO *et al.*, 1969; BYKOVSKY *et al.*, 1969; GRIMLEY *et al.*, 1968; PEDERSEN and SAGIK, 1973]. It is discussed that budding is initiated by the attachment of the nucleocapsid to the inner face of the plasma membrane or to the outer surface of CPV-2. As budding progresses, the virion is increasingly wrapped in the modified host cell membrane until it is almost entirely outside the cell, or inside the vacuole. The mature virion is released from the membrane by fusion of first the inner leaflet of the viral membrane and then the outer leaflet into a closed sphere [BROWN *et al.*, 1972]. At present, it cannot be decided whether the inner leaflet of the plasma membrane also functions in assembly of the nucleocapsids. Since the structural proteins of the virion seem to arise from a polycistronic messenger, assembly and budding of the cores may occur at the same site in the cell. This

presumption is confirmed by the fact that the nucleocapsid interacts strongly with the membrane both prior to budding and after maturation [BROWN *et al.*, 1972]. The inability to obtain membrane fractions free of nucleocapsid protein [BOSE and BRUNDIGE, 1972] suggests also that the cores arise at the modified membranes. Medium of low ionic strength was assumed to partially inhibit the nucleocapsid-membrane interaction that initiates the budding process [WAITE *et al.*, 1972]. Based on X-ray diffraction studies, HARRISON *et al.* [1971] assumed that the capsid proteins of Sindbis virus are closely associated with the lipid of the envelope. The nucleocapsid protein may therefore be considered as the protein of the inner leaflet of the viral membrane.

Morphogenesis of flaviviruses resembles that of alphaviruses in most features [MATSUMURA *et al.*, 1971; FILSHIE and ŘEHÁČEK, 1968; OTA, 1965], except that there is no accumulation of cores found around the vacuoles in flavivirus-infected cells [OTA, 1965]. The membranes of the intracellular spaces appear to be the main sites for virus assembly [DAVID-WEST *et al.*, 1972]. It is suggested that the potential existence of two virion forms is a characteristic of flavivirus replication. The normal form of virions contains one membrane glycoprotein and one membrane nonglycoprotein, whereas an abnormal form contains two glycoproteins [SHAPIRO *et al.*, 1972a].

The mode of maturation of members of the bunyavirus group involves budding from intracytoplasmic membranes into cisternae or vacuoles. Such budding was not preceded by earlier morphological evidence of viral maturation. The predominating characteristic of cells infected with bunyaviruses is the accumulation of virus particles individually in vacuoles and in masses in membranous enclosures [MURPHY *et al.*, 1968a; HOLMES, 1971].

The bulk of the maturation of vesicular stomatitis virus occurs at the plasma membranes as shown in the previous review. In some cases, especially with the New Jersey serotype, maturation has been observed at intracytoplasmic membranes [HACKETT *et al.*, 1968].

Maturation of Nodamura virus, a member of the picornavirus group, occurs in the cytoplasmic matrix in association with characteristic viroplasm. Masses of virus particles are enclosed within membrane-bound structures in the cytoplasm of infected cells. Release of virus is similar to the process which occurs after poliovirus infection [MURPHY *et al.*, 1970b].

The members of the diplornavirus group develop from a granular or reticular cytoplasmic matrix. Masses of fine filaments and tubular structures in the cytoplasm are characteristically associated with their sites of maturation. The mode of development of diplornavirus particles, which occurs entirely within a granular matrix in the cytoplasm, is a characteristic quite

distinct from that of togaviruses. The mature particles, which are devoid of envelopes, do not accumulate in the cell, but are released by extrusion [MURPHY *et al.*, 1971a; CROMACK *et al.*, 1971; HOWELL and VERWOERD, 1971].

C. Influence of Arbovirus Infection on Host Cell Macromolecular Synthesis

Infection of vertebrate cells with arboviruses can cause a progressively severe inhibition in the synthesis of cellular protein and a somewhat lesser inhibition of cellular RNA [LUST, 1966; FRIEDMAN, 1968b; STRAUSS *et al.*, 1969; MUSSGAY *et al.*, 1970; TAKEHARA, 1972]. Inhibition of protein synthesis in chick embryo cells caused by Sindbis virus infection starts as early as 90 min after infection and is dependent on the production of protein(s) coded by the viral genome [MUSSGAY *et al.*, 1970]. Concerning the flavi- and diplornaviruses, there are few reports on the inhibition of cellular protein and nucleic acid synthesis [TRENT *et al.*, 1969; HUISMANS, 1971]. Host cell protein and RNA synthesis and initiation of DNA synthesis are inhibited rapidly after infection with vesicular stomatitis virus [HUANG and WAGNER, 1965; HUANG *et al.*, 1970; YAOI and OGOTÁ, 1972]. The inhibition of cellular protein synthesis is due to new virus-coded protein synthesized after infection [WERTZ and YOUNGNER, 1972]. However, it has been recently reported that double-stranded RNA isolated from picornavirus-infected cells has an inhibitory effect on *in vitro* protein synthesis [EHRENFELD and HUNT, 1971] and *in vivo* protein synthesis [CORDELL-STEWART and TAYLOR, 1973]. Therefore, it was suggested that the inhibition of cellular protein synthesis caused by picornavirus infection is not due to newly synthesized protein but to virus-specific double-stranded RNA. Other experiments indicated that foot-and-mouth disease virus and Sindbis virus-specific double-stranded RNA caused a slight inhibition of protein synthesis in chick embryo cells, but the kinetics of this reaction were different from that determined in Sindbis virus-infected cells. Treatment of infected cells with the antibiotic Cordycepin resulted in a decreased synthesis of infectious viral RNA and viral double-stranded RNA, but inhibition of cellular protein synthesis was still initiated by the viral infection [ENZMANN, unpublished results]. It is therefore concluded that viruscoded protein directly impairs cellular metabolism. Yet it cannot be ruled out that double-stranded RNA may cause an additional inhibition of cellular synthesis.

There exist several reports concerning other alterations in host cell metabolism. Investigation of changes in the constitutive enzymes of Semliki

Forest virus-infected chick embryo cells showed that all the cytoplasmic dehydrogenases studied increased in activity [CASSELLS and BURKE, 1973], suggesting that the pathways of glucose metabolism are stimulated following virus infection. Acid phosphatase activity was found to be increased in the Golgi cisternae, vacuoles and lysosomes of nerve cells infected with Venezuelan equine encephalitis virus [GARCIA-TAMAYO, 1971]. One of the functions of the cell necessary for viral growth is the catalyzation of carbohydrate transfer to viral glycoprotein [GRIMES and BURGE, 1971]. In this regard it is interesting that virus multiplication is influenced by manipulating the hexose metabolism of the host cells. Glucosamine, 2-deoxyglucose, mannosamine and DEAE-dextran inhibit the growth of Semliki Forest virus [KALUZA *et al.*, 1972; FLEMING, 1973], possibly by affecting the specific transferases responsible for glycosylation of viral membrane proteins, thus blocking either the permitted sites of virus maturation or transport of virions through the plasma membrane. The carbohydrate moiety of the virus glycoprotein may therefore contribute to the animal host susceptibility range [FLEMING, 1973].

D. Interference

Arboviruses have been frequently used for basic studies on virus interference, mainly in connection with investigations of interferon. The vast number of publications on this topic makes it impossible to discuss within the present review. Therefore, attention is paid only to a special feature of interference with arboviruses, namely autointerference. Autointerference appears to be a special case of homologous interference that occurs under conditions of high multiplicity of infection with a population of antigenically homogeneous virus particles.

Recently, evidence has been presented for the existence of defective interfering viral particles after serial passages of Sindbis virus in BHK cells at a high multiplicity [SCHLESINGER *et al.*, 1972]. These defective particles have a density of 1.22 g/ml compared with 1.20 g/ml for infectious Sindbis virus particles [SHENK and STOLLAR, 1973]. The defective particles contain an RNA molecule indistinguishable from that of infectious particles. However, in cells infected with both infectious and defective particles a species of 12S double-stranded RNA appears in addition to the 20S species usually found in cells infected with plaque-purified virus. Several smaller single-stranded RNA molecules arise after infection with both particles [SHENK and STOLLAR, 1972]. The defective particles interfere with RNA synthesis by normal Sindbis

virus; they differ from the T particles of vesicular stomatitis virus (see below) which contain only one-third to one-half of the length of RNA found in infectious B particles.

Cells infected at high multiplicity with unfractionated vesicular stomatitis virus produce low yields of infectious virus. Under these conditions an excess of smaller, noninfectious, so-called T particles is synthesized which has been shown to interfere with the synthesis of infectious virus. These defective particles are antigenically similar to the infectious viruses [HUANG and WAGNER, 1966b]. It was suggested that the RNA of the interfering particles was competing with virus RNA for replicative sites, presumably the polymerase [WILD, 1972]. STAMPFER *et al.* [1969] found an altered pattern of viral RNA synthesized in cells which are infected at high multiplicity. Interference among different arboviruses not mediated by interferon has been described [ZEBOVITZ and BROWN, 1968]. In order to interfere with the growth of challenge virus, RNA synthesis by the interfering virus was required; blockage of the challenge virus appeared to occur before synthesis of its infectious viral RNA. A possible explanation may be the competition for replication sites or substrates.

Cultured mosquito cells can support arbovirus growth as discussed in section IV. In cell cultures of *Aedes albopictus* chronically infected with Sindbis virus, homologous interference was demonstrated by STOLLAR and SHENK [1973]. In this system, interference with the replication of superinfecting homologous virus occurs at some stage before there has been any significant replication of the RNA of the superinfecting virus. Chronically infected cultures contained double-stranded RNA with sedimentation coefficients of 12S, 15S and 20S in contrast to cultures infected for the first time with Sindbis virus which contained mainly 20S double-stranded RNA [STOLLAR *et al.*, 1972]. Superinfection with Eastern equine encephalitis virus resulted in increased amounts of 12S as well as 20S double-stranded RNA. However, it should be mentioned that an interferon-like substance was demonstrated in *A. albopictus* cell cultures persistently infected with Sindbis virus [ENZMANN, 1973].

E. Comments

Comparison of this section with the corresponding one of the previous review demonstrates the great increase of knowledge about biochemical events occurring in virus-infected cultured vertebrate cells. The structural

heterogeneity of the arboviruses is also reflected in different modes of replication. The infective cycle of RNA-containing arboviruses can be either initiated by single-stranded (+) RNA in which the RNA acts as an mRNA or by single- or double-stranded (—) RNA which serve as templates for the synthesis of mRNA. At present it is not known which factors determine the host range of a certain arbovirus. It also cannot be said which factors decide whether or not the infective cycle is accompanied by a cytopathic effect.

IV. Virus Growth in Cell Cultures of Arthropods

In recent years the growing interest in arboviruses has also been reflected by studies on the arthropod host-virus relationship. In this context the most significant advance has been the development of arthropod cell lines which have increased in number and variety during the last 10 years. The history and methods of invertebrate tissue culture were reviewed by VAGO [1967], VAUGHN [1968], GRACE [1969], and SINGH [1972]. The application of arthropod cell cultures to the study of viruses has been the topic of a symposium [WEISS, 1971]. Cell cultures derived from known virus vectors, namely members of the phyla diptera and acarina, are of main interest in this respect. In the present review only established cell lines are considered because they are the most suitable system for studying virus replication; for studies dealing with arboviruses in surviving invertebrate tissue fragments and in primary cell cultures the reader is referred to a report of YUNKER [1971].

A. Susceptibility of Several Continuous Cell Lines to Arboviruses

The susceptibility of an arthropod cell line seems to be restricted to those arboviruses that are either transmitted by the arthropod from which the cell line originated or that can infect this arthropod species under experimental conditions. In general one can say that the ability of a cell line to support virus growth reflects the natural vector-virus relationship. Exceptions from this have been documented by YUNKER and CORY [1969] and by BUCKLEY [1971]. It was found that Colorado tick fever virus multiplies in mosquito cells. In addition, several tick-borne viruses of the Kemerovo group were able to infect mosquito cell lines. In the flavivirus group mosquito-borne members grow readily in mosquito cells, but tick-borne members grow poorly or

not at all. Whether the mentioned exceptions are due to an alteration of cell susceptibility to virus infection through long-term cultivation or to a potency of the arthropod cells which is not expressed in infections of the arthropod organism cannot be said. Several cell lines derived from *A. aegypti* [GRACE, 1966; SINGH, 1967; PELEG, 1968], *A. albopictus* [SINGH, 1967] and *Antheraea eucalypti* [GRACE, 1962] used in many laboratories were shown to support the growth of arboviruses including members of the alphavirus, flavivirus, bunyavirus, rhabdovirus and diplornavirus groups, and also several ungrouped viruses [for references see YUNKER, 1971; SINGH, 1972]. Other cell lines supporting multiplication of arboviruses are quoted by SINGH [1972].

B. Replication of Certain Arboviruses in Arthropod Cells

The growth of arboviruses in *A. albopictus* and *A. aegypti* cell lines (Singh) was compared by PAUL and SINGH [1969] with that in Vero cells and in infant mice. *A. albopictus* cells supported virus growth of members of the alphavirus and flavivirus groups to a similar extent as Vero cells or mice; lower titers were measured in the arthropod cells after infection with Batai and Chandipura virus. In general, virus yields in *A. aegypti* were lower than those obtained in *A. albopictus* cells. In comparative studies with *A. albopictus* and vertebrate cells STEVENS [1970] determined similar growth curves for dengue 2 and Sindbis virus in both cell systems. In spinner cultures of *A. albopictus* cells growth kinetics of Sindbis virus at 28 °C were comparable with that in vertebrate cells at 37 °C. In contrast, ENZMANN [1973] found in albopictus cell cultures that the virus yield was only 1% compared to that of chick embryo cells. In chick embryo cells synthesis of progeny virus at 37 °C started between 90–120 min after infection; the multiplication of Sindbis virus in *A. albopictus* cells at 28 °C showed an eclipse phase of 4–5 h duration. A single growth cycle of Sindbis virus in chick embryo cells required 4 h, compared with approximately 20–24 h in *A. albopictus* cells, after which time virus titer gradually decreased. STEVEN's experiments [1970], however, showed a chronic shedding of Sindbis virus persisting at least over several days and no decrease in virus titer was observed. The replication of two other togaviruses, Semliki Forest and Kunjin virus, in *A. albopictus* cells was studied by DAVEY *et al.* [1973] who found no differences in the virus yields between the arthropod cell line and cultured vertebrate cells of mammalian and avian origin. Virus replication in the *A. albopictus* cells, however,

was not accompanied by a cytopathic effect. Virus replication appeared to be related to the optimum temperature of cell growth. In an attempt to enhance infection in *A. aegypti* cells, PELEG [1972] exposed the mosquito cultures concurrently to two viruses, Eastern equine encephalitis and Semliki Forest. Unlike vertebrate cells, no synergism was observed in cultured mosquito cells. Although no cytopathic effect appeared in the arthropod cell cultures, it was suggested that the two viruses were proliferating in different target cells. The assumption that virus may propagate only in relatively few susceptible cells was shown by FILSHIE and ŘEHÁČEK [1968] by studies on the morphology of Murray Valley encephalitis virus in *A. aegypti* cells. Virus particles were observed in only about 5% of the cells. PELEG [1969] found 8% of *A. aegypti* cells in culture involved in virus production, with an estimated yield of 1–6 PFU per infected cell. The participation in virus production of only a small proportion of cells has been noted for vesicular stomatitis virus in *Antheraea eucalypti* cells by YANG *et al.* [1969]. SINGH and PAUL [1968] and PAUL *et al.* [1969] reported appearance of a cytopathic effect when cells of *A. albopictus* were infected with Japanese B encephalitis, West Nile or dengue 1–4 viruses. This cell damage was utilized by SUITOR [1969] for developing a plaque method for Japanese B encephalitis virus in the *A. albopictus* cell system. SUITOR and PAUL [1969] described syncytia formation of *A. albopictus* cells mediated by dengue 2 virus. These authors emphasize that RNA-containing arboviruses have not been known to induce syncytial formation in vertebrate cells.

The picture which emerges from these studies is, that unlike vertebrate cells where arbovirus infections usually are short-termed and cytocidal, infections of cultured mosquito cells by these viruses are long-termed and inapparent. The virus yield in mosquito cell cultures may under certain conditions be the same as in vertebrate cells. Although a gross cytopathic effect is usually not apparent in mosquito cell cultures, some destructive processes may result from a virus infection.

V. Virus Growth in Vertebrate Hosts

A. General

In this section an attempt is made to consider in a very concentrated form (1) the spread of virus in susceptible vertebrate hosts, (2) the sites of multiplication in extraneural tissues and in the central nervous system (CNS),

and (3) the viral morphogenesis *in vivo*. Within the framework of this article the mechanisms underlying natural resistance, the influence of specific and nonspecific defense mechanisms on the course and outcome of an infection, and the relation between sites of virus multiplication and pathological changes cannot be discussed. For detailed information on these subjects the reader is referred to excellent reviews presented by MIMS [1964], JOHNSON and MIMS [1968], ALBRECHT [1968], and NATHANSON and COLE [1970].

Virus growth in vertebrate hosts is influenced by several factors: the species, the genetic differences within species, the age and physiological status of the host, the virulence and homogeneity of the virus strain, the number of infectious virus particles used for infection, and the mode of inoculation. ALBRECHT [1968] distinguishes between the following host-virus relationships: (a) virus replicates readily in the extraneural tissues but only poorly in the CNS; (b) virus replicates readily in CNS but relatively poorly in peripheral tissues; (c) virus growth is poor in extraneural tissues and CNS, and (d) virus multiplies readily in both extraneural tissue and CNS.

In the following three sections, the spread and replication of arboviruses in the extraneural tissues, the multiplication in the CNS and the influence of age and species of the host on virus growth are considered. Growth of attenuated virus in vertebrate hosts is discussed in section VII.

B. Spread and Multiplication of Virus in Extraneural Tissues

1. Viremia

After peripheral infection small amounts of virus can be demonstrated within a few minutes and up to 1 h postinfection in the blood of mice [KUNDIN, 1966]. This 'resorptive viremia' represents virus that has spilled over through damaged vessels. There is usually a lag period of several hours, or up to 2 days with small inocula, before viremia reappears, signalling the entry of newly formed virus into the blood circulation [ALBRECHT, 1968]. The cell type(s) serving as source for the virus appearing in the blood depends on the tissue specificity of the virus in a given host. With an alphavirus, Aura virus, early virus replication in suckling hamsters after subcutaneous infection was detected in the macrophages of the reticuloendothelial system, especially in the liver [PAOLA *et al.*, 1968]. MURPHY *et al.* [1970a], working with another alphavirus, Semliki Forest virus, assumed that the early viral replication detected in muscle cells and fibroblasts after intraperitoneal infection of neonatal mice accounted for the observed viremia. Immediately after

infection of guinea pigs with the TC-83 strain of Venezuelan equine encephalitis virus, viral antigen was detected in liver, spleen, lymph nodes and kidney [AIRHART *et al.*, 1969]; after inhalation infection of the upper respiratory tract, the mucosa and the lungs served as virus sources for viremia [HRUŠKOVÁ *et al.*, 1969]. Experiments performed by MÁLKOVÁ [1968] with tick-borne encephalitis virus in mice indicated that the regional lymphatic system is the first and decisive place of virus multiplication in the previremic phase, upon which the start of viremia is dependent. Bone marrow cells do not seem to play such a role in the very early phase of infection [MÁLKOVÁ and KOLMAN, 1966]. KUNDIN *et al.* [1963], working with West Nile virus in suckling mice, suggested the possibility that the blood vessel wall is one of the primary sites of virus multiplication. In the viremic phase virus is usually present in a free state and only a small proportion is bound to leucocytes [MÁLKOVÁ, 1967; SIMKOVÁ and WALLNEROVÁ, 1968]. However, persistent viremia of Colorado tick-fever virus, a diplornavirus, was found to be predominantly due to virus contained within erythrocytes, and it was assumed that erythrocyte precursor cells in the bone marrow are infected at early stages in their development, and that at least some of these infected cells continue to mature and are subsequently released into the blood stream [EMMONS *et al.*, 1972].

2. Target Organs

The ubiquitousness of virus or viral antigen as a consequence of viremic spread of virus was confirmed in several studies. In suckling mice infected with alphaviruses the pantropic character of the infection was demonstrated. Venezuelan equine encephalitis virus was detected in salivary glands, inguinal nodes and spleen [KUNDIN, 1966]; with Sindbis virus [JOHNSON, 1965] evidence for virus multiplication in striated, smooth and cardiac muscle and in vascular endothelium was obtained. In suckling hamsters Aura virus was detected in kidney and heart [PAOLA *et al.*, 1968]. Detailed studies in mice with tick-borne encephalitis virus, a flavivirus, also revealed a very rapid spread of virus by the hematogenous route and virus multiplication in a great variety of tissues [ALBRECHT, 1968]. As mentioned in the 1964 review, virus antigen was detected in cells of ectodermal, entodermal and mesodermal origin. It was concluded that the spleen is of primary importance for virus multiplication in the early viremic stage after intravenous infection [MÁLKOVÁ and SMETANA, 1966], whereas the thymus plays no important role in the distribution and multiplication of the virus during early stages of viremia [MÁLKOVÁ, 1966]. Bone marrow cells of mice can support multiplication of

tick-borne encephalitis virus [MÁLKOVÁ and KOLMAN, 1966] and yellow fever virus [BARINSKY and DEMENTIEV, 1968], but do not seem to be important for virus dissemination. Infection of mice with a member of the bunyavirus group, Uukuniemi virus, was studied and virus could be isolated from blood, liver, spleen and intestine [RAJČÁNI and GREŠIKOVÁ, 1970]. The broad range of cells capable of supporting the growth of arboviruses may demand the presence of virus-specific receptors in a wide variety of cells. This was demonstrated for Japanese B encephalitis virus; NAKAI *et al.* [1965] reported the isolation of receptors for this virus from liver parenchymal cells, kidney, spleen and erythrocytes of the mouse.

3. Transplacental Transmission

Transplacental transmission was demonstrated for Venezuelan equine encephalitis virus in human beings [WENGER, 1967], for St. Louis encephalitis virus in mice [ANDERSEN and HANSON, 1970], and for bluetongue virus in ewes [ANDERSON and JENSEN, 1969].

4. Virus Morphogenesis in Extraneural Cells

In some studies multiplication of arboviruses in extraneural tissues of vertebrate hosts was examined by electron microscopy. In mice infected with Aura virus, the cardiac myocyte revealed viral nucleoids within the cytoplasm and budding particles from the plasma membranes and from walls of sarcotubules. Cell-free viral aggregates were observed within the lumina of myocardial capillaries [LASCANO *et al.*, 1970]. MURPHY *et al.* [1970a], working with Semliki Forest virus in neonatal mice, observed the formation of viral nucleoids in muscle and fibroblast cytoplasmic matrices; the nucleoids were enveloped via passage through the sarcoplasmic reticulum or plasma membranes. Selective growth of this virus in voluntary muscle cells in young mice was seen, but virus growth was retarded after the first cycle of multiplication and it was assumed that cellular barriers are the major limitation to the spread of virus in muscles [GRIMLEY and FRIEDMAN, 1970]. In Councilman bodies found in hepatic cells of *A. s. seniculus* naturally infected with yellow fever virus no virus particles were observed, and it was concluded that the formation of Councilman bodies is not a direct consequence of the presence of virus particles [BARUCH *et al.*, 1963].

MURPHY *et al.* [1971b] followed the morphogenesis of Marburg virus in experimentally infected *Cercopithecus aethiops* monkeys. Viral maturation proceeded via budding of core structures through host cell membranes. Excess production of core structures formed elaborate intracytoplasmic

inclusion bodies which differed in structure from inclusion bodies detectable in cells infected with rhabdoviruses.

C. Spread and Multiplication of Virus in the CNS

1. Entrance into the CNS

Viruses may enter the CNS by spread from the blood or by spread along peripheral nerves. Passage across the blood-brain barrier is usual for encephalitic arboviruses and can take place in several ways. Virus in the blood might enter the cerebrospinal fluid by either passing or growing through the choroid plexus; once within the cerebrospinal fluid, movement across the meningeal and ependymal cells might occur [JOHNSON and MIMS, 1968]. Virus may also cross cerebral capillaries from blood to brain by infecting the endothelial cells of the capillary with subsequent spread into surrounding neuropil. It was reported for Sindbis virus that infection of vascular endothelial cells precedes infection of surrounding neurons and glial cells [JOHNSON, 1965]. Also, virus may move passively across the cerebral capillaries as assumed for tick-borne encephalitis virus in mice [ALBRECHT, 1968]. Spread of arboviruses along peripheral nerves is considered to be incidental and may occur via the olfactory route after aerosol infection. This was shown for Semliki Forest virus in mice and rabbits [BOULTER *et al.*, 1971], and in hamsters [HENDERSON *et al.*, 1967], for West Nile virus in mice [NIR *et al.*, 1965], and for tick-borne encephalitis virus in monkeys [MAYER and RAJČÁNI, 1968]. After intranasal infection with a rhabdovirus, vesicular stomatitis virus, viral antigen first appeared in the olfactory bulbs, pyriform lobe and hippocampus. Subsequently, clusters of cells in close proximity to the ventricular system were affected [MIYOSHI *et al.*, 1971].

2. Extraneural Infection

In mice, viral invasion of the CNS across the blood-brain barrier usually occurs during the rising slope of viremia. After crossing the vascular wall the virus may infect susceptible cells. The types of cells infected, the proportion of infected cells and the severity of infection in different CNS areas can vary considerably depending on the virus strain and the age of the infected animal. In neonatal mice infected intraperitoneally with an alphavirus, Semliki Forest virus, nearly all of the brain cells contained viral nucleoids as observed in the electron microscope [MURPHY *et al.*, 1970a]. In mice infected by the subcutaneous route with other alphaviruses, Western and Eastern

equine encephalitis viruses, examination of the brains at or before the time when encephalitis became apparent showed that almost all nerve cells in the cortex had virus-specific fluorescence [LIU *et al.*, 1970]. It was demonstrated in suckling mice infected subcutaneously with Sindbis virus, also an alphavirus, that virus was disseminated rapidly within the cerebral parenchyma infecting both neural and glial cells [JOHNSON, 1965]. In moribund hamsters infected intraperitoneally with louping ill virus, a flavivirus, almost all cerebellar neurons contained virus [DOHERTY *et al.*, 1971], and in moribund sheep the highest concentrations of louping ill virus were found in the brainstem and spinal cord [DOHERTY and REID, 1971]; this distribution of virus found in sheep infected with louping ill virus is essentially similar to that observed in monkeys inoculated with other viruses of the tick-borne encephalitis complex [MAYER and RAJČÁNI, 1968; NATHANSON *et al.*, 1967].

3. Intracerebral Infection

Intracerebral infection is widely used in studies of multiplication and dissemination of viruses in the CNS. Results obtained in such studies may differ from those in which infection of the CNS follows extraneural infection. Virus inoculated intracerebrally may invade the parenchyma both from the site of inoculation and through the ependymal and meningeal surfaces [JOHNSON and MIMS, 1968; NATHANSON and COLE, 1970]. Antigen of Western equine encephalitis virus was detected in the cytoplasm of neurons and glial cells after intracerebral infection of mice [LIU *et al.*, 1970]. After intracerebral infection of adult mice with Japanese B encephalitis virus, electron microscopy showed virus particles in the neurons of the medulla oblongata or thalamus; no virus particles have been found on the surface or within ependymal cells of the lateral ventricle wall [YASUZUMI and TSUBO, 1965]. In similar investigations virus particles were seen in 70–80% of the cortical neurons and anterior horn cells three days after infection [OYANAGI *et al.*, 1969]. Zika virus, another flavivirus, appears to have an early effect upon astroglial cells in mice infected at one day of age; microglial cells do not appear to be affected. Replication of the virus was demonstrated in both neurons and astroglial cells [BELL *et al.*, 1971]. With the flavivirus Langat in suckling mice, besides neurons, astrocytes and oligodendrocytes were found to contain virus particles [DALTON, 1972]. This is in contrast to the observation of BLINZINGER [1972], who detected virus particles of two tick-borne encephalitis virus strains in numerous cortical as well as spinal neurons of young adult mice, but not in nonneuronal elements within the CNS; this was also reported for yellow fever virus infection. In the study of BLINZINGER,

flavivirus particles were repeatedly seen in intercellular gaps of the neuropil and occasionally were also encountered inside dendrites and axons. Therefore, it was assumed that spread of these viruses can take place both along the extracellular spaces and via sequential cell-to-cell infection. In another study with yellow fever virus in adult mice, virus particles were identified in the cytoplasm of the motor neurons and also in microglia in the ventral portion of the lumbar region of the spinal cords [BARUCH, 1963]. Tribeč virus multiplies in glial cells and numerous neurons in the brains of suckling mice [GREŠIKOVÁ and RAJČÁNI, 1969]. This was also observed with Uukuniemi virus [RAJČÁNI and GREŠIKOVÁ, 1970]. Intracerebral inoculation of vesicular stomatitis virus, a rhabdovirus, into weaned mice produced only minimal neuropathological changes despite widespread distribution of viral antigen which was initially found in cells near the ventricular system and later on in most parts of the brain [MIYOSHI *et al.*, 1971]. Very interesting is the observation that when purified defective T particles of vesicular stomatitis virus are injected intracerebrally into mice along with large doses of infectious virus, they convert an otherwise rapidly fatal disease process to a slowly progressing virus infection [DOYLE and HOLLAND, 1973].

4. Virus Morphogenesis in Brain Cells

In some instances morphogenesis of arboviruses in cells of the brain was studied by electron microscopy. The general pattern of morphogenesis of Semliki Forest virus in adult [ZLOTNIK and HARRIES, 1970] and suckling [MCGEE-RUSSELL and GASZTONYL, 1967; MURPHY *et al.*, 1970a] mice closely resembles the same process as observed for different alphaviruses in tissue culture cells (section III). Morphogenesis of flaviviruses was studied in cells of the CNS with Japanese B encephalitis [YASUZUMI and TSUBO, 1965], tick-borne encephalitis [DOHERTY *et al.*, 1971; BLINZINGER and MÜLLER, 1970; BLINZINGER, 1972], louping ill [ZLOTNIK and HARRIES, 1970], yellow fever [BARUCH, 1963; DAVID-WEST *et al.*, 1972; BLINZINGER, 1972] and Zika virus [BELL *et al.*, 1971], and it was seen that the morphogenesis of these viruses is very similar to that in tissue culture cells, i.e. they bud almost exclusively from proliferating intracytoplasmic membranes, and enveloped virus particles accumulate within distended cisternae [MURPHY *et al.*, 1968a]. Studies with five California group viruses [MURPHY *et al.*, 1968b] and three other members of the bunyavirus group (Bunyamwera, Tensaw and Maguari) in mouse brain revealed that the predominating characteristic of cells infected with these viruses is the accumulation of virus particles individually in vacuoles and in masses in membranous enclosures [MURPHY *et al.*, 1968a].

D. Influence of Age and Species of the Host

The resistance to infection with most arboviruses is known to increase with age [SEAMER *et al.*, 1967]. This phenomenon can be studied in experimental models using laboratory animals. ELDADAH and NATHANSON [1967] demonstrated that suckling rats develop a fatal encephalitis after infection with West Nile virus, whereas adult rats remain asymptomatic following intracerebral inoculation, but undergo an abortive CNS infection.

With the same virus, age comparisons showed higher viremia titers in young than in older mice, which correlated with the distribution of immunofluorescence in tissues, indicating that extraneural virus replication was much more widespread and intense in young animals [WEINER *et al.*, 1970]; in these studies the high peripheral virulence of Powassan virus in older mice correlated with higher viremia titers. In experiments performed by GROSSBERG and SCHERER [1966] with Japanese B encephalitis virus, 6-month-old mice were more resistant than young mice to peripheral as well as intracranial inoculation, although infectivity was about the same in both groups. SIMON *et al.* [1966] compared infections with the JIR strain of tick-borne encephalitis virus in mice and hamsters and suggested that the low neurovirulence of the virus for hamsters is due to limited virus activity and pronounced defense reaction of the organism.

Latent infections in vertebrate hosts may serve as a reservoir to assure a continued infection chain mainly in temperate regions. Birds, wild rodents, bats and poikilothermic vertebrates have been discussed in this respect [MUSSGAY, 1964; CHAMBERLAIN, 1968]. Little is known about the site of virus multiplication and spread of virus in such animals with inapparent infections.

E. Comments

To summarize this section briefly, it can be stated that in the last ten years additional insight into the sequence of events in arbovirus infections of vertebrate hosts as outlined in the review of 1964 has been obtained. The dependence of host variables, such as age and species, virus dose, virus strain, and route of virus inoculation on the course and outcome of infection has been confirmed. It seems that in the CNS involvement of glial cells in the process of virus multiplication depends mainly on the age of the animals. Electron microscopic studies demonstrated that viral morphogenesis in neural and extraneural cells in general follows a similar pattern as in cultured

cells. It is felt that attention is now mainly focused on the factors determining whether a neurotropic arbovirus gains entrance into the CNS, and those factors decisive for the outcome of infection, e.g., death, subclinical encephalitis or an inapparent course.

VI. Virus Growth in Arthropods

Infection of arthropods with arboviruses has been accomplished by both feeding and injection, and multiplication and distribution of virus was followed in several instances. Data reviewed in the previous report [MUSSGAY, 1964] have been supplemented in the meantime by numerous experiments.

A. Dynamics and Sites of Virus Multiplication

Replication of Eastern equine encephalitis virus in salivary glands of *A. triseriatus* was studied by WHITFIELD *et al.* [1971]. Viral morphogenesis in salivary glands was similar in character to that in mammalian tissues and cell cultures. Budding from apical plasma membranes of acinar cells deposited virus directly into secretory products within the gland lumina. Venezuelan equine encephalitis virus was found by electron microscopy throughout the body of *A. aegypti* within two days after an infective blood meal [LARSEN and ASHLEY, 1971]. Virus penetrated the digestive tract and reached the brain within 48 h after the infective feeding. Virus was found in the ovaries, but not within follicle cells or eggs, and in the tissues of the malpighian tubules, but not within their lumina. No cytopathic alterations were noted. Mitochondria completely surrounded by Venezuelan equine encephalitis virus had intact cristae and appeared normal. For a long time it was thought that no pathological changes occur in infected mosquitoes. This does not seem to be without exception, since Semliki Forest virus has been shown to produce cytopathic effects in the salivary glands of *A. aegypti* [MIMS *et al.*, 1966]. These effects become histologically evident in some mosquitoes about two weeks after infection and, as they progress in severity, they are accompanied by a failure in the ability to transmit the virus and by a decline in virus titers. At this time the mosquitoes had some difficulty in obtaining a blood meal, yet no shortening of their life span could be demonstrated. The contention that the salivary glands of *A. aegypti* are actually damaged by Semliki Forest virus was supported by LAM and MARSHALL [1968a, b].

The sites of multiplication and distribution of the flavivirus, Japanese B encephalitis, in the tissues of the vector mosquito *Culex tritaeniorhynchus summorosus* was studied by DOI *et al.* [1967] and DOI [1970] using the direct fluorescent antibody staining technique. An eclipse phase during which no antigen was detected lasted until the fourth day after ingestion of an infectious blood meal. The primary site of virus multiplication was in the epithelial cells of the posterior midgut. In the second stage of spreading, fat body cells seemed to play the most important role. In the third stage the virus invaded the salivary glands after 10–12 days of infection. At the same period other organs such as the thoracic ganglia, compound eyes and the malpighian tubes became infected. Virus remained in the salivary glands up to at least 42 days. Occurrence of the virus in other organs was reduced gradually with lapse of time.

Transmission of California encephalitis virus, a bunyavirus, by *A. vexans* to rabbits was accomplished seven and nine days after the insect had imbibed an infective blood meal [CHERNESKY, 1968]. The virus was also transmitted by *A. aegypti* to newly hatched chickens. California encephalitis virus was found in the gut, thorax, legs and salivary gland of *A. vexans* mosquitoes after intrathoracic injections of $10^{1.3}$ mouse LD_{50} units per 0.003 ml of virus suspension. Salivary glands contained a maximum virus titer of $10^{5.0}$ mouse LD_{50} units five days after injection. *A. aegypti* mosquitoes yielded $10^{6.3}$ mouse LD_{50} units in the salivary gland after intrathoracic injection. The infection threshold of *A. vexans* fed with California virus was $10^{2.0}$ LD_{50} units per insect. Immediately after ingestion only the gut washings contained virus. Detection of virus was not accomplished again until four days later. The infection threshold of *A. aegypti* fed with California virus exceeded $10^{4.5}$ LD_{50} units per insect. Replication of Bunyamwera virus in mosquitoes was described by PEERS [1972]. The virus multiplied readily in *A. aegypti* after imbibing an infectious blood meal and also after intrathoracic injection. Virus spread to nonenteral tissues within four days after infection. With another virus belonging to the bunyavirus group, Ukauwa virus, eight days passed before virus spread in *A. aegypti* was detected [OGUNBI, 1967]. *A. aegypti* mosquitoes supported the replication of Bunyamwera virus following injection of $10^{3.3}$ ID_{50} units or imbibing $10^{4.6}$ ID_{50} units. After injection the virus titer of whole mosquitoes declined to $10^{1.7}$ at 12 h followed by a peak of $10^{5.2}$ at four days. After feeding, virus was first detected in the hemolymph and salivary gland at four days and attained maximum titer of $10^{5.0}$ in the salivary glands at 10 days. Transmission of virus to mice was accomplished 10 days after feeding. Electron microscopic examination of tissues from in-

fected mosquitoes showed virus particles within vacuoles in the midgut tissue samples.

BERGOLD *et al.* [1968] described the transmission of vesicular stomatitis virus by *A. aegypti*. Titers reached about 4.5 logs seven days after intrathoracic injection of the Indiana strain and about 3.5 logs after injection of the New Jersey strain. Virus particles were demonstrated by electron microscopy in salivary glands, intestine, thoracic and abdominal ganglion and midgut but not in the cerebrum and both diverticuli although these latter organs had high virus titers. Transmission of vesicular stomatitis virus by mosquitoes to baby mice after intrathoracic injection was accomplished from the second to the fourth day until at least 20 days. The minimal virus titers for efficient transmission were $10^{2.18}$ for the Indiana strain and $10^{1.16}$ for the New Jersey strain.

Electron microscopic studies of bluetongue virus, a diplornavirus, in the salivary glands of *Culicoides variipennis* were performed by BOWNE and JONES [1966], and it was seen that the virus replicates in association with a meshwork of filaments and vesicles.

For the multiplication of tick-borne encephalitis viruses in their vector, the reader is referred to a recent review by BLAŠKOVIČ and NOSEK [1972].

B. Transmission to Progeny

Transovarial transmission of vesicular stomatitis virus in experimentally infected phlebotomine sandflies to their progeny was demonstrated by TESH *et al.* [1972]. Virus was recovered from all developmental stages; mean virus titers from egg to first generation adult showed a 4-log increase, indicating that virus multiplication occurred during development of the arthropod. Infected females transmitted vesicular stomatitis virus by bite to susceptible animals.

Multiplication of a tick-borne encephalitis virus, Powassan, in *Dermacentor andersoni* ticks was studied by CHERNESKY and MCLEAN [1969]. A noninfective phase was demonstrated between the first and 11th day after an infective blood meal. Virus was demonstrated in diverticula, malpighian tubules and salivary glands of nymphs, but was detected only in diverticula of larvae. In adult ticks, similar to larvae and nymphs, Powassan virus grew initially in the epithelial cells of diverticula and subsequently gained access to salivary glands and other organs. Transstadial transfer of virus was observed through ecdysis of larvae to nymphs and from nymphs to adults. Salivary glands of adult males supported growth of virus to higher titers than those

of females. These findings were correlated with the development of salivary gland tissues, since alveoli of males did not degenerate significantly after taking a blood meal, whereas salivary glands of females atrophied upon repletion. Transovarial and also transphase transmission of tick-borne encephalitis virus by *Dermacentor pictus* was reported by WROBLEWSKA-MULARCZYKOWA *et al.* [1972]. Transovarial transmission was obtained in 28% of females and transmission from larvae to nymphs was obtained in 25% of positive larval pools.

Multiplication of Langat virus was studied by VARMA and SMITH [1972] in the tick *Ixodes ricinus*. The quiescent period of the larvae appeared to be the time when virus multiplication became stabilized in the tick. Adults remained infected for at least 285 days after they had acquired the infection as larvae.

C. Dual Infections

If a second arbovirus was introduced a week after the first infection of *A. aegypti* mosquitoes with Semliki Forest virus, it replicated, but transmission rates were greatly reduced. However, the second virus is transmitted as effectively as in normal mosquitoes if it is introduced at the same time as Semliki Forest virus. *A. aegypti* dually infected with various combinations of Sindbis, Semliki Forest, West Nile and Murray Valley encephalitis viruses at varying time intervals supported the replication of both viruses to titers equivalent to singly infected control mosquitoes and transmitted both viruses simultaneously at undiminished rates until salivary gland damage caused by Semliki Forest virus was apparent [LAM and MARSHALL, 1968a, b]. In contrast to this, ROZEBOOM and KASSIRA [1969] reported interference after dual infection of mosquitoes with several strains of West Nile virus. An infection of *Culex molestus* with a nonpathogenic strain of West Nile virus suppressed the subsequent development of the Egypt strain of West Nile virus in the arthropods. The mechanism of this resistance in mosquitoes is unknown.

VII. Growth of Attenuated Virus in Vertebrate Cells, Vertebrate–Hosts and Arthropods

A. General

In this section the term 'attenuated' is restricted to virus strains with reduced virulence for vertebrate host(s).

In the article published in 1964 [MUSSGAY, 1964], the attenuated strain of Venezuelan equine encephalitis virus obtained by serial passages in cultivated guinea pig heart cells was mentioned. The TC-83 vaccine was developed from this strain. Since then, it has been employed on a large scale for the control of Venezuelan equine encephalitis in horses in Central America and was also used during the epizootic in the United States in 1971 [MCKINNEY, 1971].

B. Attenuation

Attenuation by adaptation to experimental hosts was accomplished for the following viruses in the past ten years:

Chikungunya virus: attenuation for mice after passages in cultivated *A. albopictus* cells [BANERJEE and SINGH, 1969]. *Western equine encephalitis virus:* attenuation for mice by persistent infection of tissue cultures [SIMIZU and TAKAYAMA, 1969]. *Venezuelan equine encephalitis virus:* an attenuated virus for mice was obtained from an L cell monolayer culture that had become chronically infected with a wild strain; the virus of this culture underwent a number of serial passages in fresh L cells [HEARN and SOPER, 1967; SOPER and HEARN, 1967]. *Semliki Forest virus:* PELEG [1971] demonstrated that cultivated mosquito cells can be utilized for attenuation. *Dengue virus:* attenuation for rhesus monkeys, spider monkeys and chimpanzees by 26 passages in mouse brain and 129 passages in African green monkey kidney tissue cultures [PRICE *et al.*, 1973]. *Japanese B encephalitis virus:* attenuation for mice by 40 passages in BHK cells and 10 passages in embryonic mouse skin fibroblast cultures [INOUE, 1964]; the Mochizuki strain was passed 160 times in mouse brain and then 20 times in *M. fuscatus* kidney cultures, and human beings were inoculated with this attenuated virus [HOTTA *et al.*, 1966]. *Yellow fever virus:* attenuation for monkeys after serial passages in HeLa cell cultures [HEARN *et al.*, 1965; CONVERSE *et al.*, 1971]. *Tick-borne encephalitis virus:* human cells (line AM 57) persistently infected produced virus with lowered virulence for mice [MAYER, 1963]. *Langat virus:* attenuated for rhesus monkeys and mice by propagation in embryonated eggs [THIND and PRICE, 1966; NATHANSON *et al.*, 1968]. *Kyasanur Forest disease virus:* attenuation for mice by two passages in chick embryo tissue culture and 169 passages in monkey kidney tissue culture [BHATT and ANDERSON, 1971]. *African horse sickness virus:* attenuation for horses by chick embryo or mouse brain passages [GOLDSMIT, 1967]. *Virus of epizootic hemorrhagic*

disease of deer: attenuation for deer by propagation in suckling mice [SHOPE *et al.*, 1963; WILHELM and TRAINER, 1967].

Selection of mutants arising in nature was reported for tick-borne encephalitis virus. SMORODINTSEV *et al.* [1969] isolated virus from ticks collected in western Siberia and the Kirgiz Republic. Strains with a low extraneural pathogenicity for mice and a complete nonpathogenicity or mild pathogenicity for monkeys were obtained in areas where human disease is rare but infection of man by ticks is very frequent. MAYER and KOZUCH [1969] found a pronounced genetic heterogeneity of tick-borne encephalitis virus isolated in nature. In some variants the same combination and character of at least five markers were observed as compared with the Langat virus, and the possibility of the existence of variants with a reduced virulence in central Europe is discussed.

Attempts were also made to obtain attenuated strains by chemically-induced mutations. After treatment of Eastern equine encephalitis virus RNA with nitrous acid, mutants with reduced virulence for mice were detected [KITSAK and FOMINA, 1967]. This was also observed with Venezuelan equine encephalitis virus [SOLIANIK *et al.*, 1971]. Attenuated variants of this virus were also obtained after formaldehyde and N-nitrosomethylurea treatment [ZASUKHINA and MARININA, 1967]. A mutant with low virulence for adult mice was derived from Western equine encephalitis virus treated with N-methyl-N′-nitro-N-nitrosoguanidine [SIMIZU and TAKAYAMA, 1972]. After treatment of Eastern equine encephalitis virus with alkylating compounds, attenuated mutants were isolated [SOLIANIK *et al.*, 1972]. The mutagenic effect of the cytidine analogue, 5-azacytidine, on Venezuelan equine encephalitis virus was demonstrated [HALLE, 1968].

C. Plaque Size and Virulence

Quite a number of reports have dealt with the relation between plaque size and virulence. HEARN and SOPER [1967] were able to discern four variants of Venezuelan equine encephalitis virus based upon the properties of virulence for mice and plaque size; virus from an attenuated strain yielded only small plaques. Later it was demonstrated that during storage of the unstable attenuated virus population a minority of stable large-plaque virus increased proportionally [HEARN *et al.*, 1969]. Small-plaque virus inhibited large-plaque virus but not the reverse, and it was thought that this may provide evidence for a possible mechanism by which attenuated Venezuelan equine

encephalitis virus has a selective advantage over virulent virus *in vitro*. A relation between virulence and plaque size was reported for two variants of the Florida strain of Venezuelan equine encephalitis virus; the small-plaque variant was less virulent in mice, hamsters, and guinea pigs than the large-plaque variant [PEDERSEN *et al.*, 1972]. The shift to small-plaque character is felt to be associated with an increased sensitivity to inhibitors present in agar [TAKEMOTO, 1966]; it was shown that the plaque size of a virulent strain of Venezuelan equine encephalitis virus is reduced under agar containing a low bicarbonate concentration, presumably allowing the expression of agar inhibition [SOPER, 1966]. Interesting is the finding of SOPER and HEARN [1967] that an attenuated small-plaque variant of Venezuelan equine encephalitis virus after passage in mouse brain became virulent but still produced small plaques similar to those of the attenuated strain. This is in accordance with investigations with Venezuelan equine encephalitis [ZARATE and SCHERER, 1969; HRUŠKOVÁ *et al.*, 1970] and Sindbis virus [HANNOUN *et al.*, 1964] in which no correlation between attenuation and small-plaque size has been found. Heterogeneity with respect to the plaque size marker was reported for Western equine encephalitis virus [ZASUKHINA *et al.*, 1967]; after passaging the virus in chick embryos, the virus population had a large-plaque character whereas passages in chick embryo cell cultures resulted in the selection of small-plaque variants. The large-plaque variant was more pathogenic for mice after peripheral inoculation than small-plaque variants. TAKAYAMA [1972] found that an attenuated strain of Western equine encephalitis virus can be differentiated from its parent strain by two *in vitro* markers, namely sensitivity to sodium dextran sulfate and plaquing efficiency under acid overlay. An attenuated variant of Japanese B encephalitis virus produced smaller plaques than the virulent variant from which it was derived. The plaque size was found to depend on several apparently independent features of the plaque system: saline-soluble polysaccharide fraction in the agar, $NaHCO_3$ concentration, age of cell cultures, volume of overlay media, and temperature of incubation. Thus, the variables of the plaque system could be manipulated so that under the appropriate conditions the attenuated virus could actually exceed virulent virus plaques in diameter [FRENCH and HAMMON, 1969]. No difference with respect to pathogenicity for suckling mice was found between a small- and a large-plaque variant of Guaroa virus [TAURASO, 1969]. MÁLKOVÁ [1971b] demonstrated that a peripherally virulent variant of Tahyna virus produced no minute plaques, whereas a peripherally attenuated variant produced a mixed plaque population with a significant number of minute plaques.

D. Growth in Tissue Cultures

Comparative studies on the kinetics of growth of an attenuated and a virulent strain of Venezuelan equine encephalitis virus in tissue cultures were performed by SCHERER *et al.* [1971]. In one-step growth curve experiments, it was found that in primary hamster embryonic cell cultures the wild strain grew slightly faster than the attenuated strain, though both viruses reached equal concentrations by 10–12 h after virus adsorption. In contrast, in the BHK 21 cell line, virulent virus grew and adsorbed at a distinctly slower rate and reached significantly lower maximal titers than attenuated virus. In primary chicken embryonic cell cultures, both viruses grew and adsorbed equally fast but virulent virus reached higher maximal concentrations. Thus, the growth characteristics of the viruses in hamster and chick cell cultures were not correlated with their pathogenicity *in vivo* in hamsters and chick embryos. An attenuated strain of Western equine encephalitis virus proved unable to grow at 42 °C in chick embryo tissue culture cells, and from studies with virulent revertants, it was suggested that mutation to avirulence and temperature sensitivity occurred independently [SIMIZU and TAKAYAMA, 1972]. With tick-borne encephalitis virus, MAYER and DOBROCKA [1969] detected that cultures of suspended human leucocytes in roller tubes supported the multiplication of monkey- and mouse-virulent virus clones but not of an attenuated virus clone.

E. Growth in Vertebrate Hosts

Using Semliki Forest virus, BRADISH *et al.* [1971, 1972] studied the heterogeneity of strain populations, their influence on the expression of virulence and the relation between virulence and the pattern of virus accumulation and decay in tissues of infected animals. The virulence of several early and serologically indistinguishable strains of four original isolations was defined quantitatively through the response of mice, guinea pigs and rabbits to intraperitoneal, respiratory and intracerebral administration of virus. Different host/route combinations could be set in a gradient of susceptibility against a sharply defined gradient of strain virulence. It was found that regardless of the ultimate response – death, incapacitation or benign protection – the efficiency of initial invasion and replication of virus was uniformly high for virus strains of extreme virulence or avirulence. A significant distinc-

tion between the pattern of infection by avirulent or virulent strains of virus was found in the observation that brains and spleens showed a higher incidence and level of infectivity for the virulent strains 24 h after intraperitoneal inoculation. By 72 h following intraperitoneal infection with avirulent virus, replication in the brains was suppressed. Similar observations were made by PUSZTAI *et al.* [1971]. These results do not support the concept of a distinct peripheral invasiveness for virulent and avirulent strains, but rather a differential in the efficiency with which lethal and protective responses may be initiated. A hint for the operation of such mechanisms was also obtained with Western equine encephalitis virus in hamsters [ZLOTNIK *et al.*, 1972]. It was demonstrated that a virulent and an avirulent strain replicated equally well in the CNS and produced lesions of encephalitis, but whereas in infections with the virulent strain there was 100% mortality, infection with the attenuated strain did not produce signs of disease. Furthermore, it was shown with Venezuelan equine encephalitis virus that an attenuated strain grew equally fast and to similar titers in the brain of hamsters but in this system the attenuated virus did not produce distinctive histopathological lesions [AUSTIN and SCHERER, 1971]. In contrast to the mentioned investigations with alphaviruses, attenuated and virulent strains of flaviviruses are apparently different with regard to peripheral invasiveness. Subcutaneous application of attenuated tick-borne encephalitis virus into goats did not produce viremia and no virus was excreted into the milk [MAYER *et al.*, 1967]; in goats and mice, the attenuated strain remained confined mainly to the regional lymphatic system [MÁLKOVÁ *et al.*, 1969]. An attenuated Langat virus strain was shown to possess a lower peripheral virulence for mice and spider monkeys than the virulent parent virus; after intracerebral infection the attenuated virus was characterized by its low pathogenicity [THIND and PRICE, 1966]. Attenuated dengue virus strains, with a relative avirulence for older mice, multiplied in the brain of these animals to significant titers which then fell to undetectable levels [COLE and WISSEMANN, 1969].

F. Growth in Arthropods

In the field the extent of viremia occurring after vaccination with attenuated virus and the capacity of the attenuated virus to multiply in arthropod vectors are important for ecologic implications such as transmission of the attenuated virus to other hosts and the possibility of reversion to virulence. HARDY [1966] reported that an attenuated strain of Western equine

encephalitis virus is able to multiply in *Culex tarsalis* mosquitoes. The attenuated TC-83 strain of Venezuelan equine encephalitis virus was isolated from a pool of *Psorophora confinnis* mosquitoes collected during the 1971 epizootic in the United States [PEDERSEN *et al.*, 1971], and an attenuated strain of bluetongue virus was shown to be transmitted from vaccinated to nonvaccinated sheep by the bite of *C. variipennis* [FOSTER *et al.*, 1968].

G. Reversion to Virulence

The genetic stability of attenuated virus considered for the production of vaccines demands careful examination. With the attenuated TC-83 strain of Venezuelan equine encephalitis virus some investigators have found – as already outlined in the previous article – that it can revert to the virulent form after serial passage in mice by the intracerebral route; other studies have revealed no evidence of reversion to virulence during serial passage of the virus by subcutaneous or intraperitoneal routes in small laboratory animals [SPERTZEL, 1971]. However, during serial back-passages of the TC-83 strain in horses, clinical responses increased in frequency and severity with a corresponding higher viremia, but young adult guinea pigs did not die after inoculation with back-passaged virus or with the same virus after suckling-mouse-brain passage [LUEDKE *et al.*, 1972]. The above-mentioned attenuated Western equine encephalitis virus obtained by treating the virulent virus with nitrosoguanidine reverted to the wild-type in the mouse brain with a high incidence [SIMIZU and TAKAYAMA, 1972].

H. Comments

The possibility of obtaining relatively easily attenuated strains of various arboviruses was confirmed in numerous experiments in the past decade. This and the occasionally observed reversion to virulence indicate a plasticity of these viruses and should be kept in mind when attenuated virus is considered for vaccine production. It was confirmed that plaque size and virulence are not necessarily correlated. Experimental evidence was obtained that avirulence of a virus depends on many factors such as decreased growth potency and change in cell tropism, but it may also be based on the ability to trigger effective host defense mechanisms.

VIII. Conclusions

In the past decade it became evident that arboviruses belong in different taxons in a classification system based on properties of the virion; these taxons in turn comprise viruses other than arboviruses. Consideration of the multiplication of arboviruses of different taxons demonstrates that the heterogeneity in the structure of the virion is also reflected in some distinct patterns of virus growth, at least in vertebrate cells. These patterns are characteristic for the taxon to which an arbovirus belongs; within a taxon no differences in the mode of multiplication could be found between arbo- and nonarboviruses. This poses some questions: Which properties have arboviruses, in spite of their structural heterogeneity, in common enabling them to multiply in vertebrate and arthropod cells? Does it mean that arboviruses of a given taxon have gained new genotypic properties which are missing in nonarboviruses of the same taxon, or have the nonarboviruses lost such properties? Answers to these questions would be helpful in understanding arbovirus evolution and it would be an advancement if in the future a review on the growth cycle of arboviruses in vertebrate and arthropod cells could be focused on these topics on the basis of experimental data.

Acknowledgments

The authors wish to express their appreciation to Miss G. FREDER for her efforts with regard to the typing of the manuscript and to Dr. J. Cox for correction of the manuscript.

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