Physicochemical and Morphological Relationships of some Arthropod-borne Viruses to Bluetongue Virus—
A New Taxonomic Group. Electron Microscopic Studies

By F. A. MURPHY,* E. C. BORDEN,† R. E. SHOPE‡
AND ALYNE HARRISON*

* Centre for Disease Control, Health Services and Mental Health Administration,
Atlanta, Georgia 30333
‡ Yale Arbovirus Research Unit, Department of Epidemiology and Public Health,
School of Medicine, Yale University, New Haven, Connecticut 06520

(Accepted 15 July 1971)

SUMMARY

The morphology and mode of maturation of a number of relatively solvent resistant arboviruses were examined by thin-section and negative-stain electron microscopy of infected mouse brain and cell culture specimens. These viruses, which have physicochemical properties distinct from other arboviruses, included Colorado tick fever, Tribec, Wad Medani, Chenuda, Irituia, Palyam, Lebombo, epizootic haemorrhagic disease of deer and bluetongue. They were 65 to 80 nm in diameter and matured in the cytoplasm as unenveloped particles with an electron-dense core. Virus development occurred in association with a cytoplasmic granular matrix and was accompanied by formation of regularly substructured filaments and tubules. Surface architecture was compatible with icosahedral symmetry with T = 3 (32 capsomeres). The combination of taxonomic parameters, morphologic and morphogenetic as well as physicochemical, was distinct from that of any presently recognized virus group. The independent classification of these viruses with bluetongue as the type virus is thus proposed.

INTRODUCTION

Physicochemical studies presented in the preceding paper suggested that certain arboviruses,§ including bluetongue, Colorado tick fever and about 25 others, constitute a new taxonomic group independent of the togaviruses§ (Borden, Shope & Murphy, 1971). The distinctive physical and serological properties of these viruses include: (1) an RNA genome, for which a double-stranded structure has been demonstrated in three members; (2) relative resistance to deoxycholate and lipid solvents; (3) lability at acidic pH; and (4) antigenic independence from other known virus groups. Previous electron microscopic studies of

† Present address: Oncology Division, John Hopkins University, Baltimore City Hospital, Baltimore, Maryland 21224.
§ 'Togavirus' is the name proposed by the Vertebrate Virus Subcommittee of the International Committee on Nomenclature of Viruses for viruses having the physical character of the group A, B, and Bunyamwera supergroup arboviruses. 'Arbovirus' will be used throughout this paper in its ecological sense, not as the name of a taxonomic group.
bluetongue (Studdert, Pangborn & Addison, 1966; Bowne & Jones, 1966; Ritchie & Bowne, 1967; Els & Verwoerd, 1969), Colorado tick fever (Murphy et al. 1968a; Oshiro & Emmons, 1968), African horse sickness (Polson & Deeks, 1963; Lecatsas & Erasmus, 1967; Breese, Ozawa & Dardini, 1969; Oellerman, Els & Erasmus, 1970), Kemerovo (Reingold et al. 1964; Shestopalova et al. 1964), Tribec (Gresikova, Mrena & Vachalkova, 1969), Corripartia (Carley & Standfast, 1969), and Eubenangee (Schnagl, Holmes & Doherty, 1969) viruses have all indicated that another common property of these viruses is a 'reovirus-like' morphology and mode of morphogenesis (Vasquez & Tournier, 1962; Dales, Gomatos & Hsu, 1965; Anderson & Doane, 1966). A capsomere number of 92 was suggested in earlier studies of several of these relatively solvent resistant arboviruses, primarily on the basis of similarities to reoviruses in size, morphogenesis, and edge capsomere number. More recently, a direct study of the surface architecture of bluetongue virus by Els & Verwoerd (1969) indicated that this virus has 32 morphological units (T = 3), an icosahedral structure quite distinct from that of reoviruses.

In order to extend the morphological observations on these relatively solvent resistant arboviruses, and thereby provide a broader basis for taxonomic grouping, thin-section and negative-stain electron microscopic studies were undertaken. We sought to further delineate common morphological and morphogenetic features, confirm icosahedral symmetry, and compare directly the morphological features of these viruses with those of bluetongue virus and each other.

METHODS

Viruses. The viruses described previously (Borden et al. 1971) were mouse brain passage isolates obtained from the collections of the arbovirus Reference Laboratory, Centre for Disease Control and the Yale Arbovirus Research Unit, Yale University. They included Tribec, Chenuda, Wad Medani, Irituia, Lebombo, Palyam, epizootic haemorrhagic disease of deer (N. J. strain) and Colorado tick fever (FLORIO strain) viruses. Bluetongue virus (BT-8 strain) was kindly provided by Dr J. G. Bowne, U.S. Department of Agriculture, Denver, Colorado. Virus stocks passaged in BHK 21 cells were prepared by one growth cycle of a 10^{-1} or 10^{-2} dilution of mouse brain material in 48 hr BHK 21 monolayers.

Animals. Litters of ICR Swiss mice, 1 to 2 days of age, were inoculated intracerebrally with 10^{-1} to 10^{-2} dilutions of each of the stock viruses. Brain tissue for thin section electron microscopy was harvested when the animals were moribund. The identity of the viruses was confirmed by complement-fixation tests using reference sera and antigens prepared from brains of litter-mates of the mice examined by electron microscopy.

Cell cultures. The baby hamster kidney, BHK 21 (C-13) cell line of MacPherson & Stoker, was obtained from the American Type Culture Collection (CCL-10) and was grown as described by Murphy et al. (1968a). Monolayers, inoculated with virus stocks passaged in BHK 21 cells, at multiplicities of 0.1 to 10 suckling mouse LD 50/cell, were harvested at early signs of cytopathology (usually 48 to 72 hr).

Electron microscopy. For thin-section electron microscopy, brain tissues were cut into 1 mm³ blocks and fixed for 2 hr at 4° in 2.5 % buffered glutaraldehyde. Culture cells were scraped from bottles and centrifuged at 630 g for 15 min.; the resulting pellets were thereafter treated in the same manner as brain tissue. Specimens were fixed for 30 min. in cold-buffered 1 % osmium tetroxide, dehydrated in a standard graded ethanol series, and embedded in an Araldite-Epon mixture (Mollenhauer, 1964). Sections were stained with uranyl acetate and lead citrate.

For negative contrast microscopy, several methods of virus growth and preparation were
Electron microscopy of some arboviruses

examined in order to obtain optimal resolution of particle surface structure. Virus was grown in 0.4% bovine albumin or in 0.5, 2.0 or 10% newborn calf serum; the virus was harvested at 12, 24, 48, and 96 hr of growth and was directly examined in the supernatant medium. The tendency of these viruses to remain cell associated until late in infection (Trent & Scott, 1964; Verwoerd, 1969) made cell breakage necessary; osmotic rupture, freeze-thawing, ultrasonic treatment, and mechanical disruption were all tested. Both droplet and spray negative contrast techniques were tried with variation of proportions of reactants in the staining mixtures. Both sodium silicotungstate and uranyl formate stains were tested.

In the most uniformly successful method, cells were maintained after infection with 2.0% newborn calf serum and were harvested at the time of peak cell-associated infectivity. Cells were suspended in supernatant fluid and were disrupted by 15 to 20 strokes in a Ten-Broeck homogenizer. This and all subsequent operations were performed at 4° or less. The suspension was centrifuged at 2250 g for 15 min. to remove cell debris, and the supernatant fluid diluted 1/2 with 0.002 M-tris-HCl buffer, pH 8.8 (Verwoerd, 1969). Virus was then concentrated by centrifugation at 100,000 g for 2 hr (Colorado tick fever virus was centrifuged at 35,000 g for 15 min. because of excessive particle damage under harsher conditions). Virus pellets were dispersed in 0.05% bovine albumin and 1.0% sodium silicotungstate at pH 7.0, and were sprayed on to carbon-coated grids with a glass nebulizer (Bowne & Ritchie, 1970). Grids were examined in a Philips EM-300 electron microscope operating at 60 kV for thin-section and 80 kV for negative-contrast specimens. The microscope and photographic enlargements were calibrated by use of a diffraction grating replica (54,864 lines/inch) (Ladd Research Industries, Burlington, Vermont) at each magnification and kv step.

RESULTS

Thin section electron microscopy

Mouse brain tissues and BHK 21 cells infected with the following solvent-resistant viruses were examined: Tribec, Chenuda, Wad Medani, Irituia, Lebombo, Palyam, epizootic haemorrhagic disease of deer, Colorado tick fever and bluetongue. Since virus morphology and morphogenesis did not differ in mouse brain and cell cultures, the observations are combined. Certain common characteristics of these viruses, which are exemplified well by Tribec virus, were noted in ultrathin section.

Tribec virus was found in the cytoplasm of infected cells in intimate association with granular, rather electron dense, matrices (Fig. 1). These matrices occurred randomly and varied widely in size, were irregular in shape and unbounded, and had virus particles either throughout or concentrated at the edges. Virus particles consisted of uniformly round, electron-dense double shells with a varying core structure (Fig. 2). Cores were either electron-transmitting or irregular electron-dense central masses. The diameter of Tribec virus particles was 65 to 75 nm.; core diameter was 50 nm. The double shell surface density observed in thin section probably resulted from staining the inner and outer surfaces of the virus capsid. Almost all presumably mature virus particles were unenveloped, whether located intra- or extracellularly. Prominent tubular and filamentous structures were closely associated with Tribec virus morphogenesis (Fig. 1, 3). Filaments occurred both within cytoplasmic virus matrices and at more distant sites. Lamellae of filaments often formed linear arrays. Tubules were of varying dimensions. but usually were similar to virus particles in diameter; they were distinct from microtubules and neurofilaments of normal cells. These structures occurred both in mouse brain and cultured cells.
Fig. 1. Tribec virus in newborn mouse brain. Virus particles (V) within and at the margin of an intracytoplasmic matrix (M). Filaments (F) and tubules occurred within such matrices and free within the cytoplasm.

Fig. 2. Higher magnification of Tribec virus. Particles had an electron dense double outer shell, considered the capsid, and a core which was either transparent (e) or contained an irregular dense mass (f).

Fig. 3. Tubules (F) found in association with Tribec virus (V) infection varied in dimensions, but usually were similar to virus particles in diameter.
Electron microscopy of some arboviruses

Wad Medani (Fig. 4) and Chenuda viruses (Fig. 5) are serologically related to Tribec virus. The particle size and morphology of all three were indistinguishable. Despite widespread destruction of brain architecture, foci of infection were difficult to find. Virus particles

Fig. 4. Wad Medani virus in newborn mouse brain. Within the matrix (M) virus particles (V) developed which consist of an electron dense core and surrounding moderately dense surface layer.

Fig. 5. Chenuda virus in BHK 21 cell. Amid the debris of cytoplasmic destruction, virus particles (V) and filaments or tubules (F) were especially prominent.

Fig. 6. Lebombo virus in newborn mouse brain; virus particles maturing within a rather reticular matrix.
were intimately associated with a granular cytoplasmic matrix in cells infected with each of these viruses. Filaments and tubules were similar to those found in cells infected with Tribec virus.

Lebombo virus (Fig. 6), Irituia virus (Changuinola serological subgroup) (Fig. 7), and Palyam virus (Fig. 8) all exhibited a similar morphology and mode and site of maturation. Virus particles were 65 to 70 nm. in diameter when centred in the plane of section. In some cells the virus matrix was more reticular (Fig. 7, 8) than granular; this observation, however, was not virus-specific. The morphology of the virus of epizootic haemorrhagic disease of deer was indistinguishable from that of the other viruses studied. Unenveloped 62 nm. particles occurred in association with an intracytoplasmic matrix. Electron-transparent cores, possibly in immature virus particles, were often observed (Fig. 9). This is in accord with results obtained by Tsai & Karstad (1970). Colorado tick fever virus has a similar morphology and morphogenesis (Murphy et al. 1968a). A representative field (Fig. 10) is included for comparative purposes.

For the purpose of direct comparison, bluetongue virus was examined in thin section (Fig. 11); all aspects of this infection were similar to those described for cells infected with other relatively solvent resistant viruses. Morphogenesis took place in association with intracytoplasmic matrices. Matrices varied in character from granular to filamentous in different cells of the same culture. Cytoplasmic tubules, approximately the same diameter as virus particles, were especially numerous (Fig. 11). Other than in Colorado tick fever virus infection (Murphy et al. 1968a), no intranuclear tubules or filaments were observed with any of the viruses studied.

The brains of uninoculated control animals and cell cultures appeared normal.

**Negative contrast electron microscopy**

Irituia virus was representative of the viruses examined. The slight differences in morphological detail of the other viruses are described individually. Irituia virus particles were found in large numbers: they were round and varied in the amount of surface structure resolved (Fig. 12, 13). Particles had a moderate size range (65 to 80 nm.) with a mean of 75 nm. They had large doughnut-shaped capsomeres on their surfaces, which were 10 to 15 nm. in width with a hollow centre 2 to 3 nm. in diameter. Individual pentameric and hexameric capsomeres were identified, but it was not possible to characterize enough of these on a single surface to fulfil the criteria established by Caspar & Klug (1962) for icosahedral symmetry. Assuming icosahedral symmetry, models were constructed on a basis of the ratio of capsomere to whole particle dimensions as measured on micrographs. These resulted in a 32 capsomere icosahedron for which \( T = 3 \). Capsomeres on particle edges appeared U-shaped, 8 to 12 nm. in depth and consistent in dimensions with surface capsomeric doughnuts (Fig. 13). Each pair of adjoining edge projections represented one capsomere. Edge detail was especially well resolved on particles penetrated by the negative contrast medium; on such particles edge projections were often missing from limited areas, whereas spherical continuity was provided by an electron-transparent layer basal to the capsomere layer. Where virus degradation was further advanced, all surface continuity was destroyed and particles opened into ‘pie-shaped’ forms (Fig. 14). No evidence of internal structure was evident. Occasionally, one or two Irituia virus particles were contained within a loose-fitting membranous envelope, but the great majority of particles were unenveloped even in crude preparations.

Tribec virus (Fig. 15) was similar in size (70 nm. mean diameter) and structure to Irituia virus. Despite repeated attempts with varied techniques, capsomere structure was not
Fig. 7. Irituia virus illustrating the reticular variation of the virus matrix. This kinky thread appearance of matrix has also been observed with reoviruses.

Fig. 8. Palyam virus in newborn mouse brain. Virus particles (V) within an intracytoplasmic matrix (M) of rather reticular character.

Fig. 9. Virus of epizootic haemorrhagic disease of deer in newborn mouse brain. Unenveloped, 62 nm. virus particles (V) were found in association with granular matrices (M).

Fig. 10. Colorado tick fever virus in BHK 21 cell. Virus particle (V) morphology and morphogenesis from intracytoplasmic matrices (M) were identical to the other viruses studied.
Fig. 11. Bluetongue virus (V) in BHK 21 cell; particles were indistinguishable from the other viruses studied. Tubules (T) and filaments were especially prominent; granular and reticular matrices were also associated with sites of virus maturation.
resolved as distinctly as on Irituia particles. In addition, Tribec virus preparations contained moderate numbers of tubular structures of varying diameter and length (Fig. 16). Many of these were approximately the same diameter as virus particles, and their substructural dimensions (striation interval) were similar to those of virus capsomeres.

The virus of epizootic haemorrhagic disease of deer displayed especially pronounced...
tecture was not well resolved previously; superimposition of top and bottom surface details hindered structural analysis. Only after a briefer, lower speed centrifugation than usual were the characteristic doughnut capsomeres consistent with icosahedral symmetry resolved (Fig. 21, 22). Radial division of capsomeres into 5 or 6 units was much more evident than with any of the other viruses. As reported previously, the mean diameter was 80 nm. Under all preparative conditions, a much higher proportion of Colorado tick fever virions than of the other viruses had dark centres due to stain penetration. Particles penetrated by the

Fig. 15. Tribec virus with surface architecture similar to that of Irituia virus.

Fig. 16. Tubule in Tribec virus preparation. Its diameter is approximately that of virus particles and its cross-striation interval is similar to capsomere diameter.

Fig. 17. Virus of epizootic haemorrhagic disease of deer. Surface capsomeres probably viewed along an axis of six-fold symmetry.

Fig. 18. Large tubules in a preparation of the virus of epizootic haemorrhagic disease of deer. Repeating subunit structure with dimensions similar to that of capsomeres suggested possible construction from virus subunits.
Electron microscopy of some arboviruses

Electron microscopy of some arboviruses

contrast medium had a thin very electron-transparent layer basal to surface capsomeres; similar substructuring of Irituia and other virus particles was observed.

For a direct comparison, bluetongue virus was subjected to the same preparative and staining techniques. Particles identical to those described by Els & Verwoerd (1969) and Bowne & Ritchie (1970) were present in large numbers. Capsomere organization was identical with that of the other viruses studied (Fig. 23). The mean diameter of 100 particles measured was 68 nm. Other recurrent features common to members of the group included

stain penetration, which made the thin transparent layer beneath capsomeres especially prominent (Fig. 23), characteristic particle degradation without evidence of internal structure (Fig. 24), and tubules of regular subunit construction in all preparations examined (Fig. 25).

Several other ungrouped arboviruses which are labile in lipid solvent have been examined by thin-section and negative-contrast electron microscopy in our laboratory. None had the morphology or morphogenesis described for the solvent-resistant viruses. Some were found similar to viruses of the Bunyamwera supergroup and some were rhabdoviruses. One other arthropod-transmissible virus resistant to lipid solvents, Nodamura, has been described; however, Murphy et al. (1970) have recently shown it to be a picornavirus.
DISCUSSION

The similar morphology and mode of morphogenesis of the relatively solvent-resistant arboviruses support the common grouping originally suggested by physicochemical studies (Borden et al. 1971). As observed by thin-section electron microscopy, these viruses de-

Fig. 23. Bluetongue virus with characteristic superimposition of top and bottom images. In a direct comparison, particle morphology was very similar to that of the other viruses examined.

Fig. 24. Degraded bluetongue virus particles with missing segments.

Fig. 25. Tubules in bluetongue virus preparation, indistinguishable from those seen with several of the other viruses. Two virus particles at right.

dveloped from a granular or reticular cytoplasmic matrix, and with rare exception, were unenveloped. Filamentous and tubular structures were characteristically associated with their sites of maturation. Such virus particle maturation entirely within the cytoplasm and
Electron microscopy of some arboviruses

from a granular matrix is a characteristic quite distinct from that of togaviruses, and is more suggestive of the reoviruses. The group A togaviruses develop from a nucleoid by budding through plasma or cytoplasmic organelle membranes (Morgan, Howe & Rose, 1961). Group B togaviruses presumably also bud and accumulate within proliferated endoplasmic reticulum (Murphy et al. 1968b). Similarly, Bunyamwera supergroup viruses accumulate within distended vacuoles as a result of budding (Murphy, Harrison & Tzianabos, 1968). These morphogenetic differences represent one of the major taxonomic distinctions between the solvent-resistant arboviruses and the togaviruses. The matrix in cells infected with the solvent-resistant arboviruses appeared more discrete than that associated with reoviruses, but like reoviruses was always spatially associated with virus maturation. The intimate relationship of matrix to virus maturation in reovirus infection has been confirmed by the immuno-ferritin technique (Dales & Gomatos, 1965). Tubules and filaments in cells infected with the solvent-resistant arboviruses were also found at sites of virus maturation. The tubules were usually similar in diameter to virus particles and, considering their regular substructure observed in negative contrast, may represent virus subunits (capsomeres) assembled in an anomalous manner. Aberrant self-assembly of repeating subunits, particularly hexameric capsomeres, may result in tubular structures (Caspar & Klug, 1962); these have been described in polyomavirus (Howatson & Almeida, 1960) and herpesvirus (Murphy, Harrison & Whitfield, 1967) preparations.

The capsid architecture of all the viruses examined was strongly indicative of a common icosahedral construction. By direct comparison, capsomere arrangement was indistinguishable from bluetongue virus. Els & Verwoerd (1969) concluded that the bluetongue virus particle is an icosahedron with T = 3 (32 capsomeres). As recognized by Els & Verwoerd, direct visualization of surface structure of viruses such as those studied here is hindered by the rather small number of morphological units visible on a single surface of individual particles, by superimposition of capsomeres from both surfaces, and by virus particle fragility and the resultant subtle or frank degeneration. Our most valuable tool was the construction of models based upon ratios of capsomere to total particle dimensions. In lieu of establishing the rigorous requirements for relationships between neighbouring pentamers as set forth by Caspar & Klug (1962), this approach in each case served to confirm the T = 3 (32 capsomere) structure and to eliminate the T = 4 (42 capsomere) structure, the only serious alternative. Precise structural characterization may depend upon a three-dimensional analysis, such as that recently described using a computerized Fourier analysis (Crowther et al. 1970).

Although physicochemical and morphological features of the solvent-resistant arboviruses have been quite consistent, there are conflicting interpretations of certain details. Enveloped forms of bluetongue (Bowne & Jones, 1966; Ritchie & Bowne, 1967; Bowne & Ritchie, 1970) African horse sickness (Breese et al. 1969), Eubenangee (Schnabl et al. 1969), and Colorado tick fever (Murphy et al. 1968a) viruses have been described. Els & Verwoerd (1969) interpreted surrounding membranes in bluetongue virus preparations as ‘pseudoenvelopes’ since they occurred on a very small proportion of particles. Only rarely were enveloped particles found in these thin section or negative contrast specimens. The predominant mode of virus release appeared to be by cell lysis; reoviruses are released in an analogous manner. The viruses examined ranged from 60 to 80 nm.; in general, particles with fuzzy surfaces were slightly larger than those more definitely structured. Overall, our size estimates are larger than those of others: Ritchie & Bowne (1967) found bluetongue virus particles to be 55 to 110 nm. in diameter with the larger particles covered by additional surface layers. Els & Verwoerd (1969) reported that the same virus had a mean diameter of 54 nm. and a very narrow size
range. Bluetongue virus in our study had a diameter of 68 nm. and a very narrow size range. We are unable to reconcile this interlaboratory variation of about 15%. Recalculation of the diameter of Colorado tick fever showed no variation from the 80 nm. mean reported previously (Murphy et al. 1968a).

Some virus particles in all of the preparations examined were deeply penetrated by the negative contrast medium, but no internal structure was ever visualized. However, a thin electron transparent zone basal to the doughnut capsomeres was resolved by stain penetration. This zone, especially prominent in Colorado tick fever virus particles, is also seen in the micrographs of Irituia (Fig. 12, 14) and bluetongue viruses (Fig. 23). It was previously considered analogous to the inner capsid (subcapsid) of reoviruses but, until better resolved, might alternatively be considered an elaboration of a single outer capsid.

<table>
<thead>
<tr>
<th>Virus group</th>
<th>RNA*</th>
<th>Enveloped</th>
<th>Symmetry</th>
<th>Capsomers</th>
<th>Lipid solvents</th>
<th>pH 3</th>
<th>Arthropod cycle in nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reovirus</td>
<td>ds</td>
<td>No</td>
<td>Cubic</td>
<td>92</td>
<td>Absolutely resistant</td>
<td>Resistant</td>
<td>-</td>
</tr>
<tr>
<td>Togavirus</td>
<td>ss</td>
<td>Yes</td>
<td>Cubic (†)</td>
<td>32 (†)</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>+</td>
</tr>
<tr>
<td>Bluetongue-like</td>
<td>ds</td>
<td>No</td>
<td>Cubic</td>
<td>32</td>
<td>Relatively resistant</td>
<td>Sensitive</td>
<td>+</td>
</tr>
</tbody>
</table>

* ds, Double-stranded; ss, single-stranded.
† Shown for only one virus (Horzinek & Mussgay, 1969).

We conclude that the combination of taxonomic parameters of the solvent resistant arboviruses is distinct from that of presently existing virus groups (Table 1). Current taxonomic schema group together viruses having similar nucleic acid type, capsid size, symmetry, and capsomere number, and morphogenetic features as manifested by envelopment and assembly site. We therefore propose, in agreement with Verwoerd and his colleagues (Els & Verwoerd, 1969; Verwoerd, 1969; Verwoerd, 1970), that these viruses be placed in a new virus group with bluetongue as the type virus. Placing the bluetongue-like viruses into a new group maintains the present precise definition of reoviruses and confirms the efficacy of a precise definition of togaviruses. Exact placement in the animal virus classification schemata currently in use depends upon the importance assigned to the various taxonomic characteristics. However, the probably double-stranded nature of the genome of the bluetongue-like viruses would seem to be the most fundamental property. It suggests a parallel taxonomic, and possibly evolutionary, relationship of these viruses to reoviruses.

We are indebted to Mr. G. William Gary, Jun. for his excellent assistance in these studies. The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. Use of trade names is for identification and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education and Welfare.
REFERENCES


(Received 21 May 1971)