Preparation of Noninfectious Arbovirus Antigens

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Noninfectious arbovirus antigens were prepared from borate-saline suspensions of infected sucking mouse brain buffered with tris(hydroxymethyl)aminomethane and treated with β-propiolactone (BPL). The activity and stability of these antigens were enhanced by altering the buffering system, by passing the virus seed through a series of four or more continuous passages in the brains of sucking mice, or by a combination of these procedures. The titers of group A and B arbovirus antigens were comparable to titers of antigens extracted by the conventional sucrose-acetone-BPL (SA-BPL) method. Antigens prepared from some ungrouped and Bunyamwera arboviruses by either the borate-saline-BPL or SA-BPL method produced inconsistent results and will require the development of more unique procedures to obtain suitable hemagglutinating antigens.

In our serological investigations of arboviruses, it has been necessary to prepare and maintain numerous hemagglutinating (HA) and complement-fixing (CF) antigens. With many viruses, this has been a difficult task, because some arbovirus suspensions do not yield HA antigens without cumbersome and hazardous extraction procedures. The sucrose-acetone (SA) extraction procedure described by Clarke and Casals (2) has been one of the most successful methods devised for preparing arbovirus antigens, but the method involves the use of copious volumes of a potentially explosive solvent and risks of infecting unprotected personnel. French and McKinney (3) improved upon methods for antigen production by rendering antigens noninfectious through treatment with β-propiolactone (BPL). In our laboratory, the SA method had been the primary procedure used to prepare hemagglutinins, but, in rendering these preparations noninfectious by treatment with BPL, we were not always successful in obtaining a stable antigen. Antigen instability appeared to result from a decrease in pH and was associated with a granular precipitation. HA patterns with some BPL-treated SA antigens were poorly defined and sometimes difficult to interpret.

Efforts to improve the stability and reactivity of the noninfectious antigens led to an investigation of the pH stability after the addition of BPL. Tris(hydroxymethyl)aminomethane (Tris) buffer was substituted for phosphate buffer, and the alkaline saline procedure was substituted for SA extraction to yield a simple method for producing HA and CF antigens for numerous arboviruses. This report describes this simplified procedure and its application for several arboviruses.

MATERIALS AND METHODS

Viruses. Viral seeds were prepared as 10% suspensions of infected infant mouse brains. Strain designations and passage level (P), if known, for the respective viral seeds were: Venezuelan encephalitis, Trinidad donkey brain, P14; Eastern encephalitis, Louisiana-SC7, P13; Western encephalitis, McMillan, P7; chikungunya, Halstead BAH 306, P3; Mayaro, strain unknown, P11; Semiliki Forest, strain unknown, P4+; Bebaru, AMM 2354, P7; Getah, AMM 2021, P5; O'nyong-nyong, Osege, P6; Sindbis, AR339, P27; Una, BT1495-3, P10; Middelburg, strain unknown, P3+; Aura, BeAr10315, P7; yellow fever, Asibi, P4+; St. Louis encephalitis, Hubbard, P102; St. Louis encephalitis, strain unknown, P2+; Japanese B encephalitis, Peking, P4+; dengue type II, New Guinea-B, P44; Powassan, M794, P7; Rio Bravo, 4912, P10; Langat, TP-21, P6+; West Nile, Eg101, P8; louping ill, H3636, P19; Rift Valley fever, van Wyk, P6, van Rift Valley fever (neurotropic), Smithburn, P102.

Hemagglutination test. Virus hemagglutinins were titrated with microtiter equipment by use of the procedures for arboviruses described by Clarke and Casals (2). Each new virus antigen was examined for optimal pH and temperature during testing. All HA titers were recorded as the highest dilution showing greater than 50% agglutination.

CF tests. Antigens were assessed for CF activity by checkerboard titrations against homologous immune serum, with the use of 2 exact units of complement and 3 units of hemolysin. CF end points were defined as the greatest dilution of antigen showing 50% hemolysis in the presence of a constant dilution of antiserum containing at least 8 units of antibody.

Borate-saline-BPL (BS-BPL) extraction procedure.
Although variations of the BS-BPL extraction procedure were tried, the procedure adopted was as follows. (i) Infectious brain was triturated and suspended in BS (pH 9.3) to 10 to 15% concentration. (ii) To this preparation, 0.1 volume of 1.0 M Tris buffer was added, (iii) and BPL was added to a 3% concentration (may be omitted in subsequent extractions of pellet). (iv) The suspension was held at 4°C for 18 to 24 hr with intermittent shaking, and then (v) was centrifuged at 12,000 × g for 1 hr. (vi) The supernatant fluid was decanted and tested for HA titer. (vii) The pellet was suspended in one-half to two-thirds the original volume of BS, and steps ii through vi were repeated.

The suspending diluent for infectious mouse brains was buffered BS, pH 9.3 or 9.0 (2), to which were added 100 units of sodium penicillin per ml and 100 μg of streptomycin sulfate per ml. The pellet obtained at step v could be re-extracted as many as four times before being discarded. Successive BS extractions may also be treated with Tris and BPL to disperse aggregates, but additional BPL is detrimental for some viral antigens.

SA extraction procedure. The SA method for preparing hemagglutinins was included in these investigations for comparison with the BS-BPL method. The SA procedures were essentially those described by Clarke and Casals (2), up to and including the step where the final acetone precipitate is resuspended. This precipitate was resuspended in BS (pH 9.0), held at 4°C overnight, and centrifuged at 12,000 × g for 1 hr; the resulting supernatant liquid was saved, and the pellet was resuspended as before. Each resuspension and centrifugation constituted one saline extraction of the acetone precipitate. Various treatments of these extracts with BPL and Tris buffer are described below.

RESULTS

Interaction of BPL and Tris buffer in BS-BPL extraction method. Past experience with BPL indicated that 0.15% BPL was sufficient to kill most arboviruses in 10 to 20% (v/v) suspensions of infected tissues. However, concentrations of BPL greater than 0.15% appeared to improve the dispersion or deaggregation of viruses. Increasing the concentration of BPL to 0.2 or 0.3% also required an increase in buffer to maintain an alkaline pH during the hydrolysis of BPL. A shift to an acid pH was often associated with a decrease in antigen titer, and the retention of the pH between 8.0 and 9.0 appeared to be necessary for antigen stability. When the concentrations of phosphate buffer recommended by French and McKinney (3) were increased necessarily to control pH, a precipitate often formed and hemagglutinin titers were lost or reduced. Therefore, phosphate buffer was replaced with Tris buffer, which proved to have a greater stabilizing effect on the hemagglutinins. The concentration of Tris buffer necessary to maintain the pH above 8.0 was determined empirically. Figure 1 shows the pH shift with respect to time of 20% suspensions of normal brain in BS (pH 9.0) after the addition of BPL to a concentration of 0.3%. Each suspension was held at 4°C during the 96-hr assay, except for brief intervals when it was warmed to 10°C for pH measurement. The pH of the sample without Tris buffer dropped below 7.0 within 30 min and to pH 5.4 by 96 hr. It was apparent from these data that at least 0.1 M Tris buffer was required to maintain a pH of 8.0 to 9.0.

Fig. 1. Buffering capacity of Tris in 20% normal suckling mouse brain treated with 0.3% BPL.
**Group A arboviruses.** Antigens of several group A viruses that were prepared by BS-BPL extraction are listed in Table 1. Viruses such as Venezuelan equine encephalitis, Eastern equine encephalitis, Western equine encephalitis, chikungunya, Una, Middelburg, Getah, and Aura yielded antigens with acceptable HA titers after a single passage in suckling mouse brain. These viruses have designated avid HA producers. In our hands, other group A viruses such as Mayaro, Semliki Forest, Bebaru, Sindbis, and O’nyong-nyong yielded poor or inconsistent HA titers unless they were subjected to four or more successive passages in suckling mouse brain. This was particularly true of Mayaro and Bebaru, which were carried through 10 continuous passages in suckling mice to obtain a suitable hemagglutinin.

Venezuelan encephalitis virus is one of the more avid HA producers and a good model to illustrate a comparison between the SA-BPL and BS-BPL extraction methods. The pellet of the initial BS-BPL extraction was re-extracted three more times with BS (pH 9.0); the second acetone precipitate of the SA extraction was extracted by resuspending the precipitate in BS (pH 9.0) containing 0.1 M Tris, adding 0.2% BPL, holding it for 16 to 20 hr at 4°C, and centrifuging it at 12,000 × g for 1 hr. The supernatant liquid was designated the first saline extract. Tris and BPL were omitted in subsequent saline extractions of the SA pellet to yield the second, third, and fourth saline extracts. All extracts were assessed for HA titers (Table 2). There was a fourfold decrease in titer of each successive extract of the SA-BPL method and a similar rate of decrease after the second saline extraction of the BS-BPL method. The increase in titer between the first and second saline extraction was observed with Venezuelan encephalitis and several other viruses subjected to BS-BPL extractions, but was seldom observed with the SA extraction method. The difference in HA titers between corresponding extracts obtained by the two methods was not a function of the volumes, because the total volume of all four BS-BPL extracts was 258 ml versus only 75 ml for the combined volume of the SA extracts. Since both procedures started with equal portions of a single lot of mouse brain, the BS-BPL method yielded more hemagglutinins than did the SA method.

The effect of mouse age on yields of HA and CF antigens from brains was investigated with Venezuelan encephalitis virus. Mice between 4 and 21 days of age appeared to be equally susceptible to Venezuelan encephalitis virus inoculated intracerebrally, but the antigen yields by the BS-BPL method from 21-day-old mice were definitely poorer than from 14- or 4-day-old mice, as indicated by the titers presented in Table 3. Virus infectivity titers in these brains were not appreciably different among these age groups, but apparently the expression of antigens was suppressed in preparations of brains from the older mice.

A first suckling mouse brain passage of O’nyong-nyong virus was prepared by the BS-BPL method. This antigen showed no HA activity, but had a CF titer of 1:64. The same virus seed was carried through four successive passages in suckling mice, and the brains of the fourth passage were subjected to three successive extractions by the BS-BPL method. All three extracts had acceptable HA titers of 1:256, and these were maintained after lyophilization.

**Group B arboviruses.** Table 4 shows the HA and CF titers of many of the group B arbovirus antigens we have prepared by the BS-BPL method. Most group B viruses proved to be avid antigen producers, but some, like dengue type II virus, required several continuous passages in suckling mice before an acceptable HA antigen was obtained. Although these viruses were gen-

| Virus antigen                  | HA titer | CF titer |
|-------------------------------|----------|----------|
| Venezuelan encephalitis       | 4,096    | 48       |
| Eastern encephalitis          | 2,048    | 64       |
| Western encephalitis          | 512      | 16       |
| Chikungunya                   | 128      | 32       |
| Mayaro                        | 512      | 32       |
| Semliki Forest                | 128      | 40       |
| Bebaru                        | 1,024    | 16       |
| Getah                         | 64       | 16       |
| O’nyong-nyong                 | 256      | 64       |
| Sindbis                       | 512      | 16       |
| Una                           | 32       | NDb      |
| Middelburg                    | 64       | ND       |
| Aura                          | 128      | 32       |

a Virus passed through four or more continuous passages in suckling mice; all other viruses were passed only once.

b Not done.

**Table 2.** Venezuelan encephalitis antigens prepared by BS-BPL and SA-BPL extraction

| Saline extraction | HA titer (reciprocal) |
|-------------------|-----------------------|
|                   | BS-BPL extraction     | SA-BPL extraction |
| First             | 1,024                 | 2,048             |
| Second            | 4,096                 | 512               |
| Third             | 1,024                 | 128               |
| Fourth            | 256                   | 32                |

The increase in titer between the first and second saline extraction was observed with Venezuelan encephalitis and several other viruses subjected to BS-BPL extractions, but was seldom observed with the SA extraction method. The difference in HA titers between corresponding extracts obtained by the two methods was not a function of the volumes, because the total volume of all four BS-BPL extracts was 258 ml versus only 75 ml for the combined volume of the SA extracts. Since both procedures started with equal portions of a single lot of mouse brain, the BS-BPL method yielded more hemagglutinins than did the SA method.

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TABLE 3. Effect of mouse age on Venezuelan encephalitis virus antigen

| Age of suckling mice inoculated | Maximal HA titer | CF Titer |
|---------------------------------|-----------------|---------|
| days                            |                 |         |
| 4-6                             | 4,096           | 48      |
| 14                              | 1,024           | 24      |
| 21                              | 256             | 4       |

* Extracted by the BS-BPL method.

TABLE 4. Group B arbovirus antigens prepared by BS-BPL extraction

| Virus antigen          | Maximal HA titer | CF titer |
|------------------------|------------------|---------|
| Yellow fever           | 2,048            | 20      |
| St. Louis (Price strain)* | 128   | ND      |
| St. Louis (Hubbard strain) | 64 | 16      |
| Japanese B (Peking strain) | 256 | 16      |
| Dengue II Ha*          | 128              | 16      |
| Powasson               | 2,048            | 64      |
| Rio Bravo              | 2,048            | 16      |
| Langat, TP-21          | 4,096            | 20      |
| West Nile             | 32               | ND      |
| Louping ill*           | 512              | 4       |

* Three or more continuous passages in suckling mice.

* Not done.

Generally avid hemagglutinin producers, the HA antigens were quite sensitive to inactivation by excessive BPL treatment. One BPL treatment during the initial BS-BPL extraction was sufficient to render the antigens noninfectious. Further BPL treatment of subsequent saline extractions eliminated all HA activity.

The effects of excessive BPL treatment were best exemplified by the Asibi strain of yellow fever virus. Brains from yellow fever-infected suckling mice were divided into two equal lots, A and B. These lots were triturated and suspended in BS (pH 9.0) containing 0.07 M Tris. Lot A received no BPL on either the first or second saline extraction. Lot B was treated with 0.3% BPL on both the first and second saline extractions. As the HA titers presented in Table 5 indicate, first saline extracts of both A and B had high HA titers. The second extraction of preparation A was a much poorer antigen than the first, and there was no HA activity at all in the second BS-BPL extract of preparation B. When the first BS-BPL extract of antigen B was retreated with BPL, all HA activity was lost. It was concluded from these results that one BS extraction with BPL and a second without BPL might yield high titers of noninfectious yellow fever hemagglutinin. This was tried with a third lot of infected brains with the use of 0.1 M instead of 0.07 M Tris, and an excellent antigen was obtained (Table 6). Lyophilization of this antigen caused a substantial loss of titer, but the dry product maintained an acceptable level of HA activity.

Other arboviruses. The BS-BPL extraction method was tested on two viruses of the Bunyamwera group, Bunyamwera and Germiston, and with an ungrouped arbovirus, Rift Valley fever. The initial passage of Bunyamwera virus produced a 1:128 HA titer and a CF titer of 1:8 from the original saline extraction. Additional saline extractions had unacceptable HA titers. The virus seed was then subjected to four successive passages in suckling mice in an effort to improve the yield of hemagglutinins. For reasons not presently understood, no HA activity was obtained from infected brains of any of these additional passages.

Germiston virus had undergone three successive passages in suckling mice. Antigens prepared from brains of each passage by the BS-BPL method yielded no HA activity until they were treated with protamine sulfate according to procedures described by Clarke and Casals (2). HA titers of 1:256 were obtained from each passage level, but this antigen could not be used in the CF test because of its anticomplementary activity.

Pantropic and neurotropic strains of Rift Valley fever virus were examined for hemagglutinins.

TABLE 5. HA activity of yellow fever virus with and without BPL treatment

| Saline extraction | HA titers | Antigen A, no BPL | Antigen B, 0.3% BPL |
|-------------------|-----------|-------------------|-------------------|
| First             | 2,048     | 1,024             |
| Second            | 128       | <2                |
| First antigen B extraction retreated with BPL | NR | <2 |

* Antigens extracted from 20% suckling mouse brains in borate saline + 0.07 M Tris buffer.

* Not run.

TABLE 6. HA activity of yellow fever virus after a single BPL treatment

| Saline extraction | HA titer |
|-------------------|----------|
| First             | 2,048    |
| Second            | 1,024    |
| First and second pooled | 2,048 |
| Lyophilized pooled antigen | 256 |

* Antigen extracted from 15% suckling mouse brains in borate saline + 0.1 M Tris + 0.3% BPL.
tinin production. Several variations of the BS-BPL method were tested, but all failed to produce HA activity. Suitable hemagglutinins were prepared by a modification of the SA-BPL method; these will be described in another publication.

SA-BPL antigens have not been prepared from all of the virus seeds listed for the BS-BPL antigens. However, antigens prepared by either method appear to perform at the same optimal pH and temperature. Both antigen preparations have been compared in routine hemagglutination-inhibition antibody studies, and similar hemagglutination-inhibition titers against 8 units of hemagglutinin were observed. These comparisons have been limited to the more avid hemagglutinin-producing viruses of groups A and B arboviruses.

**DISCUSSION**

The BS-BPL procedure has three fundamental advantages: (i) it eliminates the hazards associated with the use of volatile, flammable lipid solvents for the removal of nonspecific inhibitors, (ii) it reduces the chances of denaturing antigens during extraction with lipid solvents and removal of volatile residues, and (iii) it greatly reduces handling of infectious material during the extraction period.

A second successive extraction with borate-buffered saline, with or without BPL, frequently led to a considerable increase in antigen yield from the brain material. Subsequent extractions of the same material led to continuously decreasing HA titers. Two possible processes might explain the observed change in HA titers: (i) the first extraction may remove or dilute inhibitors of viral hemagglutination, and (ii) aggregates of antigen may be dispersed by the action of BPL or products of its hydrolysis.

The group A virus antigens sustained, and in many cases required, more than one BPL treatment to improve HA activity. On the other hand, more than one BPL treatment of the group B viruses destroyed all HA activity. Results of a similar nature were observed by French and McKinney (3), and indicate that group B hemagglutinins and inhibitors are less stable to BPL treatment than those of group A. BS-BPL preparation of Bunyamwera, Germiston, and Rift Valley fever viruses was not entirely successful, and multiple BPL treatment of the virus extracts failed to improve the antigen. The above observations support the hypothesis of Nicoli (5) and Salminin (6) that more than one type inhibitor is associated with mouse brain suspensions, and that viruses differ in their sensitivities to these inhibitors; thus, extraction procedures must be defined according to the sensitivities of the virus. Tris buffer has a pKₐ of the pH of the mid-point of buffering range) of 8.3 at 20 C. This is a suitable pH for stability of arbovirus antigens during the hydrolysis period. Tris proved to be a suitable buffer and was never accompanied by interfering precipitates or unfavorable shifts in pH during antigen preparation.

Some strains of virus show a greater ability to produce hemagglutinin than do others. The use of successive virus passage proved useful for enhancement of less avid antigen-producing strains of group A and B arboviruses, but had an adverse effect on Bunyamwera and Germiston virus. Although, the relationship between virus passage and antigen production was not studied, it was empirically determined that optimal HA titers were frequently obtained on the 4th, 8th, and 12th passage of virus, suggesting a cyclic alteration of the virus with regard to expression of hemagglutinin. This was observed particularly with Mayaro, Bebaru, Semliki Forest, and O'nyong-nyong viruses. Similar results were recorded by Chanock and Sabin (1) in their study of St. Louis encephalitis virus.

The BS-BPL antigens appear to be reasonably stable during storage in the wet state at -30 C or at 4 C after freeze-drying. Some deterioration was noted by the first week in the quality of HA patterns or titers observed from stored or freeze-dried antigens, particularly among the less avid hemagglutinin producers of group B. This problem was overcome by adding sucrose to the suspending medium prior to BPL treatment, by passing the virus seed through three to four more passages in suckling mice, or by a combination of these procedures. The sucrose appeared to protect the antigen in a manner similar to its action in the SA-BPL method, but the enhancement of stability of passage of the virus in mice is unexplained.

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