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TECHNICAL MANUSCRIPT 509

PREPARATION OF NONINFECTIOUS ARBOVIRUS ANTIGENS

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APRIL 1969

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PREPARATION OF NONINFECTIOUS ARBOVIRUS ANTIGENS

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BIOLOGICAL SCIENCES LABORATORIES

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

ABSTRACT

Noninfectious arbovirus antigens were extracted from saline suspensions of infected suckling mouse brain that were buffered with tris(hydroxymethyl)aminomethane and treated with beta-propiolactone (BPL). The activity and stability of several antigens were enhanced by altering the buffering system and by rapid, continuous passage of the virus in suckling mice. For several viruses of groups A and B, the titers of antigens extracted with borate saline-BPL (BS-BPL) were comparable to titers of antigens extracted with sucrose-acetone-BPL. Results from some ungrouped and Bunyamwera arboviruses required the development of unique and more sophisticated procedures for the preparation of suitable antigens.

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I. INTRODUCTION*

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[1] has been one of the most successful methods devised for preparing arbovirus antigens, but the method involves copious volumes of a potentially explosive solvent and risks of infecting unprotected personnel. French and McKinney[2] have improved upon methods for antigen production by rendering antigens noninfectious through treatment with beta-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 following the addition of BPL. Tris buffer [tris(hydroxymethyl)aminomethane] 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.

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II. MATERIALS AND METHODS

A. 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 (VE), Trinidad donkey brain, P14; eastern encephalitis (EE), Louisiana-SC7, P13; western encephalitis (WE), McMillan, P7; chikungunya, Halstead BAH 306, P3; Mayaro, strain unknown, P11; Semliki 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 (SLE), Hubbard, P102; SLE, strain unknown, P2+; Japanese B encephalitis (JBE), 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; and Rift Valley fever (neurotropic), Smithburn, P102.

B. HEMAGGLUTINATION TEST

Virus hemagglutinins were titrated with microtiter equipment using the procedures for arboviruses described by Clarke and Casals.[1] 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.

C. COMPLEMENT FIXATION TESTS

Antigens were assessed for CF activity by checkerboard titrations against homologous immune serum, using two exact units of complement and three units of hemolysin. CF endpoints were defined as the greatest dilution of antigen showing 50% hemolysis in the presence of a constant dilution of antiserum containing at least eight units of antibody.

D. BORATE SALINE - BPL (BS-BPL) EXTRACTION PROCEDURE

Although variations of the BS-BPL extraction procedure were tried, the defined schema outlined in Figure 1 was followed. The suspending diluent for infectious mouse brains was borate buffered-saline (BS) , 9.3 or 9.0[1] to which was added 100 units of sodium penicillin per and 100 µg streptomycin sulfate per ml. The pellet obtained at Step 5 could be re-extracted as many as four times before being discarded. Successive BS extractions also may be treated with Tris and BPL to disperse aggregates, but additional BPL is detrimental for some viral antigens.

Step 1	Infectious brain triturated and suspended in BS (pH 9.3) to 10 to 15% concentration.
Step 2	One-tenth volume of 1.0 M Tris buffer added.
Step 3	BPL added to 0.3% concentration (may be omitted in subsequent extractions of pellet).
Step 4	Hold at 4 C for 18 to 24 hours with intermittent shaking.
Step 5	Centrifuge at 12,000 x g for 1 hour.
Step 6	Decant supernatant fluid and test for HA titer.
Step 7	Resuspend pellet in ½ to ⅔ the original volume of BS and repeat Steps 2 through 6.

FIGURE 1. Schema of Borate Saline - BPL Extraction for Preparing Noninfectious Arbovirus Antigens.

E. SUCROSE-ACETONE (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[1] 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 x g for 1 hour; 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 in the text below.

III. RESULTS

A. 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 to 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. It soon became obvious that the phosphate buffer used by French and McKinney[2] would not suffice for these higher concentrations of BPL. Tris buffer proved to be a satisfactory substitute. The concentration of Tris buffer necessary to maintain the pH above 8.0 was determined empirically. Figure 2 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-hour 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 hours. 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.

B. GROUP A ARBOVIRUSES

Antigens of several group A viruses that were prepared by BS-BPL extraction are listed in Table 1. Viruses such as VE, EE, WE, chikungunya, Una, Middelburg, Getah, and Aura yielded antigens with acceptable HA titers following a single passage in suckling mouse brain. These viruses we 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 rapid, successive passages in suckling mouse brain. This was particularly true of Mayaro and Bebaru, which were carried through ten continuous passages in suckling mice to obtain a suitable hemagglutinin.

VE 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 16 to 20 hours at 4 C, and centrifuging at 12,000 x g for 1 hour. 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

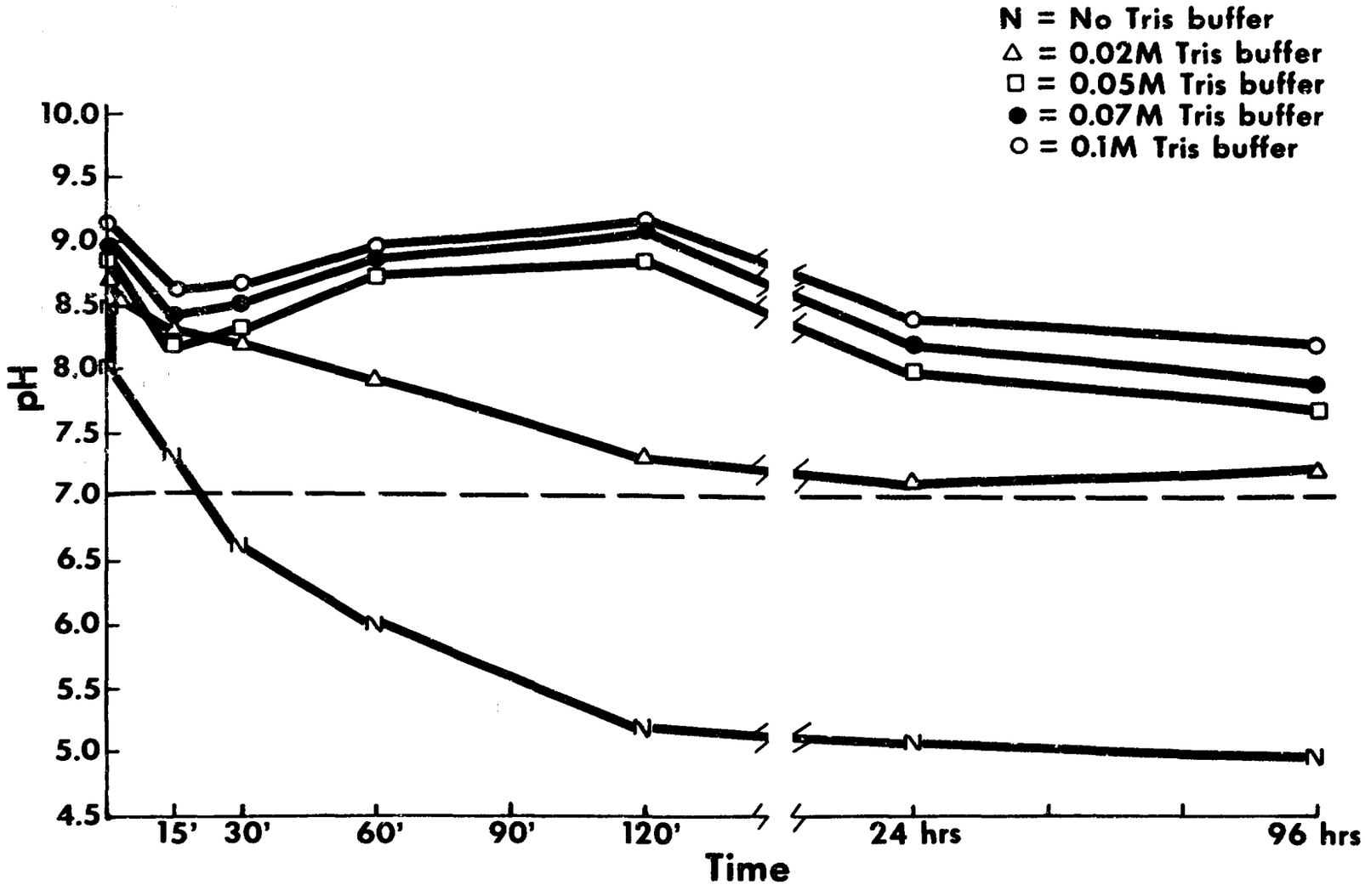

FIGURE 2. Buffering Capacity of Tris in 20% Normal Suckling Mouse Brain Treated with 0.3% BPL.

similar rate of decrease following the second saline extract of the BS-BPL method. The increase in titer between the first and second saline extraction was observed with VE 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 of 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.

TABLE 1. GROUP A ARBOVIRUS ANTIGENS PREPARED BY BS-BPL EXTRACTION

Virus Antigen	HA Titer	CF Titer
VE	4,096	48
EE	2,048	64
WE	512	16
Chikungunya	128	32
Mayaro[a]	512	32
Semliki Forest[a]	128	40
Bebaru[a]	1,024	16
Getah	64	16
O'nyong-nyong[a]	256	64
Sindbis[a]	512	16
Una	32	ND[b]
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. ND = Not done.

TABLE 2. VE ANTIGENS PREPARED BY BS-BPL AND SA-BPL EXTRACTION

Saline Extraction	HA Titer (Reciprocal) BS-BPL Extraction	SA-BPL Extraction
1st	1,024	2,048
2nd	4,096	512
3rd	1,024	128
4th	256	32

The effect of mouse age on yields of HA and CF antigens from brains was investigated with VE virus. Mice from age 4 to 21 days appeared to be equally susceptible to VE virus inoculated intracerebrally, but the antigen yields 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.

TABLE 3. EFFECT OF MOUSE AGE ON VE VIRUS ANTIGEN[a/]

Age of Suckling Mice Inoculated, day	Maximum HA Titer	CF Titer
4 to 6	4,096	48
14	1,024	24
21	256	4

a. Extracted by the BS-BPL method.

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. As the results indicate in Table 4, all three extracts had acceptable HA titers of 1:256, and these were maintained after lyophilization.

TABLE 4. HA ACTIVITY OF O'NYONG-NYONG VIRUS[a/]

Saline Extraction & Subsequent Treatment	HA Titer
1st + Tris + BPL	1:256
2nd + Tris + BPL and stored at -60 F	1:256
3rd + Tris + BPL	1:256
1st, lyophilized	1:256
3rd, lyophilized	1:256

a. Antigen prepared from fourth rapid passage in suckling mice by extraction from 15% SMB in borate-saline (pH 9.0) with 0.1 M Tris and 0.3% BPL.

C. GROUP B ARBOVIRUSES

Table 5 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 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.

TABLE 5. GROUP B ARBOVIRUS ANTIGENS PREPARED BY BS-BPL EXTRACTION

Virus Antigen	Maximum HA Titer	CF Titer
Yellow fever	2,048	20
St. Louis (Price strain)[a]	128	ND[b]
St. Louis (Hubbard strain)	64	16
Japanese B (Peking strain)	256	16
Dengue II H_3[a]	128	16
Powasson	2,048	64
Rio Bravo	2,048	16
Langat, TP-21	4,096	20
West Nile	32	ND
Louping ill[a]	512	4

a. Three or more continuous passages in suckling mice.
b. ND = Not done.

The effects of excessive BPL treatment were best exemplified by the Asibi strain of yellow fever (YF) virus. Brains from YF-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 6 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 YF hemagglutinin. This was tried with a third lot of infected brains using 0.1 M Tris instead of 0.07 M,

and an excellent antigen was obtained (Table 7). Lyophilization of this antigen caused a substantial loss of titer, but the dry product maintained an acceptable level of HA activity.

TABLE 6. HA ACTIVITY OF YELLOW FEVER VIRUS WITH AND WITHOUT BPL TREATMENT[a]

Saline Extraction	HA Titers	
	Antigen A No BPL	Antigen B 0.3% BPL
1st	2,048	1,024
2nd	128	<2
1st antigen B extraction retreated with BPL	-	<2

a. Antigens extracted from 20% SMB in borate saline + 0.07 M Tris buffer.

TABLE 7. HA ACTIVITY OF YELLOW FEVER VIRUS AFTER A SINGLE BPL TREATMENT[a]

Saline Extraction	HA Titer
1st	2,048
2nd	1,024
1st and 2nd pooled	2,048
Lyophilized pooled antigen	256

a. Antigen extracted from 15% SMB in borate saline + 0.1 M Tris + 0.3% BPL.

D. 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. With an initial passage of Bunyamwera virus in suckling mice a single BS-BPL extraction of the brains yielded an antigen with an HA titer of 1:128 and a CF titer of 1:8. 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.[1] 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.

Both a pantropic and a neurotropic strain of Rift Valley fever virus were examined for hemagglutinin 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.

IV. DISCUSSION

The BS-BPL method described in this report provides the investigator with a simple, safe procedure for preparing large or small volumes of noninfectious arbovirus antigens. The procedure has three fundamental advantages: (i) it reduces the use of hazardous, flammable chemicals necessary to remove nonspecific inhibitors; (ii) it reduces the chances of antigen denaturation, which may result during purification and dehydration; and (iii) it reduces the handling of infectious material during the extraction period.

Differences observed between antigen preparation procedures were most noticeable between successive borate-saline extractions of the pellet; these were employed to recover as much antigen as possible from the treated pellet. One of the consistent differences noted in the BS-BPL procedure was the higher HA titer obtained from the second, rather than the first, borate-saline extraction of the brain pellet. This phenomenon was seldom observed in SA-BPL - prepared antigens. This suggested that nonspecific inhibitors may not be completely eliminated by the original BPL treatment, which means that the inhibitors are removed from the pellet during the first saline extraction, allowing greater expression of the antigen from the second saline extraction. It is also suggested that a second BPL treatment of the original BS-BPL extraction acts to break up the antigen aggregates and further remove the residue inhibitor. However, it should be noted that antigen titers of the first saline extraction, even after a second BPL treatment, rarely equaled or exceeded HA titers of the second saline extraction.

The BS-BPL antigens appear to be quite stable for storage in either the wet state at -30 C or lyophilized at 4 C. Working stock antigens (antigen diluted 1:5 in borate saline) are fairly stable for 2 weeks at 4 C or at -30 C for longer periods. Freezing and thawing of stock antigens did not reduce the HA activity of the antigen, and in many cases the HA

titer increased. These stability characteristics are also comparable to those of SA-BPL preparations, which are remarkably stable at 4 C for long periods of time.

All results examined thus far have indicated that Tris buffer is a compatible buffer, capable of maintaining the desired pH against the volume of BPL necessary to disinfect the antigen and inactivate the antigen inhibitor. Other buffers may be utilized in place of Tris in this procedure, possibly with equal success; however, Tris has performed so well in our tests that we have not found it necessary to examine other buffers.

The assurance of maintaining a good antigen during BPL treatment rests with the ability of the buffer to prevent any dramatic changes in pH during the hydrolysis period. This means that a buffer must be physiologically compatible with the infectious tissue used for antigen preparation and be able to maintain a basic pH. Tris has a pKa (the pH of the midpoint of buffering range) of 8.3 at 20 C,[3] indicating that its greatest buffering range is above 8.0, which is a stable range for arbovirus antigens.

Although the buffer is responsible for maintaining a stable environment for the antigen during the inactivation period, BPL is the indispensable ingredient of the procedure. Its action upon the antigen and antigen inhibitors influences the HA titer of the final product.

Trial examinations have fairly well established that 0.15% BPL is sufficient to render almost all the arboviruses noninfectious. Increasing the quantity of BPL to 0.3% by volume improved the antigen preparations in three ways: first, it assured better and safer sterilization of the infectious SMB; second, it promoted further breakup of antigen aggregates, which is indicated by an increase in HA titer and expansion of the pH range; and third, it assured a more nearly complete elimination of the antigen inhibitor.

The relationship between the quantity of BPL used and the serological group to which the virus antigen belongs is also important. The group A virus antigens sustained, and in many cases required, additional BPL treatment to improve HA activity. On the other hand, more than one BPL treatment of the group B viruses destroyed all HA activity. Similar results were also demonstrated by French and McKinney.[2] BS-BPL preparations of Bunyamwera, Germiston, and RVF viruses were not entirely successful, and multiple BPL treatment of the virus extracts failed to improve the antigen. These results suggest that differences in the nature of the HA inhibitors may be associated with each virus or virus group, and that extraction procedures unique to each virus would be required to unmask the hemagglutinin. Such procedures are now being examined for the Bunyamwera and ungrouped arboviruses.

Successive virus passage in suckling mouse brain also appeared to be most successful for the group A and B arboviruses. Successive passage of Bunyamwera virus appeared to have an adverse effect on antigen production, in that no HA activity was obtained from antigens prepared from the last four of five passages. The relationship between virus passage and antigen production is not understood, and one can only speculate on the results of the past observations. The results suggest two possibilities: (i) that there may be an increase in virus titer during passage, and (ii) that there is a host-virus adaptation, causing an increase in the hemagglutinin titer. If the second possibility is true there would not necessarily be an increase in virus titer. Also with some viruses both possibilities may be true.

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Noninfectious arbovirus antigens were extracted from saline suspensions of infected suckling mouse brain that were buffered with tris(hydroxymethyl)aminomethane and treated with beta-propiolactone (BPL). The activity and stability of several antigens were enhanced by altering the buffering system and by rapid, continuous passage of the virus in suckling mice. For several viruses of groups A and B, the titers of antigens extracted with borate saline-BPL (BS-BPL) were comparable to titers of antigens extracted with sucrose-acetone-BPL. Results from some ungrouped and Bunyamwera arboviruses required the development of unique and more sophisticated procedures for the preparation of suitable antigens.

14. Key Words

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*Antigens
*Mice
Beta-propiolactone
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