Inactivation of viruses in labile blood derivatives

I. Disruption of lipid-enveloped viruses by tri(n-butyl)phosphate detergent combinations

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Use of the organic solvent, tri(n-butyl)phosphate (TNBP), and detergents for the inactivation of viruses in labile blood derivatives was evaluated by addition of marker viruses (VSV, Sindbis, Sendai, EMC) to anti-hemophilic factor (AHF) concentrates. The rate of virus inactivation obtained with TNBP plus Tween 80 was superior to that observed with ethyl ether plus Tween 80, a condition previously shown to inactivate greater than or equal to $10^{6.9}$ CID50 of hepatitis B and greater than or equal to 10^4 CID50 of Hutchinson strain non-A, non-B hepatitis. The AHF recovery after TNBP/Tween treatment was greater than or equal to 90 percent. Following the reaction, TNBP could be removed from the protein by gel exclusion chromatography on Sephadex G25; however, because of its large micelle size, Tween 80 could not be removed from protein by this method. Attempts to remove Tween 80 by differential precipitation of protein were only partially successful. An alternate detergent, sodium cholate, when combined with TNBP, resulted in almost as efficient virus inactivation and an 80 percent recovery of AHF. Because sodium cholate forms small micelles, it could be removed by Sephadex G25 chromatography. Electrophoretic examination of TNBP/cholate-treated AHF concentrates revealed few, if any, changes in protein mobility, except for plasma lipoprotein(s). TRANSFUSION 1985;25:516-522.

THE TRANSMISSION OF viral hepatitis by coagulation factor concentrates and other plasma derivatives remains a serious health concern. The recent worldwide outbreak of acquired immunodeficiency syndrome (AIDS) that is caused by a blood-borne virus[1,2] further necessitates the development of effective methods for virus inactivation in labile blood derivatives. Although the pasteurization of albumin at 60°C for 10 hours in the presence of fatty acid ligands has been reported to inactivate 10^4 infectious doses of hepatitis B,[3] application of this method to antihemophilic factor (AHF) results in the complete loss of biologic activity. More recent approaches to virus inactivation in blood products include heating in the presence of low molecular weight stabilizers[4] or in the lyophilized state[5] and treatment with beta-propiolactone and ultraviolet (UV) irradiation.[6]

We recently reported[7] the susceptibility of hepatitis B and Hutchinson strain of non-A, non-B hepatitis viruses to inactivation by ethyl ether and Tween 80, a method known to disrupt lipid-enveloped viruses. Greater than $10^{6.9}$ chimpanzee infectious dose (CID)50 of type B and 10^4 CID50 of Hutchinson strain non-A, non-B hepatitis viruses were inactivated, and the recovery of AHF was 70 percent. Because of the hazardous nature of ethyl ether, an alternative was sought. This report explores the use of tri(n-butyl)-phosphate (TNBP) as an alternative to ethyl ether through the addition of easily quantifiable marker viruses to an AHF concentrate. Previous work with TNBP substantiated its effectiveness in the disruption of lipid-enveloped viruses.[8]

Materials and Methods

Reagents

Chemicals were reagent grade unless otherwise stated. TNBP, sodium deoxycholate, Tween 80, and Triton X-100 were obtained commercially (Fisher Scientific, Springfield, NJ). Sodium cholate was also obtained commercially (Eastman Kodak, Rochester, NY), as was PEG 4000 (Union Carbide Corp., Moorestown, NJ), Aerosil 380 (Degussa, Atlanta, GA), and antisera (Calbiochem-Behring Corp., La Jolla, CA).

From The New York Blood Center, New York, New York.

This work was supported, in part, by award No. NO1-HB-3-7009 from the National Heart, Lung, and Blood Institute.

Received for publication October 30, 1984; revision received February 1, 1985, and accepted February 4, 1985.

BAYP0000024_063_0001

Viruses and virus assays

Vesicular stomatitis virus (VSV) was obtained from Dr. William Stewart, II, and cultured in mouse L929 cells. Encephalomyocarditis virus (EMC) was obtained from Dr. Emilio Emini, and stocks were prepared by culturing the virus in mouse L929 cells. Sindbis virus was obtained from the collection of Dr. William Scherer, and stocks were prepared in primary chicken embryo cells. Sendai virus (Cantell strain) was purchased from Flow Laboratories.

The biologic activity of all viruses was assayed by endpoint dilution assay. Ten-fold serial dilutions of virus preparations were made in culture medium (MEM). For VSV, EMC, and Sindbis virus, each dilution was used to inoculate eight replicate wells of cells in 96-well microtiter plates. Human A549 cells were used for VSV and EMC, and primary chicken embryo cells were used for Sindbis virus. Virus-induced cytopathology was scored after 72 hours of incubation at 37°C in 5 percent CO2. For Sindbis virus, each dilution was inoculated into the allantoic sac of three 10-day-old embryonated chicken eggs. Following incubation for 3 days at 37°C, allantoic fluid was harvested from each egg and a 1 to 20 dilution was assayed for hemagglutination activity using avian red cells. For all assays, the ID50 value was calculated using the Spearman-Karber method.[14,19]

Inactivation of viruses added to AHF

Virus and virus inactivating agents under evaluation were added to an AHF concentrate (New York Blood Center) and mixed continuously, usually by rocking, under the conditions stated. For assessment of virus inactivation, the reaction was stopped by 100-fold dilution into Eagle's Minimal Essential Medium (Gibco Laboratories, Grand Island, NY) containing 5 percent (vol/vol) fetal calf serum (M.A. Bioproducts, Walkersville, MD). The lack of virus inactivation at this dilution was confirmed for each of the inactivation conditions studied. Samples were sterile filtered (Swinnex Millipore filters, Bedford, MA) and frozen at −70°C or below, until assay.

AHF activity was assayed by determining the degree of correction in APTT time (General Diagnostics, Morris Plains, NJ) of factor VIII-deficient plasma (George King, Overland Park, KS).[9]

Chromatography (Biogel A15m, Bio-rad Labs, Rockville Center, NY) was performed at ambient temperature on a column (1.5 × 96 cm) equilibrated with 0.02 *M* Tris-HCl, pH 7.2, containing 0.15 *M* NaCl, and operated at a flow rate of 5.4 ml per cm² per hr. Discontinuous electrophoresis under nondenaturing conditions was performed essentially as described,[10] and proteins were stained with Coomassie blue. Crossed-immunoelectrophoresis was performed essentially as described[11] using 1 percent agarose (Seakem ME; FMC Corp, Rockland, ME) in tricine buffer IV (BioRad, Rockville Center, NY) applied to Gelbond film (10 × 15 cm; FMC Corp.). Electrophoresis in the first dimension was at 20 mA per plate and proceeded until a bromophenol blue marking dye migrated two-thirds of the plate length. Agarose above the sample lane was removed and replaced with agarose containing antibody. Electrophoresis in the second dimension was at 10 mA per plate and proceeded overnight. The agarose was soaked in normal saline to remove soluble proteins, air dried, and stained with Coomassie blue.

Tween 80 concentration was determined after hydrolysis of samples with 25 percent (wt/vol) NaOH at 100°C for 18 hours, by the method of Skoog.[12] Sodium cholate concentration was determined after extraction of samples with 4 volumes of ethanol at ambient temperature and clarification by centrifugation. The clear supernatant was mixed with an equal volume of 2 percent anisaldehyde and 5 volumes of 50 percent H2SO4 at 60°C for 30 minutes. Absorbance at 570 nm was determined.[13] TNBP concentration was determined as follows: to 1.0 ml of sample was added 0.5 ml of 1.5 N HClO4. After vigorous mixing, 4 ml of CHCl3 was added, and after mixing, the sample was clarified by centrifugation. The organic phase was evaporated under a stream of air, the residue was dissolved in 50 μl of hexane, and TNBP was quantified by gas chromatography.

Results

Assessment of virus inactivation with organic solvent/detergent

The rates of virus inactivation observed at 4°C with 20 percent (vol/vol) ethyl ether and 1 percent (vol/vol) Tween 80 were compared with that observed with 0.1 percent (vol/vol) TNBP and 1 percent (vol/vol) Tween 80 using viruses added to an AHF concentrate (Fig. 1). For each of the lipid-enveloped viruses studied (VSV, Sindbis, Sendai), the rate of inactivation was more rapid with TNBP/Tween than with ether/Tween. Treatment with TNBP/Tween for 6 hours inactivated 4.0, greater than or equal to 5.8, and 3.5 logs of VSV, Sindbis, and Sendai viruses, respectively. The infectivity of EMC, the non-lipid-enveloped virus, was unaffected by either treatment. Raising the temperature from 4°C to ambient (22–24°C) accelerated virus inactivation markedly by both ether/Tween and TNBP/Tween (Table 1, experiments 1 and 2), increasing the inactivation velocity constant, k*, for TNBP/Tween by 8- to 12-fold for

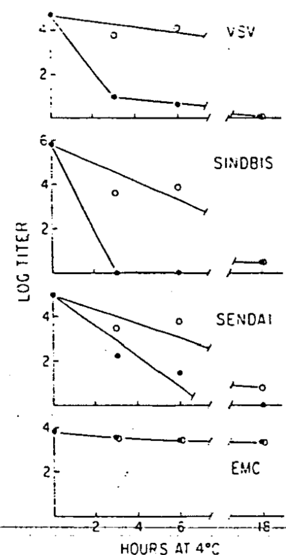

FIG. 1. Comparison of TNBP/Tween to ether/Tween. Viruses were added to separate aliquots of an AHF concentrate followed by addition of ethyl ether (20% vol/vol) and Tween 80 (1% vol/vol) (*open circles*) or TNBP (0.1% vol/vol) and Tween 80 (1% vol/vol) (*closed circles*) and incubated at 4°C. Residual infectious virus was assayed at the times indicated after a 100-fold dilution.

* $k = \frac{2.303 \log C/C_o}{t - t_o}$, where Co is the initial concentration of virus at time, t_o, and C is the concentration of virus at time, t.

Table 1. *Inactivation of viruses by organic solvents and detergent*

Experiment	Conditions			Log Inactivation			
	Additive	Temperature	Hours	VSV	Sindbis	Sendai	EMC
1	Ether (20%), Tween 80 (1%)	4°C	3	0.8	2.0	1.5	0.2
			6	0.5	1.7	1.2	0.2
		ambient	1	3.1	≥5.4	≥4.0	0.0
			3	≥4.1	≥5.4	≥4.0	0.5
2	TNBP (0.1%), Tween 80 (1%)	4°C	1	0.4	0.3	0.0	−0.2
			3	3.7	1.0	2.8	0.2
		ambient	1	3.3	3.7	1.6	0.7
			3	4.5	≥5.5	3.0	0.4
3	Tween 80 (1%) alone	4°C	3	0.3	0.0	0.0	0.4
		ambient	18	—	−0.1	−0.3	0.0
4	Ether (20%) alone	4°C	3	—	1.3	0.8	0.0
			18	—	2.0	≥5.0	—
5	TNBP (0.3%) alone	ambient	4	0.8	4.6	4.9	−0.1
	TNBP (2.0%) alone	ambient	4	0.8	3.6	≥5.1	0.1

the first hour, although just 1.1- to 1.2-fold for the first 3 hours. Raising the TNBP concentration from 0.1 to 0.3 percent (vol/vol) in the presence of 1 percent Tween 80 improved the reproducibility of virus inactivation (data not shown). Tween 80 (1 percent) used without an organic solvent was ineffective in inactivating VSV, Sindbis, Sendai, or EMC at 4 or 22°C (Table 1, experiment 3); however, the organic solvent without detergent did inactivate virus. Ether (20%) at 4°C inactivated 2 logs of Sindbis and greater than or equal to 5.0 logs of Sendai virus in 18 hours (Table 1, experiment 4), and treatment with TNBP (0.3 or 2.0%) at ambient temperature for 4 hours inactivated 0.8, 3.6 to 4.6, and greater than or equal to 4.9 logs of VSV, Sindbis, and Sendai virus, respectively (Table 1, experiment 5).

When combined with TNBP (0.3%), the effect on virus inactivation and AHF recovery of reduced concentrations of Tween 80 and of substitutes was evaluated (Table 2). Decreasing the concentration of Tween 80 from 1 to 0.1 or 0.01 percent reduced the degree of inactivation of virus, especially VSV. This is in accord with the observation that, of the three lipid-enveloped viruses used, VSV inactivation is accelerated most by addition of detergent to TNBP (Table 1, experiments 2 versus 5, ambient temperature). Other nonionic detergents, e.g., Triton X-100, could substitute for Tween 80; however, polyethylene glycol 4000, a nondetergent analog of Tween and Triton, could not. Use of TNBP plus either nonionic detergent resulted in greater than or equal to 90 percent recovery of AHF at all concentrations of detergent used. Addition of sodium deoxycholate or sodium cholate, naturally occurring bile salts, to TNBP also

Table 2. *Inactivation of viruses by TNBP and several detergents*

Experiment	Conditions			Log Inactivation				AHF Recovery (%)
	TNBP (0.3%) and Detergent	Detergent Concentrate (%)	Hours	VSV	Sindbis	Sendai	EMC	
1	Tween 80	1	1	3.5	4.6	4.5	−0.1	—
		0.1	1	1.4	4.6	5.2	0.1	—
		0.01	1	0.7	3.6	4.7	0.0	—
2	Tween 80	1	4	4.2	≥5.6	4.7	0.0	114
	Triton X-100	1	4	≥4.3	≥5.6	≥5.8	−0.2	92
		0.2	4	≥4.3	≥5.6	≥5.8	0.1	99
		0.04	4	4.2	≥5.6	≥5.8	0.1	99
	Sodium deoxycholate	0.2	4	4.3	≥5.6	≥5.8	−0.4	2.5
		0.05	4	3.3	≥5.6	4.8	0.0	73
		0.01	4	0.1	4.6	5.2	0.5	97
	Polyethylene glycol 4000	1	4	0.1	3.6	3.5	0.0	87
	TNBP alone	—	4	0.6	3.0	3.8	0.1	90
3	Sodium cholate	1	4	≥4.6	≥5.3	≥5.0	0.0	2
		0.2	4	2.3	3.7	≥5.5	−0.1	83
		0.05	4	0.5	3.1	3.5	0.0	—
		0.01	4	−0.1	2.5	3.2	0.0	—

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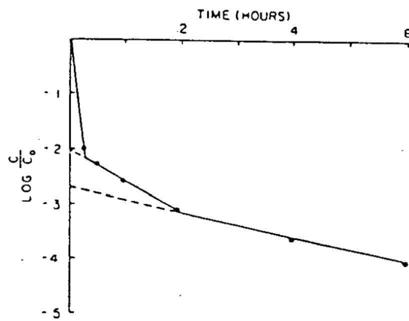

FIG. 2. Inactivation of VSV in presence of TNBP and sodium cholate. VSV was added to an AHF concentrate, followed by addition of TNBP (0.3% vol/vol) and sodium cholate (0.2% wt/vol), and incubated at ambient temperature. Residual virus was assayed as in Fig. 1.

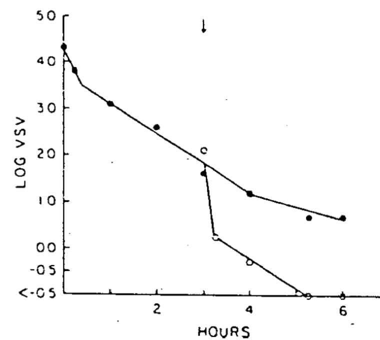

FIG. 4. Sensitivity of residual, infective VSV to a second TNBP-cholate treatment. VSV added to an AHF concentrate was treated with 0.3 percent TNBP/0.2 percent sodium cholate at 24° C. At the arrow, an aliquot was removed, subjected to chromatography on Sephadex G25, and then the treatment started anew (*open circles*).

accelerated virus inactivation, although concentrations of sodium deoxycholate and sodium cholate above 0.05 and 0.2 percent (1 m*M* and 4.6 m*M*), respectively, inactivated AHF substantially (Table 2). Use of sodium cholate (0.2%) without TNBP did not inactivate the four viruses used in this study (data not shown).

Because VSV was relatively resistant to inactivation by TNBP cholate, its inactivation was examined more closely. The rate of VSV inactivation by 0.3 percent TNBP and 0.2 percent sodium cholate was not first order. Starting with a VSV titer of 3.6 logs (after a 100-fold dilution to stop the reaction), the inactivation velocity constant, k, was estimated at 18.4 per hour for the first 0.25 hour, 1.2 per hour for the period between one-half and 2 hours, and 0.5 per hour for the period between 2 and 6 hours (Fig. 2). One common explanation for a decay in the velocity constant is heterogeneity of the virus being studied. If this was the case, the data in Figure 2 indicate that the resistant fractions were 0.9 and 0.2 percent, respectively, estimated by extension of the second and third components to the X axis. Alternatively, the decrease in the velocity constant could be ascribed to other factors affecting the reaction, such as reassociation of virus particles or consumption of reagent.

Several experiments were conducted to distinguish among these models. To determine if the reduced rate of inactivation resulted from reagent consumption or other factors specific to the progress of the reaction besides heterogeneity of the virus, 1 hour after the inactivation reaction had begun, the mixture was divided in two, an additional 1.5 logs of VSV were added to one of the aliquots, and the rate of inactivation was monitored. If the decline in rate resulted from consumption of reagents, then the decline in virus titer in each of the two aliquots would be parallel. Alternately, if the decline in rate constant represented virus heterogeneity, inactivation of the newly added virus would be rapid. The results (Fig. 3) indicate that the inactivation rate of virus

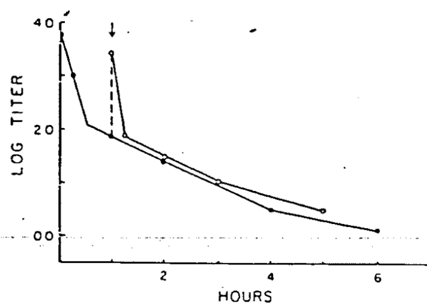

FIG. 3. Effect of readdition of VSV on its rate of inactivation by TNBP and sodium cholate. VSV was added to an AHF concentrate, followed by addition of TNBP (0.3% vol/vol) and sodium cholate (0.2% wt/vol), and incubated at ambient temperature for the time indicated. After 1 hour, the mixture was divided in two and an additional 1.5 logs per ml of VSV were added to one of the aliquots (*open circles*).

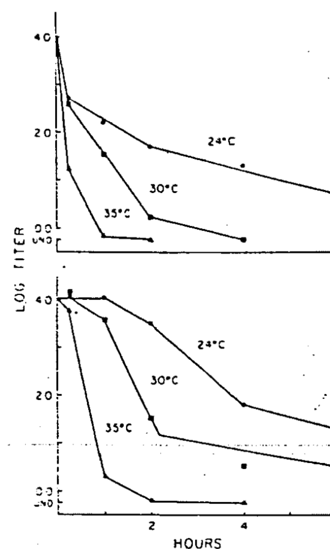

FIG. 5. Inactivation of VSV at elevated temperatures. VSV was added to an AHF concentrate followed by TNBP (0.3% vol/vol) and sodium cholate (0.2% wt/vol) (*upper panel*) or TNBP (0.3% vol/vol) alone (*lower panel*). Incubations were at the temperatures indicated, and other details were as described in Fig. 1.

added after 1 hour was rapid and similar to the initial rate of inactivation at time zero.

If the change in inactivation rate resulted solely from virus heterogeneity, then residual infectious VSV present at the conclusion of the initial inactivation reaction would be expected to inactivate slowly on reexposure to fresh TNBP/cholate. However, VSV subjected to two consecutive treatments with TNBP/cholate with only a Sephadex G25 step intervening showed essentially identical initial rates of inactivation (Fig. 4). This suggests that the normally observed decline in inactivation rate was an extrinsic and not an intrinsic property of the virus.

The rate of inactivation of VSV by 0.3 percent TNBP in the presence of 0.2 percent sodium cholate was substantially increased by raising the temperature from 24 to 30 or 35°C (Fig. 5, upper panel). When sodium cholate was omitted, an initial lag in inactivation was apparent, the duration of which appeared to be inversely dependent on temperature. The recovery of AHF following 6 hours of reaction at 24, 30, and 35°C was, respectively, 82, 79, and 55 percent for TNBP/cholate, and greater than or equal to 90 percent for TNBP alone at each of the temperatures.

Analysis of protein in AHF concentrate treated with TNBP/detergent

Following treatment with 0.3 percent TNBP and 0.2 percent sodium cholate, examination of the proteins present by discontinuous gel electrophoresis under nondenaturing conditions did not show any alteration in band pattern (Fig. 6). AHF appeared to remain associated with von Willebrand's factor as judged by their elution profile when chromatographed (Biogel A15m) (Fig. 7). The proteins present in an AHF concentrate also were examined by crossed immunoelectrophoresis following treatment with 0.3 percent TNBP and 0.2 percent sodium cholate for 6 hours at ambient temperature. Using agarose as the separation medium and anti-normal human serum as antibody, at least 19 protein peaks were detected (Fig. 8). The great majority of these were unchanged as a consequence of treatment, while two peaks, marked 1 and 2, differed. Peak 1 was identified as alpha-1-lipoprotein using monospecific antiserum. The identity of peak 2 is unknown.

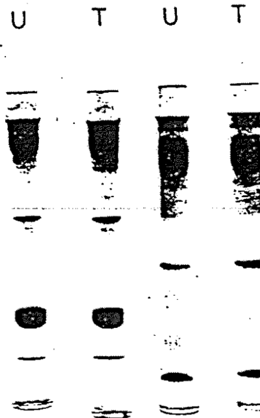

FIG. 6. Electrophoresis of AHF treated with TNBP/cholate. An AHF concentrate was treated with TNBP (0.3% vol/vol) and sodium cholate (0.2% wt/vol) for 6 hours at 24°C after which it was chromatographed on a column of Sephadex G25. The eluted protein (T) and untreated AHF concentrate (U) were each electrophoresed under nondenaturing conditions as described under Methods. Electrophoresis of the right two gels was prolonged to better resolve the slower migrating proteins.

FIG. 7. Gel exclusion chromatography of AHF treated with TNBP/cholate. The AHF concentrates described in Fig. 6 were chromatographed on a column of Biogel A15m. The absorbance at 280 nm of the eluate was monitored continuosly, and AHF procoagulant activity and von Willebrand factor antigen were measured in individual fractions.

Reagent removal

TNBP could be removed by dialysis or gel exclusion chromatography on Sephadex G25 to a level below the sensitivity of our assay (0.5 ppm); however, Tween 80, which

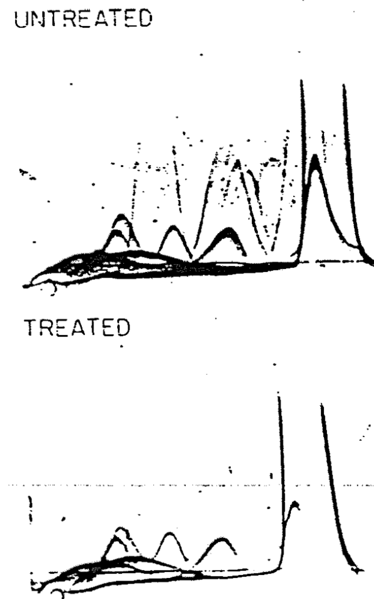

FIG 8. Crossed immunoelectrophoresis of AHF treated with TNBP/cholate. The AHF concentrates described in Fig. 6 were analyzed by crossed immunoelectrophoresis using rabbit anti-normal human serum.

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FIG. 9. Removal of TNBP and cholate from an AHF concentrate. An AHF concentrate treated as described in Fig. 6 was chromatographed on a column (2.6 × 29 cm) of Sephadex G25 equilibrated with 0.02 M Tris-HCl, pH 7.2, containing 0.02 M sodium citrate and 0.10 M NaCl at ambient temperature. The flow rate was 10 cm per hr, and the sample volume was 11 percent of the column volume. Individual fractions were monitored for absorbance at 280 nm, AHF procoagulant activity, TNBP, and sodium cholate.

forms large micelles, could not be removed by these methods (data not shown). Precipitation of AHF by addition of 2.2 molal glycine and 2.1 molal NaCl resulted in a recovery of 90 to 100 percent of the initial AHF and 5 to 10 percent of the initial Tween in the precipitate. Under the conditions used, 1000 units of AHF would be contaminated by 237 mg of Tween. Reducing the initial concentration of Tween 80 during the inactivation reaction to 0.1 or 0.01 percent (vol/vol) reduced its contamination of precipitated AHF to 102 and 12 mg per 1000 units, respectively. Alternately, following treatment with 0.3 percent TNBP and 1 percent Tween 80 and precipitation as described above, the residual Tween could be reduced by adsorption with 0.2 percent Aerosil 380 to 11 mg per 1000 units of AHF, although these conditions resulted in an additional 30 percent loss of AHF activity.

Sodium cholate, because of its small micelle size, was removed completely by Sephadex G25 (Fig. 9).

Discussion

Elimination of viral infectivity from blood products requires denaturing conditions capable of rendering viruses noninfective while preserving the biological activities and nonimmunogenic properties of proteins. Thermal inactivation is inherently denaturing to both virus and protein, although viruses are inactivated more rapidly, probably as a consequence of their complexity. Furthermore, conditions developed to stabilize proteins to heat also stabilize viruses.[15]

The use of organic solvents to disrupt, and thereby inactivate, lipid-enveloped viruses appears to provide the necessary discrimination between virus inactivation and maintenance of protein structure. Previously, we demonstrated that ethyl ether combined with Tween 80 inactivated greater than or equal to $10^{6.9}$ CID50 of hepatitis B and greater than or equal to $10^{4.0}$ CID50 of Hutchinson strain non-A, non-B hepatitis, and that AHF, factor IX, and fibronectin activity was largely or completely retained.[7] However, introduction of ethyl ether into the work environment is hazardous. The inactivation of hepatitis viruses by chloroform also has been reported.[16]

One alternative to ethyl ether is TNBP, a nonexplosive organic solvent previously shown to be useful in the disruption of lipid-enveloped viruses[8] but not demonstrated until now to preserve the biological activity of proteins. We evaluated its use in preparation of blood derivatives through the addition of easily quantifiable viruses to an AHF concentrate. Virus inactivation by TNBP/Tween was found to be superior to ether/Tween when reacted at 4°C; however, comparison of inactivation rates at ambient temperature showed little difference. EMC virus, a non-lipid-enveloped virus, was unaffected by either treatment. The rate of virus inactivation was reduced when organic solvent was used without detergent, although the magnitude of rate reduction depended on the virus being studied and the temperature of incubation. An initial lag in VSV inactivation was observed when incubated with TNBP (0.3% vol/vol) at 24°C without detergent. The lag period was shortened considerably by raising the temperature to 35°C and was eliminated by detergent addition. Perhaps detergent, or working at higher temperature, opens up the virus structure making it more accessible to organic solvent extraction.

Although the disruption of viruses with organic solvents is restricted to lipid-enveloped viruses, hepatitis B, Hutchinson strain non-A, non-B hepatitis, and HTLV-III,[1] implicated in AIDS transmission, are lipid-enveloped. One non-A, non-B hepatitis virus isolated from a chimpanzee has been reported to be resistant to treatment with chloroform[17] and would not be expected to be lipid-enveloped; however, this report has yet to be confirmed.

Treatment of AHF concentrate with TNBP/Tween appears to have little or no effect on AHF activity or on the association between AHF and von Willebrand's factor. Other proteins present as contaminants, with the exception of lipoproteins, appear to be unaffected. Other results, not reported here, indicate that factor IX coagulant activity and fibronectin opsonic activity, measured by the uptake of gelatin-coated lipid emulsion by human peripheral leukocytes,[18] are fully retained. Use of sodium cholate in place of Tween 80 is favored both because it is a naturally occurring detergent and because it can be removed by gel exclusion chromatography on Sephadex G25; however, the recovery of AHF activity is reduced from greater than or equal to 90 percent to approximately 80 percent.

Acknowledgments

Appreciation is given to Alfred M. Prince, MD, for his insight and valuable advice, and to Eugene Maxwell, Raju Amin, Eleanor

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Dusci, Stephen Rubino, Lori Walakovits, Rose Shaffer, and Jayne Chin for their diligence in quantifying virus titers.

References

1. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome. Science 1983; 220:868-71.
2. Gallo RC, Salahuddin SZ, Popovic M, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 1984; 224:500-3.
3. Shikata T, Karasawa T, Abe K, Takahashi T, Mayumi M, Oda T. Incomplete inactivation of hepatitis B virus after heat treatment at 60 C for 10 hours. J Infect Dis 1978;138:242-4.
4. Heimburger VN, Schwinn H, Gratz P, Luben G, Kumpe G, Herchenhan B. Faktor VIII-Konzentrat, hochgereinigt und in Lösung erhitzt. (Arzheim-Forsch). Drug Res 1981;31:619-22.
5. U. S. Patent #4,456,590.
6. Prince AM, Stephan W, Brotman B. Beta-propiolactone/ultraviolet irradiation: a review of its effectiveness for inactivation of viruses in blood derivatives. Rev Infect Dis 1983; 5:92-107.
7. Prince AM, Horowitz B, Brotman B, Huima T, Richardson L, van den Ende MC. Inactivation of hepatitis B and Hutchinson strain non-A, non-B hepatitis viruses by exposure to Tween 80 and ether. Vox Sang 1984;46:36-43.
8. Neurath AR, Vernon SK, Dobkin MB, Rubin BA. Characterization of subviral components resulting from treatment of rabies virus with tri(n-butyl)phosphate. J Gen Virol 1972;14:33-48.
9. Lenahan JG, Phillips GE. Some variables which influence the activated partial thromboplastin time assay. Clin Chem 1966;12:269-73.
10. Davis BJ. Disc electrophoresis—II. Method and application to human serum proteins. Ann NY Acad Sci 1964;121:404-27.
11. Laurell CB. Antigen-antibody crossed electrophoresis. Anal Biochem 1965;10:358-61.
12. Skoog B. Determination of polyethylene glycols 4000 and 6000 in plasma protein preparation. Vox Sang 1979;37:345-9.
13. Kritchevsky D, Martak DS, Rothblat GH. Detection of bile acids in thin-layer chromatography. Anal Biochem 1963; 5:388-92.
14. Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. (Naunyn-Schmiedeberg's) Arch Exp Pathol Pharmak 1931;162:480-3.
15. Horowitz B, Wiebe ME, Lippin A, Vandersande J, Stryker MH. Inactivation of viruses in labile blood derivatives. II. Physical methods. Transfusion 1985;25:523-7.
16. Feinstone SM, Mihalik KB, Kamimura T, Alter HJ, London WT, Purcell RH. Inactivation of hepatitis B virus and non-A, non-B hepatitis by chloroform. Infect Immunol 1983;41:816-21.
17. Bradley DW, Maynard JE, Popper H, et al. Posttransfusion non-A, non-B hepatitis: Physicochemical properties of two distinct agents. J Infect Dis 1983;148:254-65.
18. Chang, M-D Y, Chin S, Horowitz B. A new assay for fibronectin opsonic activity and its application to plasma and plasma fractions. Vox Sang 1985;48:217-28.
19. Spearman C. The method of 'right and wrong cases' ('constant stimuli') without Gauss's formulae. Br J Psychol 1908;2:227-42.

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BAYP0000024_063_0007