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TECHNICAL MANUSCRIPT 434

EFFECT OF YOLK-SAC FRACTIONS ON VIRAL AND RICKETTSIAL INFECTIVITY

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EFFECT OF YOLK-SAC FRACTIONS ON VIRAL AND RICKETTSIAL INFECTIVITY

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Project 1C014501B71A

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ABSTRACT

The rickettsia of Rocky Mountain spotted fever, Rickettsia rickettsii, under certain conditions was more stable in partially purified suspensions than in crude yolk-sac suspensions. To demonstrate the presence in yolk sac of substances detrimental to infectivity, and to learn something of their nature, yolk sacs of normal embryonated chicken eggs were fractionated with lipid solvents. Fractions obtained were tested for their effect on the infectivities of R. rickettsii and the viruses of eastern and Venezuelan equine encephalitis (EEE and VEE).

The water-soluble fraction of an acetone extract contained substances that were inactivating to both rickettsial and viral infectivity, and the water-soluble fraction of an ethanol extract stabilized viral infectivity. Thin-layer chromatographic analyses indicated that the inactivating fraction contains, in addition to cholesterol, a mixture of bile acids, including deoxycholate, that probably are responsible for the viral inactivation. The inactivating effect was partially neutralized by 0.3% bovine serum albumin. Thin-layer chromatography of the stabilizing fraction produced several bands that fluoresced under ultraviolet light. The stabilizing and UV-absorbing properties of this fraction were dialyzable. Qualitative tests for protein and carbohydrate were negative for both the stabilizing and inactivating fractions. Results of tests for purines and pyrimidines in the stabilizing fraction are inconclusive. When the two fractions were mixed and tested with partially purified VEE virus, each fraction modified the effect of the other; rapid inactivation was prevented, but the inactivating fraction markedly reduced the effect of the stabilizing fraction.

I. INTRODUCTION

In an earlier report,[1] we showed that the etiologic agent of Rocky Mountain spotted fever, Rickettsia rickettsii, could be stabilized by glutamine more effectively in purified or partially purified suspensions than in crude yolk-sac preparations. The addition of bovine serum albumin or other serum proteins was required, together with glutamine, to overcome the poor stability of the rickettsia in yolk-sac suspensions. Our results indicated that yolk-sac material might contain a substance or substances detrimental to rickettsial infectivity. An apparent specific requirement for serum proteins in the stabilization of yolk-sac suspensions of R. rickettsii could be related to a known interaction of serum albumins and globulins with fatty acids, sterols, and phospholipids.[2-4]

To determine if inactivating substances could be separated from yolk sac, yolk sacs of normal embryonated chicken eggs were extracted with lipid solvents, and the fractions obtained were tested for their effect on R. rickettsii and on eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE) viruses. This report presents the results of these tests.

II. MATERIAL AND METHODS

Partially purified suspensions of R. rickettsii were prepared by homogenizing infected yolk sacs in potassium phosphate buffer of pH 7.5 (K7.5) and applying one cycle of low and high speed centrifugation to sediment the rickettsiae, which were then resuspended in K7.5 buffer. Suspensions of EEE virus (Louisiana strain[5]) were prepared by subjecting infected Maitland-type chick embryo culture fluids to one cycle of differential centrifugation. Sediments from the high-speed centrifugation were resuspended in phosphate buffer, pH 7.8. VEE virus (Trinidad strain[6]) was used in these tests as 1:100 dilutions of mouse brain suspensions.

To obtain the various fractions, yolk sacs of normal, 11-day, embryonated eggs were homogenized in a microblender with 80% ethanol. Following low-speed centrifugation, the alcoholic supernatant fluid was retained and the sediment was extracted in a Soxhlet apparatus with either chloroform-methanol (2:1) or acetone. The ethanol, chloroform-methanol, and acetone extracts were evaporated to dryness (or to a thick syrup) by passing nitrogen over the extracts held in a water bath of appropriate temperature. Residues from the extracts were redissolved in chloroform; these solutions were then vigorously shaken with water and centrifuged to obtain water-soluble components of each. The chloroform layers were again evaporated to dryness

under nitrogen and the residues were emulsified with Tween 80 solution (0.5%) for testing purposes, as was the water wash from the chloroform-methanol extract.

Samples of the various fractions were mixed with the viral or rickettsial suspensions in rubber-stoppered glass bottles and held at 4 during the test period. At indicated intervals, assays for infectivity were performed in 7-day embryonated eggs for rickettsiae (yolk-sac route) and in 14-day eggs for viruses (amniotic route).

III. RESULTS

In initial tests with R. rickettsii, Tween 80 was added to most of the fractions tested, and, because this emulsifying agent was itself inactivating to the rickettsiae, the effect of the fractions themselves was difficult to assess. However, it appeared that one fraction, the water wash of the chloroform-methanol extract, caused a more rapid inactivation than that produced by Tween 80 (Table 1). The fact that this fraction (sample 5 in Table 1) contained less Tween 80 (0.25%) than samples 2, 3, and 4 (each 0.5%) tends to increase the significance of the lower titers obtained with this fraction.

TABLE 1. EFFECT OF YOLK-SAC FRACTIONS ON THE INFECTIVITY OF RICKETTSIA RICKETTSII

Sample Number	Added to Rickettsial Suspension	Infectivity, $\log_{10}$ yolk-sac LD_{50}/ml[a] Days at 4 C		
		1	2	3
1	Buffer (control)	6.5	6.3	5.0
2	0.5% Tween 80 (control)	6.0	3.6	0
3	Water-insoluble fraction of C_2H_5OH extract	6.3	4.2	3.6
4	Water-insoluble fraction of $CHCl_3$-CH_3OH extract	6.2	5.0	4.4
5	Water-soluble fraction of $CHCl_3$-CH_3OH extract	5.1	0	0
6	Water-soluble fraction of C_2H_5OH extract	6.6	6.1	4.9

a. Starting titer: $1 \times 10^{7.6}$ yolk-sac LD_{50} per ml.

When the fractions extracted from yolk sac were tested with partially purified EEE virus, the fraction that appeared inactivating to R. rickettsii also caused a more rapid inactivation of the virus than did the Tween 80 control; in tests with this virus, a very definite stabilizing effect was observed for the water-soluble fraction of the ethanol extract.

When these fractions were tested with a mouse brain suspension of VEE virus (diluted 1:100), Tween 80 had essentially no effect on the virus, and, therefore, the effect of the fractions could be seen more clearly than in the tests with R. rickettsii and EEE virus. Infectivity titers of the virus samples after 4, 12, and 19 days at 4 C are presented in Table 2. Inactivation by the water-soluble fraction of the chloroform-methanol extract was very apparent with this type of virus suspension. Note the stabilizing effect of the water-soluble fraction of the ethanol extract.

TABLE 2. EFFECT OF YOLK-SAC FRACTIONS ON THE INFECTIVITY OF VEE VIRUS

Added to Virus Suspension	Infectivity, $\log_{10}$ amniotic LD_{50}/ml[a]		
	Days at 4 C		
	5	12	19
Buffer (control)	4.7	4.4	4.6
0.5% Tween 80 (control)	5.7	3.9	3.6
Water-soluble fraction of $CHCl_3$-CH_3OH extract	3.0	0	0
Water-soluble fraction of C_2H_5OH extract	6.5	6.6	5.9

a. Starting titer: $1 \times 10^{6.6}$ amniotic LD_{50}/ml.

The water wash of the chloroform-methanol extract was gel-like, and Tween 80 had been used in mixing it with the viral and rickettsial samples. To obtain a cleaner separation (i.e., to eliminate the gel-like character of this water wash), acetone was employed instead of chloroform-methanol for Soxhlet extraction of the residue from the ethanol extraction. The aqueous fraction obtained from the acetone extract, unlike that from the chloroform-methanol extract, was relatively clear and was tested for activity without the addition of Tween 80. That this fraction contained inactivating compound(s) is indicated by the data in Table 3. These results

were obtained with samples composed of two parts of a 1:100 dilution of a mouse brain suspension of VEE virus and one part of the aqueous wash.

TABLE 3. EFFECT OF WATER-SOLUBLE FRACTION OF ACETONE EXTRACT OF YOLK SAC ON VEE VIRUS

Preparation[a]	Infectivity $\log_{10}$ amniotic LD_{50}/ml			
	Days at 4 C			
	0	1	2	3
Virus + buffer	6.5	5.9	6.0	5.5
Virus + water-soluble fraction of acetone extract	6.5	4.5	3.7	<2.5

a. Mouse brain suspension of VEE virus, diluted 1:100; one volume of buffer or water-soluble fraction added to two volumes of virus suspension.

Because bovine serum albumin was required for maximum stability of R. rickettsii in yolk-sac suspensions in earlier experiments, we were interested in determining whether the effect of the inactivating fraction of yolk sac could be modified by the addition of bovine albumin. A 1:100 dilution of a mouse brain suspension of VEE virus was mixed with either the yolk-sac fraction (the water wash of the acetone extract) or an appropriate volume of the fraction to which bovine serum albumin had been added so as to give a final concentration of 0.3%. Duplicate samples, together with control samples, were placed at 4 C and assayed for infectivity after 1, 2, and 3 days. As shown in Table 4, the inactivating effect of the yolk-sac fraction was not completely neutralized but was decreased by bovine albumin. In addition to the reports of other workers[2-4] on the tendency of serum proteins to complex with various lipids, we had previously found[3] that the rapid inactivation of R. rickettsii by 20 µg of sodium deoxycholate/ml could be prevented by 0.3% bovine albumin. However, albumin at this concentration was not effective against 200 µg of deoxycholate/ml.

TABLE 4. PROTECTIVE EFFECT OF BOVINE SERUM ALBUMIN ON THE INACTIVATION OF VEE VIRUS BY YOLK-SAC EXTRACT[a/]

Preparation	Infectivity, $\log_{10}$ amniotic LD_{50}/ml[b/] Days at 4 C		
	1	2	3
Virus + buffer	6.1	5.8	4.7
Virus + water-soluble fraction of acetone extract	4.4	3.5	2.8
Virus + water-soluble fraction and bovine albumin	5.1	4.4	4.1

a. Extract is the water-soluble fraction obtained from an acetone extract of yolk sac.
b. Starting titer: $1 \times 10^{6.8}$ amniotic LD_{50}/ml.

Examination of the inactivating fraction by thin-layer chromatography revealed that this fraction contained several substances (Fig. 1). This chromatogram was obtained on a silica gel G plate by development with butanol:acetic acid:water (10:1:1), followed by spraying with phosphomolybdic acid. The most prominent band (6) is cholesterol. The spot to the left of the numeral was given by a cholesterol standard, and the spot and the band were both purple. The rather faint band 5 was the same light blue as the spot to its right, but the spot was slightly lower. This spot was given by a sodium deoxycholate standard.

Another thin-layer plate, with the inactivating fraction applied across its entire width, was run in parallel with the one shown in Figure 1. Bands were located by spraying the edge of this plate. Bands 5 and 7 were removed by scraping the silica gel from the plate, and the components were recovered from the silica gel by extraction with acetone. After evaporation of the acetone, the compounds were dissolved or suspended in phosphate buffer. Components 5 and 7 were then tested for their effect on suspensions of partially purified EEE virus. After 4 hours at 4 C, component 5 reduced the titer 2.0 $\log_{10}$ below the control titer, and after 24 hours, 4.0 $\log_{10}$ or more. Component 7 was somewhat less inactivating than component 5.

These results suggest that the inactivating fraction extracted from yolk sac contains, in addition to cholesterol, a mixture of bile acids, including deoxycholate, that probably are responsible for the viral inactivation.

Figure 1. Thin-Layer Chromatogram of the Inactivating Fraction of Yolk Sac.

Investigations into the nature of the inactivating fraction were suspended at this point to assess the effectiveness of the stabilizing fraction (i.e., the water-soluble fraction of the ethanol extract) and to determine some of its characteristics. Its stabilizing potential was tested with partially purified VEE and EEE viruses. Table 5 shows the results obtained in one test with this fraction. Partially purified EEE virus was employed, and the storage temperature was 4 C. The titer was as high at 22 days as at 7 days, and at a level of 1 x $10^{4.1}$ at 42 days.

TABLE 5. EFFECT OF STABILIZING FRACTION OF YOLK SAC ON INFECTIVITY OF EEE VIRUS

	Infectivity, $\log_{10}$ amniotic LD_{50}/ml				
	Days at 4 C				
Preparation	0	7	16	22	42
Virus + buffer	8.3	0	-	-	-
Virus + fraction 6[a/]	-	7.0	6.9	7.1	4.1

a. Stabilizing fraction of yolk sac from 10-day eggs.

We were interested in determining whether the effect of the inactivating fraction on viral infectivity would be modified by the stabilizing fraction. As shown in Table 6, each fraction apparently exerts an effect upon the other; rapid inactivation is prevented, but the inactivating fraction markedly reduces the effect of the stabilizing fraction.

TABLE 6. LIMITED INTERACTION OF INACTIVATING AND STABILIZING FRACTIONS OF YOLK SAC: EFFECT ON VEE VIRUS INFECTIVITY

	Infectivity, $\log_{10}$ amniotic LD_{50}/ml		
	Days at 4 C		
Added to Virus Suspension	1	4	13
Buffer (control)	3.3	0	0
Inactivating fraction	<2.0	0	0
Stabilizing fraction	7.2	7.4	6.4
Inactivating and stabilizing fractions	6.3	4.6	0

Chromatography of the stabilizing fraction on plates of silica gel G, using the system butanol:acetic acid:water (10:1:1) for development, produced a number of bands that fluoresced under ultraviolet (UV) light. Figure 2 indicates the appearance of a chromatogram viewed under UV. Material recovered from four of these bands (A, B, D, E) showed some stabilizing activity for EEE virus, with most of the activity present in band A (material that remained at the origin). Although the UV absorption curves of these components showed differences when initially determined, all possessed essentially the same UV spectrum after being held in solution (80% ethanol) for 2 months at 4 C, suggesting that a mixture of related compounds had changed to the same end product or products. Both the stabilizing activity and the UV-absorbing property of the intact stabilizing fraction are dialyzable, but a relationship between these two properties has not been established.

Qualitative tests for protein and carbohydrate were negative for both the stabilizing and inactivating fractions. Colors obtained on spraying chromatograms of the inactivating fraction with phosphomolybdic acid were suggestive of the presence of bile acids. Results of tests for purines and pyrimidines in the stabilizing fraction are, at present, inconclusive.

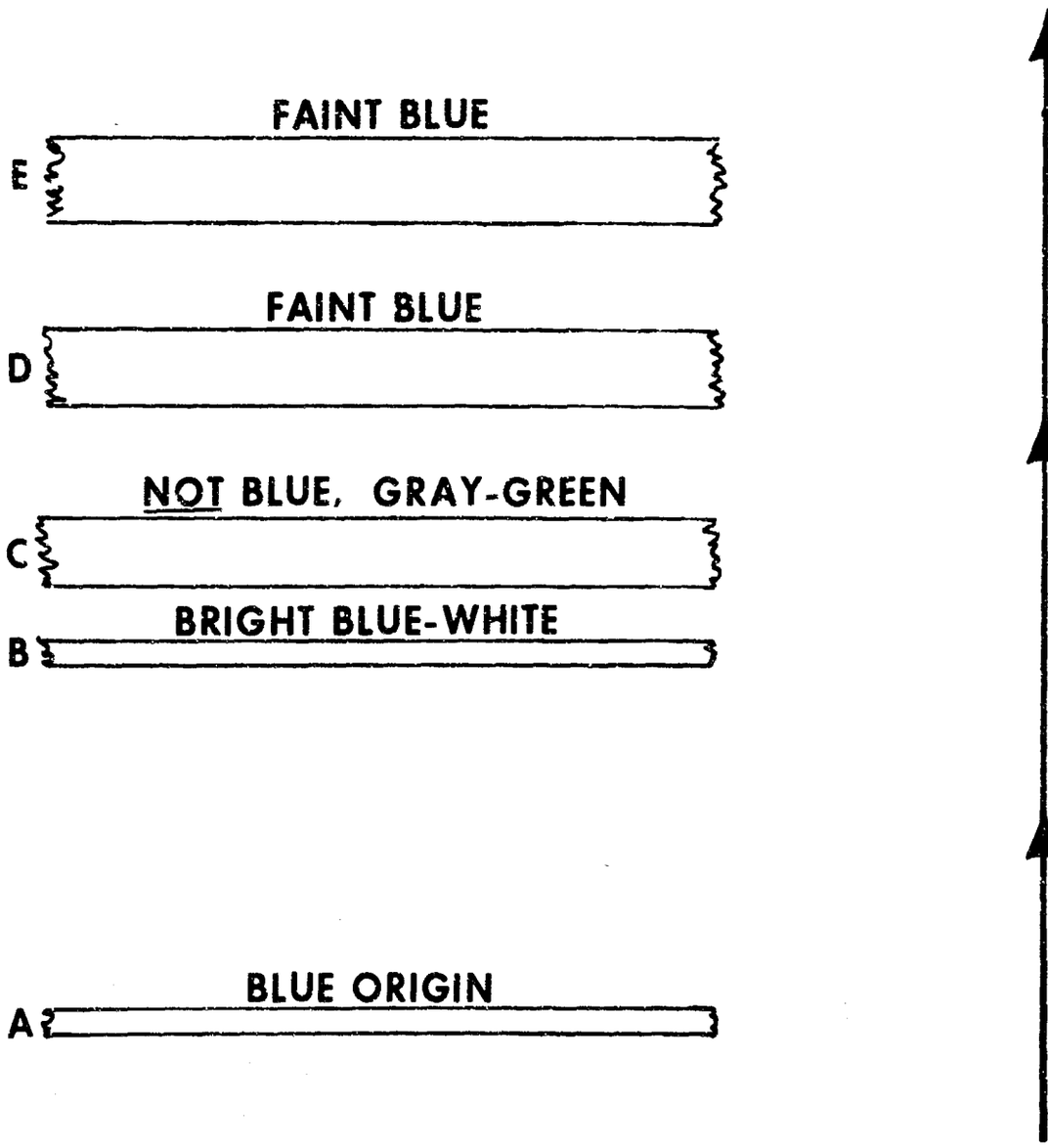

Figure 2. Schematic Drawing of Thin-Layer Chromatogram of the Stabilizing Fraction of Yolk Sac.

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13. ABSTRACT

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Chromatography						
*Yolk sac						
*Infectivity						