of the sterile preparations brought about fixation by the rhizobia or by mixtures of rhizobia and radiobacter, a frequent contaminant of nodules. The positive results with *Azotobacter* and *Clostridium* demonstrate that fixation could occur under the experimental conditions. As shown in *Experiment* 5 fixation is not always obtained with *Clostridium* probably because of the presence of free oxygen and soluble organic nitrogen. Neither of these would prevent fixation by *Azotobacter*. To reduce the level of soluble available nitrogen, one experiment was made using a purified hemoprotein solution and also hemoglobin from blood. Neither induced fixation by the rhizobia, nor did the addition of oxalacetate alter this result.

Nonsterile prepartions did not consistently induce fixation, the positive results being concentrated in two experiments. The results with the nonsterile filtrates are significant for the argument that lack of fixation by pure cultures of the rhizobia arises through loss of this physiological function after laboratory cultivation. In these experiments preparations were obtained that, although filtered, contained numerous rhizobia. These were identified by microscopic examination, cultural characteristics, physiological tests in litmus milk and veal infusion broth, and by inoculation of the host plant. Such organisms, tested for fixation immediately after recovery from the nodule and prior to any transfer on artificial medium, were unable to fix even the minute quantity of nitrogen that can be detected by the isotopic method (Experiment 7, 8, and 9). In the positive trials with nonsterile filtrates (Experiment 6 and 10) species of *Clostridium* demonstrated to be capable of fixing nitrogen were isolated.

Summary. When tested by the sensitive isotopic method, neither an aqueous extract of nodules nor a purified preparation of hemoprotein could induce fixation of atmospheric nitrogen by free-living *Rhizobium*.

Cultures of the root nodule bacteria taken directly from the nodules and tested prior to cultivation on laboratory media did not fix nitrogen even though hemoprotein was present.

In a few experiments, fixation of molecular nitrogen was obtained, but the nodular preparations were shown to contain nitrogen-fixing clostridia.

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Effect *in vitro* of Specific Lipid Fractions of Animal Sera on Psittacosis Virus.

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Casals and Olitsky[1] have reported that a number of neurotropic viruses were inactivated when mixed with various animal sera and incubated at 37°C. Furthermore when these sera were successively extracted with acetone, ether, and hot alcohol-ether, each extract was effective in inactivating virus. This report attempts to confirm these results —using psittacosis virus—and to isolate that lipid fraction having the virus-inactivating property.

Method and materials. Virus: Psittacosis virus originally isolated by Dr. Dorland Davis from a fatal disease in a parrot ("4" strain) was propagated in the allantoic sac of 8-day-old chick embryos. Allantoic fluid was

TABLE I.
Fifty Percent End-point of Psittacosis Virus Incubated at 37° in Presence of Serum, Saline and Sterile Water.

	1 hr	2 hr	4 hr	24 hr
Serum	$10^{-6.0}$	$10^{-6.0}$	$10^{-5.8}$	$10^{-3.6}$
Water	$10^{-6.0}$	$10^{-6.0}$	$10^{-4.2}$	$10^{-1.1}$
Saline	$10^{-6.1}$	$10^{-5.8}$	$10^{-2.4}$	10^{-0}

[1] Casals, J., and Olitsky, P. K., *Science*, 1947, **106**, 267.

TABLE II.
Average of 50% End-points a Single Pool of the "4" Strain of Psittacosis Virus Incubated for 1 Hour at 37°C in Presence of Serum and Egg Yolk Extracts and Lecithin.

Flow sheet	Avg of LD_{50} of psittacosis in serum extr.	Avg of LD_{50} of psittacosis in egg yolk extr.	Avg of LD_{50} of egg yolk lecithin*	Avg of LD_{50} of vegetable lecithin†
Serum or egg yolk	$10^{-6.1}$	$10^{-6.0}$		
Bloor's reagent → filtrate	$10^{-6.2}$	$10^{-6.1}$		
ppte—denatured protein				
petrol ether → ppte	$10^{-4.0}$	$10^{-5.8}$		
filtrate —total lipid				
acetone → filtrate				
$MgCl_2$ → ppte	$10^{-4.3}$	$10^{-6.1}$		
filtrate—phospholipid free-				
ether → ppte—sphyngomyelin-cerebroside	$10^{-4.6}$			
filtrate - - - - - lecithin cephalin	$10^{-1.3}$	$10^{-5.8}$		
$CdCl_2$ → ppte	$10^{-1.8}$			
filtrate —cephalin-like				
NH_3 → filtrate—lecithin-like	$10^{-0.8}$			
Control of egg and vegetable lecithin —			$10^{-5.9}$	$10^{-6.0}$

* Mr. Theriault of National Institute of Health.
† Pfanstiehl vegetable lecithin.

pooled, cultured for contaminants, titrated in mice and quick frozen in sterile ampules in 2 cc lots. The 6BC and Gleason strains of psittacosis virus obtained from Dr. Orville Golub of Camp Detrick were also used.

Serum extracts. Total lipid extracts by Man's method[2] were made of sera from normal chicken, rabbit, horse, sheep and human bloods. The petroleum ether solution of the serum lipids was then concentrated under negative pressure of 28 inches of mercury at a temperature of 35-40°C in a constant small stream of dry nitrogen. Seven volumes of acetone were then added to this concentrated extract and allowed to stand overnight at 4°C. The precipitate was centrifuged at 1500 r.p.m. in a standard centrifuge for 5 minutes, the supernatant poured off and to it was added 1 drop of a saturated solution of magnesium chloride.[3] This was again centrifuged and the supernatant was saved as the phospholipid-free fraction of the total lipid constituents of serum. The precipitate in acetone was then divided into the ether-soluble and ether-insoluble fractions, the latter being saved as the sphyngomyelin-cerebroside fractions of serum. The lecithin-like fraction was isolated from the ether-soluble fraction using Maltaner's[4] modification of the technic of Levene and Rolf.[5] The cephalin-like fraction was recovered in the ether washing of the cadmium lecithin. Each of these 4 fractions—phospholipid-free, sphyngomyelin-cerebroside, lecithin and cephalin—was concentrated and to each was added an amount of sterile distilled water

2 Man, E. B., *J. Biol. Chem.*, 1937, **117**, 183.

3 Sinclair, R. G., Dolan, M., *J. Biol. Chem.*, 1942, **142**, 659.

4 Maltaner, F., *JACS*, 1930, **52**, 1718.

5 Levene, P. A., and Rolf, I., *J. Biol. Chem.*, 1927, **72**, 587.

equal to the amount of original serum. The organic solvent was then bubbled off with nitrogen gas. The extracts were in some cases dried completely with nitrogen gas and then taken up with water with no difference in the final titer. No further steps were taken to identify the fractions.[6] Except for the manifestly-necessary last step, great care was taken to avoid exposure to air, temperatures over 40°C, and water.

Titrations. The infected allantoic fluids were made up in 10-fold dilutions in each of the above extracts as well as in controls of distilled water, saline, non-infected allantoic fluids and egg yolk extracts. After incubation at 37°C for times varying from 1-24 hours, 0.03 cc were injected intracranially into 6-week-old mice of the NIH stock strain (10-20 mice for each dilution), and the LD_{50} computed by the method of Reed and Muench.

Results. Since the virus was gradually inactivated in any diluent when incubated at 37°C for longer than 4 hours (Table I), the various fractions were compared on the basis of one-hour results. Under these conditions no virus inactivation could be demonstrated by normal sera. Virus-inactivating properties could be demonstrated, however, in lipid extracts of the same sera. Table II, presenting the averages of 15 extractions, demonstrates that the phospholipid-free and sphyngomyelin-cerebroside fractions have a 10-100 fold inactivating effect, that the lecithin-like and cephalin-like fractions decreased the titer 10.000-100,000 fold, and that the lecithin-like fraction was about 10 times more effective than the cephalin-like fraction. Comparable results were obtained with the 6BC and Gleason strains. Controls of water; saline; egg yolk extracts; commercial lecithin, prepared in concentration equal to that expected in serum extracts by bubbling off the organic solvent in the presence of water; all failed to exert any such inactivating effect.

Discussion. This work fails to demonstrate that animal sera can inactivate psittacosis virus, but tends to confirm findings of others[1] that virus inactivation can occur in isolated lipid fractions peculiar to serum. It should be pointed out that different yields with different titres were frequently obtained from

TABLE III.

Average of 50% End-points of a Single Pool of the "4" Strain of Psittacosis Virus Incubated for 1 Hour at 37° in the Presence of Serum and Egg Yolk Extracts and Lecithin.

	Chicken 1	Sheep 1	Sheep 2	Human 1	Human 1	Chicken 2	Sheep 3	Sheep 4	Sheep 4	Sheep 5	Sheep 5	Sheep 6	Horse	Pooled	Pooled	Avg	Egg yolk 1	Egg yolk 2
Serum	$10^{-6.0}$	$10^{-5.8}$	$10^{-6.4}$	(1)		(1)	(1)						(1)		(1)	$10^{-6.1}$	$10^{-6.2}$	$10^{-5.8}$
Denatured protein	$10^{-6.4}$	$10^{-6.2}$	$10^{-5.9}$			(2)										$10^{-6.2}$	$10^{-6.3}$	$10^{-5.8}$
Total lipid	$10^{-4.0}$	$10^{-4.0}$	$10^{-4.1}$													$10^{-4.0}$	$10^{-5.9}$	$10^{-5.7}$
Phospholipid free	$10^{-4.0}$	$10^{-3.0}$	$10^{-4.3}$	$10^{-3.5}$	$10^{-4.5}$	$10^{-4.1}$	$10^{-4.5}$	$10^{-5.0}$	$10^{-3.5}$	$10^{-4.6}$	$10^{-5.2}$	$10^{-5.0}$	$10^{-4.8}$	$10^{-4.6}$		$10^{-4.3}$	$10^{-6.2}$	$10^{-6.0}$
Sphyngomyelin cerebroside	$10^{-4.6}$	$10^{-5.4}$	$10^{-3.8}$						(3)							$10^{-4.6}$		
Cephalin lecithin	$10^{-1.5}$	$10^{-3.0}$	$10^{-1.8}$	$10^{-3.8}$	$10^{-2.9}$	10^{-0}	$10^{-1.5}$	10^{-0}	10^{-0}	10^{-0}	10^{-0}	10^{-0}	$10^{-3.0}$			$10^{-1.3}$	$10^{-6.0}$	$10^{-5.6}$
Cephalin													$10^{-2.1}$	$10^{-1.8}$	$10^{-1.5}$	$10^{-1.8}$		
Lecithin													10^{-0}	$10^{-1.0}$	$10^{-1.5}$	$10^{-0.8}$		
Egg yolk lecithin			$10^{-5.9}$															
Vegetable lecithin			$10^{-6.0}$															

(1) Greater than 10^{-4}; 100% mortality at 10^{-4}.
(2) Greater than 10^{-6}; 80% mortality at 10^{-6}.
(3) Greater than 10^{-5}; 90% mortality at 10^{-5}.

equal aliquots of the same sera. This may be due to the difficulties in extracting a pure substance that is not contaminated or decomposed.[6]

Summary. Specific lipid fractions of certain animal sera appear capable of inactivating psittacosis virus *in vitro* though the original sera exerts no such effect. The virus inactivating property appears to be in the lecithin-like and cephalin-like fractions, and peculiar to serum lipids.

[6] Bull, H. B., Biochemistry of the Lipids, 1937.