

NOTE TO USERS

This reproduction is the best copy available.

UMI[®]

UNIVERSITY OF CINCINNATI

May 17, 1951

I hereby recommend that the thesis prepared under my supervision by Wilton A. Rightsel

entitled SEROLOGICAL STUDIES ON BACTERIUM TULARENSE

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

Lea P. Orkey
Melvin A. Logan

SEROLOGICAL STUDIES ON BACTERIUM TULARENSE

A dissertation submitted to the
Graduate School of Arts and Sciences
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1951

by

Wilton A. Rightsel

A. B. Indiana University 1942

M. S. Indiana State 1946

AUG 23 1951

UMI Number: DP16753

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.



UMI Microform DP16753

Copyright 2009 by ProQuest LLC

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Acknowledgement

The author expresses his appreciation to those who so generously gave of their time and counsel, and especially to Dr. Lee Foshay for his unfailing interest, technical advice, and constructive criticism in the preparation of the manuscript.

The material presented in this thesis is based upon work sponsored in part by the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, under Contracts No. W-18-035-CM-134, W-18-064-CM-233, and USADA-18-064-CML-446 with the University of Cincinnati.

TABLE OF CONTENTS

	<u>Page</u>
Introduction.....	1
Materials and Methods.....	5
Experimental Results	
Part A: Precipitin Studies on Tularenses Systems....	30
Part B: Rat Protection Studies Employing Anti-tularenses Goat Serums.....	64
Discussion.....	75
Summary.....	91
46 Tables	
7 Figures	
References	

INTRODUCTION

Tularemia was recognized as a disease of rodents and described by McCoy (1) in 1911. The causative organism, a small gram-negative rod, was named Bacterium tularense by McCoy and Chapin (2) who first isolated the agent and performed the first serologic studies. Wherry and Lamb (3) made a bacteriological diagnosis of the first human case, recognized by Vail (4) as the cause of an ocular disease, and tularemia became established as an acute, febrile disease in man.

The immunogenic properties of killed B. tularense vaccines were demonstrated in man by Foshay et al. (5) and Kadull et al. (6), but such vaccines did not produce the solid immunity observed after infection with the organism. Foshay et al. (5), studying the use of killed virulent organisms as a vaccine for man, found a lowered incidence of disease in exposed groups and also a decrease in severity of symptoms. The further observation was made that vaccination of man with one strain protected against infection with other strains. In these studies immunogenic differences were not detected among the various strains.

The value of active or passive immunization in tularemia has been difficult to demonstrate chiefly because immunologic studies are hindered by the lack of a satisfactory experimental animal. It was demonstrated by Ruchman and Foshay (7) that white mice are highly susceptible to tularemia and that they

are protected only to a slight degree, if at all, by the injection of killed vaccines made from a virulent strain of B. tularensis. Downs and Woodward (8) studied the immunogenic properties of living organisms of lowered virulence for the white mouse. They demonstrated that mice were highly resistant to infection with a virulent strain and disposed of the organisms very rapidly after immunization by infection with a number of strains of lowered virulence. It was thus shown that certain strains would elicit a high degree of immunity in the white mouse when inoculated in sublethal concentrations.

Foshay (9) previously advocated the use of immune serum as a therapeutic measure in human tularemic infections. His findings were verified by Larson (10) who found that both immune and convalescent human serums contained protective antibody. Foshay et al. (11) demonstrated a correlation between the amount of polysaccharide precipitable antibody and the protective antibody titer for rats in serums from hyperimmune goats and horses. Aware that protective antibody may exist in the serum of man after vaccination as well as after infection, Alexander (12) utilized quantitative chemical methods to measure the immune response. This was indicated by a measurement of antibody present in serum which is reactive with the polysaccharide antigen described by Nicholes (13).

Immunologic relationships between B. tularensis and mammalian hosts have been extensively studied. However serologic

or antigenic differences among the various strains have never been established. The literature to date does not attempt to differentiate the strains by any serological or immunological methods except for a comparison of 4 strains by reciprocal agglutinin-adsorption tests performed by Francis and Evans (14), and studies by Downs and Woodward (8) on immunologic properties of living organisms of graded virulence. Strains of B. tularensis differ in virulence and, since antigenic homogeneity in any group of organisms is the exception rather than the rule, the relation of virulence to antigenic structure and serological relationships seems worthy of further investigation. Numerous studies on morphology, agglutinin absorption and protective antibody content have failed to reveal any qualitative differences between virulent and avirulent strains of the organism.

The discussion to follow derives from a study of the quantitative relationships existing between 2 tularemic antigens and their respective antibodies in immune serums of various animal species. The work includes immunochemical studies for differences that might be present in serologic antigen:antibody reactions as disclosed by the quantitative precipitin reaction, between antigens and antiserums made from and produced by various strains of B. tularensis ranging in degrees of virulence from zero to about maximal for the white mouse. The investigations utilize quantitative chemical methods for measuring the amount of immune globulin, which at present afford a more

accurate measurement of antibody, and its reactivity with antigen, than other available methods. An attempt was also made to detect qualitative differences between tularemic protective antibodies that had been produced by a stimulus with (a) a virulent strain and (b) an avirulent strain.

MATERIALS AND METHODS

There are numerous ways of describing the intensity of precipitin reactions and measuring the relative potency of immune serums. However only those based upon the determination of the amount of specific precipitate either by direct weighing (15) or by chemical analysis (16) yield absolute results and meet requirements of quantitative analytical chemistry. Other methods are often useful when quantitative results are not required. Techniques such as the Martin serum dilution method (17), the optimal proportions method of Dean and Webb (18), and the neutralization method of Culbertson (19), all give results in relative terms rather than in absolute units, and are much less precise. These methods have been investigated. Furthermore, Heidelberger (20) has developed a quantitative theory of the precipitin reaction and has applied it to the study of various antigen-antibody systems. Since relationships of the theory were found to apply to each of several systems studied by Heidelberger and associates (16, 20, 21) it seemed of interest to extend these studies and to test their applicability to various tularensis systems. The superiority of the quantitative method for the determination of precipitins over other methods for determining antigenic differences in closely related bacteria suggested its application to tularensis antigen-antibody systems as a means of investigating the

relationships of antigenicity to virulence among this group of organisms.

Before discussing the precipitation technique and the methods for determination of nitrogen content of specific precipitates, a description of each component of the reacting mixture, its preparation, treatment, and storage will be discussed.

1. Antigens. Several antigenic components were extracted from 2 strains of B. tularensis and used as the precipitating antigens throughout the investigation. The antigens were prepared from the typical highly virulent strain SCHU and the avirulent strain 38. These strains were selected because they represent the antipodes in degrees of virulence. The methods for isolation of the fractions will be described.

(a) Palmer and Gerlough Fraction. Polysaccharides derived from strains SCHU and 38 were prepared by the phenol extraction method of Nicholes (13) who employed the method of Palmer and Gerlough (22) and first demonstrated the antigenicity of the polysaccharide in vitro. The first lots of polysaccharides were prepared by and supplied through the courtesy of Drs. H. T. Eigelsbach and P. S. Nicholes at Camp Detrick, Maryland. Subsequently, similar samples were prepared here. From each substance a stock solution containing exactly 100 µg/ml was prepared. Improved preservation stability was secured by dispensing these stock solutions in 8-ml capacity lusteroid

tubes, storing a years's supply of antigen in the deep freeze unit, and withdrawing small samples only as needed. This eliminated the need for a preservative and its possible consequent chemical changes in the polysaccharides. These preparations have been designated as "PG SSS-SCHU" and "PG SSS-38" respectively.

(b) Boivin Fraction. Mass cultivation and harvesting of each strain was necessary prior to extraction of the Boivin fraction. The liquid medium employed for cultivation of strain 38 was a 2 per cent, old Merck meat peptone (purchased about 18 years ago) supplemented only with cystine and glucose. The media were usually dispensed in 1-liter quantities, and cultures were incubated at 37 C with constant shaking for 48 hours. The 48-hour cultures were harvested by centrifugation and washed twice with saline. The washed organisms were then extracted with 75 per cent acetone for 2 hours at 37 C, then with 100 per cent acetone for 24 hours at the same temperature. After centrifugation, the acetone-extracted organisms were dried in vacuo over CaCl_2 for 48 hours. The constant dry weight of organisms from each lot of medium was determined and the material stored in a desiccator at room temperature. The following figures show yields of strain 38 obtained from each lot of mass culture.

<u>Vol. Medium</u> <u>Liters</u>	<u>Dry Weight</u> <u>Grams</u>
1.0	1.1078
1.0	1.2278
0.5	0.4782
1.5	1.1078
1.0	0.7915
1.0	0.9536
2.5	2.5372

Seven lots of mass culture of strain 38 gave a total yield of 8.2039 grams of dried organisms, representing an average of 0.965 grams of acetone dried cells per liter of culture.

The virulent strain SCHU M₃₃ R₂₂ was grown in the gelatin hydrolyzate blood cell extract cysteine medium first employed by Gibby et al. (23) for obtaining massive cell yields from large inocula. The medium was again dispensed in 1-liter quantities and the cultures, initiated with an inoculum of 0.5 billion cells per ml, were incubated at 37 C with constant shaking for 36 to 48 hours. The yield of organisms was harvested as described above for strain 38 and the acetone dried material was stored in a desiccator at room temperature until used. Four 1-liter lots of mass culture gave a total yield of 4.6478 grams of dried organisms. This represents an average of 1.1619 grams of acetone dried cells per liter of culture.

An antigenic fraction was isolated from the 2 strains of B. tularensis by the Boivin trichloroacetic acid (TCAA) extraction technique (24, 25) as modified by Freeman, Challinor, and Wilson (26). The original procedure of Boivin, with one exception, was followed for extracting the antigenic component. The technique employed was as follows: Acetone extracted cells were suspended in 5 times their weight of cold distilled water. The suspension was cooled to 0 C and an equal volume of 0.5 N TCAA was added. The mixture was held for 3 hours at 0 C with occasional shaking. It was then centrifuged at high speed to remove insoluble bacterial residue which was discarded. The opalescent TCAA centrifugate was dialyzed against running tap water for 24 hours. The TCAA extract was centrifuged at high speed to remove any precipitate formed during dialysis. The polysaccharide was precipitated from solution by addition of 3 times its volume of acetone. The white precipitate was harvested by centrifugation and the supernatant discarded. The precipitate was redissolved in a minimum of distilled water and cleared of a small quantity of insoluble matter by centrifugation at high speed. The solution was acidified with a trace of HCl and dialyzed against a large volume and frequent changes of distilled water. The 38 polysaccharide was isolated from solution by precipitating with ethyl alcohol to a concentration of 68 per cent by weight with a trace of sodium acetate added as electrolyte. However the polysaccharide from strain SCHU

was isolated by precipitating with acetone in the presence of a trace of HCl. In each instance the precipitate was washed once with alcohol and ether, then dried in vacuo over drierite.

Acetone-extracted cells from B. tularense, strain 38, in a quantity of 3.52 grams yielded 54.8 mg of an off-white, brittle substance, representing 1.55 per cent of the dry weight of organisms. The preparation was readily soluble to a concentration of 200 µg/ml and gave no color development with the Folin-Ciocalteu phenol reagent (27) for the determination of nitrogen in protein. This material was designated "Boivin SSS-38(R-68)".

B. tularense, strain SCHU M₃₃ R₂₂, type D, in a quantity of 4.0 grams, yielded 7.8 mg of a snow white, fluffy substance, representing only 0.2 per cent of the dry weight of organisms. This was labeled "Boivin SSS-SCHU".

The dried polysaccharide preparations, from strain SCHU and 38, were kept in a desiccator at room temperature until used. A stock solution of each Boivin SSS was made in 0.85 per cent saline to a concentration of 200 µg/ml. The solutions were stored at refrigeration temperature since they were used so rapidly.

(c) Sonic Extract Antigen. An effort also was made to prepare a predominantly protein antigen by sonic disintegration of cells for comparison with the 2 polysaccharide fractions. In this way the combining power of each antigenic

preparation for the same antibody could be compared. Preliminary studies of a sonic extract as antigen were made only with the highly virulent SCHU strain. Again the preparation of a sonic extract necessitated growing organisms in sufficient volume to obtain a maximal yield in a short time. For this purpose a synthetic amino acid medium devised by Dr. J. Tamura was used (28). Constituents of the medium were as follows:

	<u>mg/L</u>
L-cysteine	400
L-glutamic acid	5,000
DL-threonine	1,000
L-leucine	400
DL-isoleucine	400
L-lysine	400
L-proline	800
DL-serine	800
DL-valine	500
L-tyrosine	80
L-aspartic acid	150
L-arginine	150
L-histidine	100
L-methionine	100
thiamin	0.1
ATP	50 µg/ml
biotin	0.05 µg/ml
monobasic potassium phosphate	0.05 M
NaCl	1%
"B" salts (MgCl ₂ 2 x 10 ⁻⁴ M	
CaCl ₂ 2 x 10 ⁻⁴ M	
MnSO ₄ 2 x 10 ⁻⁷ M	
FeSO ₄ 2 x 10 ⁻⁷ M)	0.5 mg/100 ml

pH adjusted to 6.7 by adding 10 per cent NaOH. Sterilized at 10 pounds for 10 minutes. Add sterile glucose to make final concentration of 2 per cent.

For inoculating the medium the SCHU strain (SCHU M₃₃ R₂₂, type D, was grown on glucose cysteine blood agar slants.

A 24-hour culture from a 24-hour culture on 6 GCBA slants was suspended in 20 ml of saline. Five ml of the suspension were used to inoculate 2 liters of medium. The cultures were continuously shaken at 37 C and 20- to 22-hour cultures gave yields of 16 to 20 billions of organisms/ml with a terminal pH varying between 7.6 and 7.9. These cells were packed by centrifugation at 4,000 rpm for 1½ hours at 4 C. The supernatants were decanted, and the packed cells were washed and suspended in saline. Washing and resuspending were repeated twice and, after the last washing, the packed cells were pooled and suspended in a small volume of distilled water to give a final concentration of 40 to 80 billions of cells/ml. The concentrated suspensions were subjected to treatment at 9,000 cycles/sec in a Raytheon magnetostriction oscillator. The largest volume subjected to treatment at any one time was 40 ml and sonic treatment was continued until maximal clearing and opalescence were attained, which took about 45 minutes. Portions of sonicized extract were then stored in bulk at refrigeration temperature until needed.

The sonic extract to be used as a precipitating antigen required some form of standardization of the stock solution. To arrive at a standard procedure an investigation was made of (a) turbidities and number of organisms before sonic disintegration, (b) dry weight of organisms before sonic treatment, (c) nitrogen content of whole organisms, and (d) nitrogen de-

terminations on the sonicized material.

Data for standardization of 2 yields of organisms for an antigen from SCHU M₃₃ R₂₂, type D, are given as follows:

	<u>Sample I</u>	<u>Sample II</u>
<u>Before Sonic Disintegration:</u>		
Turbidity	: T- 13,230	29,010
No. of Organisms	: B/ml 40.05	87.60
Dry Weight	: Mg/ml 9.35	11.28
Nitrogen	: Mg/ml 1.0064	1.3477
<u>After Sonic Disintegration :</u>		
Nitrogen	: Mg/ml 1.0436	1.3315

These figures show that turbidities and numbers of organisms are inadequate as a reference standard. Readings for these figures were made on a photometer. Sources of error may occur when dilutions of the stock suspension are made in order to take readings or when 2 cultures vary significantly in age. Sample II showed more than 2 times the turbidity of sample I, yet sample II contained only a fraction more nitrogen than did sample I. Likewise, the dry weight of sample II was but 1.83 mg greater than sample I. However the dry weights and nitrogen contents of the 2 samples exhibited good agreement. The ratio of dry weight/N in sample II was 0.118 and it was 0.112 for sample I. Therefore it appeared that the more accurate method of standardization was by nitrogen determinations or dry weight of organisms in the original suspension. It is difficult to adjust an unknown suspension of organisms to a reference standard in terms of dry weight but the nitrogen content of any

suspension may easily be adjusted by dilution and promptly checked by a micro-Kjeldahl nitrogen determination. The data above also show that the nitrogen content of the suspension before and after sonic disintegration was essentially the same. Sample II varied only by 6 μ g nitrogen and sample I by 37 μ g. Therefore an accurate nitrogen determination on the suspension either before or after sonic disintegration provided a satisfactory reference standard for the antigen. However in view of the fact that the sonic extract was to be used as the quantitating antigen, the nitrogen content of the sonicized material was adopted as the standard. Accordingly all stocks of sonicized antigen were then adjusted by dilution to a single reference containing a given quantity of nitrogen per ml.

2. Antisera. Hyperimmune antitularense serums were utilized throughout the first portion of the study. Antisera from each strain, the virulent SCHU strain and the avirulent 38 strain, were prepared in goats, rabbits, and a horse (SCHU only). Also various process-preserved animal serums were used that had been prepared previously by Dr. L. Foshay and associates. A brief description of each serum, together with its source and specification will be given:

Goat antiserum GLV_{Schu}-43 was lyoprocessed in June 1944 from bleedings of the previous winter from goats inoculated intravenously (I-V) with living SCHU cultures of maximal virulence. This serum was adopted as the provisional standard

antiserum. The rehydrated serum agglutinated the homologous antigen completely in dilution 1:10,240 and about 75 per cent in 1:20,480.

Goat antiserum G₁LV_{Schu}-46-A was prepared by inoculating goat No. 1 I-V with living suspensions of the virulent SCHU strain.

Goat antiserum G₄LA₃₈-46 was prepared by inoculating goat No. 4 I-V with living suspensions of the totally avirulent 38 strain.

Goat antiserum G₇LA₃₈-46-A was prepared by inoculating goat No. 7 as goat No. 4 but this animal received much more antigen over a longer period of time.

Goat antisera G₁LV_{Schu}-46-B and G₇LA₃₈-46-B were obtained from goats No. 1 and No. 7, respectively, by a second bleeding, made 25 days after the initial bleeding.

Samples of each of the above sera were held for 1 year or more as (a) continuously frozen in dry ice, (b) lyophilized, and (c) in liquid bulk at 4 C.

Horse antiserum HLV_{Schu}-43 was prepared in 1943 and 1944 by inoculating a horse I-V with living virulent SCHU strain. After use from bulk storage at 4 C for about 1 year, the remaining serum was lyophilized and stored at 4 C.

All of the above sera had been on hand for some time and were supplied through the courtesy of Dr. L. Foshay and associates. However the supply of some of these sera

was low and a greater variety of various hyperimmune animal serums was desired. Therefore 3, new, young female goats were procured to prepare more hyperimmune goat antiserums. Two were bled to provide a supply of strictly normal serum. Thereafter all were started on a series of I-V inoculations, two with the living SCHU strain, and one with the living 38 strain. The inoculation schedule given in table 1 is indicative of the course of immunization and the cautious approach with the virulent strain to avoid possible delays caused by excessive pulmonary infiltrations.

Since the Heidelberger theory of the precipitin reaction (21) was developed from experimental work carried out chiefly with rabbit antiserums it was deemed advisable to investigate the tularensis system with the same animal species. Accordingly 2 rabbits were obtained and antiserum was prepared by giving 3 I-V inoculations at weekly intervals, of the same numbers of bacterial cells, one receiving strain SCHU killed with 0.5 per cent formalin, the other the living 38 strain. The inoculation schedule given in table 2 illustrates the plan followed together with the serum designations.

After detailed quantitative studies of the previous serums were made, it was realized that investigations had been limited to antiserums prepared against only 1 highly virulent strain of B. tularensis and an avirulent strain. Since this represented a maximal range in degree of virulence, and each

Table 1

Goat Inoculation Schedules
(1949)

<u>Date</u>	<u>SCHU Antigen</u>		<u>38 Antigen</u>
	Goat No. 12	Goat No. 14	Goat No. 13
	Using SCHU M ₃₀ R ₂₁		
2/ 4	1.0 Mill.	1.0 Mill.	1.0 Mill.
2/ 7	Omit	1.0 "	1.0 "
2/ 9	1.0 Mill.	Omit	300 "
2/11	1.0 "	"	300 "
2/14	1.0 "	"	300 "
2/16	1.0 "	"	450 "
2/18	1.0 "	"	450 "
2/21	1.0 "	1.0 Mill.	416 "
	Using SCHU M ₃₁ R ₂₁		
2/23	1.0 Mill.	1.0 Mill.	300 "
2/25	1.0 "	1.0 "	600 "
2/28	1.0 "	1.0 "	830 "
3/ 2	5.0 "	5.0 "	1.66 Bill.
3/ 4	5.0 "	5.0 "	2.42 "
3/ 7	10.0 "	10.0 "	4.05 "
3/ 9	20.0 "	20.0 "	5.00 "
3/11	30.0 "	30.0 "	5.00 "
3/14	21.2 "	21.2 "	6.10 "
	Using SCHU M ₃₂ R ₂₁		
3/16	40.0 Mill.	40.0 Mill.	7.80 "
3/18	60.0 "	60.0 "	10.90 "
3/21	80.0 "	80.0 "	Omit
3/23	150 "	150 "	3.42 Bill.
3/25	250 "	250 "	9.09 "
3/28	300 "	300 "	8.96 "
3/30	400 "	400 "	6.38 "
4/ 1	600 "	600 "	5.90 "
4/ 4	758 "	758 "	7.28 "
4/ 6	150 "	150 "	8.40 "
4/ 8	200 "	200 "	12.06 "
4/11	250 "	250 "	13.92 "
4/13	300 "	300 "	11.80 "
4/15	300 "	300 "	5.70 "
4/18	300 "	300 "	11.80 "
4/20	400 "	400 "	10.92 "
4/22	455 "	455 "	10.00 "
4/25	500 "	500 "	10.00 "
4/27	600 "	600 "	13.65 "
4/29	600 "	600 "	16.59 "
5/ 2	600 "	600 "	14.73 "
5/ 4	600 "	600 "	24.75 "
5/ 6	600 "	600 "	18.72 "
5/ 9	-	-	12.12 "

(Continued)

Table 1 (Continued)

<u>Date</u>	<u>SCHU Antigen</u>		<u>38 Antigen</u>
	<u>Goat No. 12</u>	<u>Goat No. 14</u>	<u>Goat No. 13</u>
5/10	Bled 625 cc	Bled 450 cc	Bled 725 cc
Labeled:	Serum pooled. GLV Schu-49-A		G ₁₃ LA ₃₈ -49-A
5/11	100 Mill.	100 Mill.	7.26 B111.
5/13	150 "	150 "	10.92 "
5/16	300 "	300 "	13.05 "
5/18	300 "	300 "	13.65 "
5/20	300 "	300 "	12.06 "
5/23	300 "	300 "	12.06 "
5/25	-	-	15.81 "
5/27	-	-	14.72 "
5/31	Bled 750 cc	Bled 600 cc	Bled 850 cc
Labeled:	Serum pooled. GLV Schu-49-B (Goat died 1 hr. after bleeding.)		G ₁₃ LA ₃₈ -49-B

1950

7/10	Bled 700 cc	Bled 700 cc
Labeled:	G ₁₄ LV Schu-49-C50a	G ₁₃ LA ₃₈ -49-C50a

- Storage: 1) One portion continuously frozen in deep freeze.
 2) Other portion held in liquid bulk at 4 C with merthiolate preservative 1:30,000.

Table 2
Rabbit Inoculation Schedule
(1948)

<u>Date</u>	<u>SCHU Antigen</u> Rabbit No. 1	<u>38 Antigen</u> Rabbit No. 2
	Using SCHU M ₂₈ R ₁₅	
4/10	3.48 Bill.	3.48 Bill.
4/17	4.91 "	4.91 "
4/26	3.80 "	3.80 "
5/6	Bled 40 cc	Bled 25 cc
Labeled:	R ₁ KV _{Schu} -48	R ₂ KA ₃₈ -48
Storage:	1) One portion continuously frozen in deep freeze. 2) One portion in liquid bulk at 4 C.	

strain was specifically selected, it was recognized that the same procedure should be followed using various other strains chosen to represent a graded series with respect to virulence. In addition, bleedings of the animals were planned to be made during the course of inoculations so that detectable changes occurring in the blood serum during the process of immunization could be followed. Studies of this kind were not attempted during the 1949 schedule. With these facts in mind 4 female goats were obtained. Only one of the goats, No. 7, had been used previously. This is the same goat that supplied serum G7LA38-46-A in 1946-47 and it therefore was bled prior to any inoculation. This goat was again selected for immunization with the avirulent 38 antigen. The remaining goats were normal animals; each inoculated with a different strain of B. tularense. The mouse LD₅₀ titers of the various strains varied from less than 10⁻⁰ for the 38 strain to 10^{-8.67} for the SCHU D strain. The other strains, JAP and CHUR, provided intermediates in the graded virulence series. All goats were inoculated I-V with living suspensions of organisms. The complete inoculation schedule is given in table 3, which also indicates the various bleeding intervals, the identifications of serums, and the weights of the goats during the courses of immunization. For the bleeding procedure the goats were given a pentothal sodium anesthetic in a dosage of 10 mg per kg body weight. This amount was adequate for the short time required.

Table 3

**Goat Inoculation Schedules
(1950)**

Goats Used: No. 7: Used previously in 1946-47 for inoculation I-V with living 38 antigen.
New Goats: No. 10, No. 17, No. 18: No previous inoculations.

Procedure: Goats inoculated I-V with living strains of B. tularensis grown on DCBA slants and suspended in saline.

Antigens Used and Goat Identification				
	CHUR M ₁₈ LD50-10-8.45	JAP M ₁ LD50-10-4.25	SCHU M ₃₃ R ₂₂ (type D) LD50-10-8.67	38 Avirulent
Date	No. 17	No. 18	No. 10	No. 7
4/ 6				Bled 500 cc serum: G ₇ LA ₃₈ -AA Pre- Inc. 44 kg
4/ 7	29.3 kg	27.2 kg	29 kg	
4/ 8	5 Mill.	5 Mill.	5 Mill.	
4/11	10 "	10 "	10 "	10 Mill.
4/14	30 "	30 "	30 "	30 "
4/17	Bled 150 cc	Bled 200 cc	Bled 200 cc	Bled 200 cc
Serum	G ₁₇ LVChur-50-A	G ₁₈ LVJap-50-A	G ₁₀ LVSchur-50-A	G ₇ LA ₃₈ -50-A
4/18	75 Mill.	75 Mill.	75 Mill.	75 Mill.
4/20	150 "	150 "	150 "	150 "
4/22	200 "	200 "	200 "	200 "
4/24	250 "	250 "	250 "	250 "
4/25	29.1 kg	26.8 kg	27.7 kg	49.1 kg
4/27	Bled 200 cc	Bled 200 cc	Bled 200 cc	Bled 200 cc
Serum	G ₁₇ LVChur-50-B	G ₁₈ LVJap-50-B	G ₁₀ LVSchur-50-B	G ₇ LA ₃₈ -50-B
4/29	500 Mill.	500 Mill.	500 Mill.	2.40 Bill.
5/ 2	600 "	600 "	600 "	4.32 "
5/ 4	758 "	758 "	758 "	4.84 "
5/ 6	1.06 Bill.	1.06 Bill.	1.06 Bill.	9.63 "
5/ 8	1.52 "	1.52 "	1.52 "	13.65 "
5/12	26.1 kg	25 kg	26.8 kg	49.1 kg
5/12	Bled 200 cc	Bled 200 cc	Bled 200 cc	Bled 200 cc
Serum	G ₁₇ LVChur-50-C	G ₁₈ LVJap-50-C	G ₁₀ LVSchur-50-C	G ₇ LA ₃₈ -50-C
5/13	1.82 Bill.	1.82 Bill.	1.82 Bill.	17.14 Bill.
5/16	2.12 "	2.12 "	2.12 "	22.12 "
5/18	2.50 "	2.50 "	2.50 "	27.04 "
5/20	2.73 "	2.73 "	2.73 "	27.26 "
5/22	3.03 "	3.03 "	3.03 "	26.35 "
5/24	3.48 "	3.48 "	3.48 "	16.05 "
5/26	3.48 "	3.48 "	3.48 "	16.25 "

(Continued)

Table 3 (Continued)

Antigens Used and Goat Identification				
	CHUR M ₁₈	JAP M ₁	SCHU M ₃₃ R ₂₂	38
	LD ₅₀ -10-8.45	LD ₅₀ -10-4.25	LD ₅₀ -10-8.67	Avirulent
Date	No. 17	No. 18	No. 10	No. 7
5/31	Bled 200 cc	Bled 200 cc	Bled 200 cc	Bled 200 cc
Serum	G ₁₇ LVChur-50-D	G ₁₈ LVJap-50-D	G ₁₀ LVschu-50-D	G ₇ LA38-50-D
6/ 1	4.02 Bill.	4.02 Bill.	4.02 Bill.	25.50 Bill.
6/ 3	4.55 "	4.55 "	4.55 "	15.38 "
6/ 6	5.02 "	5.02 "	5.02 "	29.51 "
6/ 8	5.53 "	5.53 "	5.53 "	20.10 "
6/10	6.06 "	6.06 "	6.06 "	25.80 "
6/12	7.11 "	7.11 "	7.11 "	57.27 "
6/12	22.6 kg	26.6 kg	29.8 kg	50.4 kg
6/14	8.15 Bill.	8.15 Bill.	8.15 Bill.	25.45 Bill.
6/16	9.09 "	9.09 "	9.09 "	46.59 "
6/17	Bled 600 cc Died during bleeding.		Found dead.	
Serum	G ₁₇ LVChur-50-E			
6/19				66.48 Bill.
6/21				70.14 "
6/23		Bled 700 cc		Bled 700 cc
Serum		G ₁₈ LVJap-50-E		G ₇ LA38-50-E
7/ 7		27 kg		52 kg
7/10		Bled 550 cc		Bled 900 cc
Serum		G ₁₈ LVJap-50-F		G ₇ LA38-50-F
8/ 1		Bled 600 cc		Bled 850 cc
Serum*		G ₁₈ LVJap-50-G		G ₇ LA38-50-G

Storage: All serums in deep freeze.

* "G" bleedings represent final bleedings.

The only rabbit serums which were available and tested were 1 anti-SCHU and 1 anti-38 serum. Both serums were obtained after only 3 inoculations, and thus they were not comparable with the hyperimmune goat serums. Consequently it was elected to prepare hyperimmune rabbit serums in the same fashion that goat serums had been made. For this procedure 8 new, young rabbits, each weighing between 5 to 6 pounds were used. The rabbits were divided into 4 pairs, each pair being inoculated with a different strain, representing a series of graded virulence, as in the 1950 goat inoculation schedule. The rabbits were inoculated I-V with formalin-killed suspensions of organisms. They were bled from the heart at frequent intervals during the course of immunization. The serums from each pair of rabbits were pooled and stored until analyses could be made. The rabbit inoculation schedule of 1950, together with bleeding intervals and serum designations are given in the accompanying table 4.

In processing each of the antiserums obtained from the goats and rabbits the freshly drawn blood was allowed to stand at room temperature for about 2 hours, then stored in the refrigerator at 4° C overnight to allow for complete clot retraction. The next day the serums were separated from the clots, cleared by centrifugation, and stored in the deep freeze unit with merthiolate preservative added to a concentration of 1:30,000. Portions of each serum were removed only as needed

Table 4

**Rabbit Inoculation Schedules
(1950)**

Rabbits Used: 2 young rabbits for each strain of B. tularensis, each weighing 5-6 lbs. and never used previously for any experimental work.

Procedure: Rabbits inoculated I-V with killed strains of B. tularensis. Organisms killed by suspending in 0.5% formalin in NaCl.

Antigens Used and Rabbit Identification				
	SCHU M ₃₃ R ₂₂ (type D) 2 Rabbits	CHUR M ₁₈ 2 Rabbits	JAP M ₁ 2 Rabbits	38 2 Rabbits
Date	No. 1	No. 3	No. 5	No. 11
4/12	.10 Bill.	.10 Bill.	.10 Bill.	.10 Bill.
4/14	.50 "	.50 "	.50 "	.50 "
4/17	1.06 "	1.06 "	1.06 "	1.06 "
4/21	Bled 60 cc	Bled 75 cc	Bled 60 cc	Bled 65 cc
Serum	R ₁ KVSchu-50-A	R ₃ KVChur-50-A	R ₅ KVJap-50-A	R ₁₁ KA38-50-A
4/22	1.52 Bill.	1.52 Bill.	1.52 Bill.	1.52 Bill.
4/24	2.12 "	2.12 "	2.12 "	2.12 "
4/26	2.50 "	2.50 "	2.50 "	2.50 "
4/28	3.03 "	3.03 "	3.03 "	3.03 "
5/2	Bled 75 cc	Bled 70 cc	Bled 85 cc	Bled 85 cc
Serum	R ₁ KVSchu-50-B	R ₃ KVChur-50-B	R ₅ KVJap-50-B	R ₁₁ KA38-50-B
5/4	4.02 Bill.	4.02 Bill.	4.02 Bill.	4.02 Bill.
5/6	4.02 "	4.02 "	4.02 "	4.02 "
5/9	4.91 "	4.91 "	4.91 "	4.91 "
5/11	6.06 "	6.06 "	6.06 "	6.06 "
5/13	7.58 "	7.58 "	7.58 "	7.58 "
5/17	Bled 90 cc	Bled 60 cc	Bled 90 cc	Bled 90 cc
Serum	R ₁ KVSchu-50-C	R ₃ KVChur-50-C	R ₅ KVJap-50-C	R ₁₁ KA38-50-C
5/18	9.09 Bill.	9.09 Bill.	9.09 Bill.	9.09 Bill.
5/20	9.09 "	9.09 "	9.09 "	9.09 "
5/22	12.12 "	12.12 "	12.12 "	12.12 "
5/24	12.12 "	12.12 "	12.12 "	12.12 "
5/29	15.15 "	15.15 "	15.15 "	15.15 "
5/31	15.15 "	15.15 "	15.15 "	11.76 "
6/2	15.15 "	15.15 "	15.15 "	15.15 "
6/7	Bled 140 cc	Bled 105 cc	Bled 130 cc	Bled 80 cc
Serum	R ₁ KVSchu-50-D	R ₃ KVChur-50-D	R ₅ KVJap-50-D	R ₁₁ KA38-50-D
6/8			1 found dead.	
6/9	18.18 Bill.	18.18 Bill.	18.18 Bill.	18.18 Bill.
6/12	18.18 "	18.18 "	18.18 "	18.18 "
6/15	18.18 "	18.18 "	18.18 "	18.18 "
6/19	18.18 "	18.18 "	18.18 "	18.18 "
6/21	18.18 "	18.18 "	18.18 "	18.18 "

Table 4 (Continued)

<u>Antigens Used and Rabbit Identification</u>				
	<u>SCHU M33 R22</u> (type D) 2 Rabbits	<u>CHUR M18</u> 2 Rabbits	<u>JAP M1</u> 1 Rabbit	<u>38</u> 2 Rabbits
<u>Date</u>	<u>No. 1</u>	<u>No. 3</u>	<u>No. 5</u>	<u>No. 11</u>
6/29	Bled 120 cc	Bled 95 cc	Bled 60 cc	Bled 120 cc
Serum	R1KVSchu-50-E	R3KVChur-50-E	R5KVJap-50-E	R11KA38-50-E
6/30	Inoculated 1 rabbit of each pair with living organisms as follows:			
	5 Mill.	5 Mill.	-	5 Mill.
7/3	Bled 1 - 80 cc	1 found dead.		
	(Sacrificed)			
Serum	R1aKVSchu-50-F*			
7/15	Bled 120 cc	Bled 120 cc	Bled 65 cc	Bled 245 cc
Serum	R1KVSchu-50-F*	R3KVChur-50-F*	R5KVJap-50-F*	R11KA38-50-F*

Storage: All in deep freeze.

* "F" bleedings represent final bleedings.

for analytical procedures.

3. Quantitative Antibody Determinations. Of all available serological in vitro tests for the quantitative determination of the amount of antibody precipitated with a polysaccharide antigen, the quantitative precipitin method of Heidelberger and Kendall (29), modified to be applicable to human serum by Heidelberger and MacPherson (30) was selected as the method of choice. The validity of this quantitative precipitation method is attested to by its general use for determining antibody against a variety of antigens. Since its introduction by Heidelberger and MacPherson, it has been used by Kabat et al. in estimating antibody in human serums precipitated by meningococcal polysaccharide (31) and for calculation of isoantibodies in human serum (32). Alexander utilized the method to measure the antibody response after infection or vaccination with B. tularensis (12). Therefore this quantitative precipitin technique was used for a study of the relative combining powers of the various antigenic preparations in both homologous and heterologous antigen-antibody systems.

(a) The Precipitin Test. The usual procedure was to add increasing increments of antigen to a constant amount of dilute antibody solution in a series of 12-ml pyrex centrifuge tubes. The volume and dilution of serum was dependent upon the relative concentration of antibody as determined by preliminary screening tests. Appropriate aliquots of serum were used so

that the amount of antigen would eventually precipitate all of the antibody in the selected volume of diluted serum.

The antigen was added first, usually in ascending 10 or 20 μ g increments, followed by addition of the same amount of diluted serum to each tube, and all finally brought up to the same volume, usually 2.5 ml, with saline. Control tubes were run with each series with saline in place of the antigen solution. The contents of the tubes were mixed, placed in a 37 C water bath for 2 to 4 hours then held in the refrigerator at 4 C for 48 hours. Following incubation at 4 C, the tubes were centrifuged at 3,000 rpm in a refrigerated centrifuge maintained at 0 to 4 C. The supernatant fluids were decanted into separate tubes and kept for subsequent detection of excess of reactants, antigen or antibody. The tubes were inverted and allowed to drain on filter paper for a few minutes, then each precipitate was washed twice with chilled saline, using 3.0 ml of saline for each washing. Care was taken not to lose the precipitate but at the same time adequately to disperse it so as to remove traces of occluded nonspecific protein. After each washing the precipitates were repacked and drained as before.

(1) Examination of Supernatants for Reactant in Excess. The supernatant fluids from the antigen-antibody reaction mixtures were tested for the presence of antigen or antibody by dispensing 0.5 ml of the fluid into each of 2 precipitation tubes. To 1 tube was added an equal volume of a

hyperimmune goat serum usually diluted 1:10 as a source of antibody, and to the other tube was added an equal volume of polysaccharide antigen containing 20 µg/ml. The tubes were mixed, incubated in a 37 C water bath for 2 to 3 hours, then placed at 4 C for 48 hours. The tubes were examined and readings recorded as positive, negative or a trace for the presence of precipitate.

(2) Determination of Antibody Nitrogen. The specific precipitates were analyzed for nitrogen by a colorimetric determination which utilizes the Folin-Ciocalteu phenol reagent for estimation of nitrogen in proteins (27). This method was modified by Herriot (33), and first applied to antigen-antibody precipitates by Heidelberger and MacPherson (30). The standard reagent was prepared as described in the original paper of Folin and Ciocalteu (27). Final readings were made with a Klett-Summerson photoelectric colorimeter which was developed and described by Summerson (34).

The twice washed precipitates were dissolved in 0.5 ml of N/10 NaOH. The tubes containing these precipitate solutions were gently agitated and examined at intervals for total dissolution of the precipitate. The volume was made up to 2.5 ml with distilled water. After mixing, the tubes were tightly stoppered and refrigerated overnight. The next morning 2.0 ml aliquots from each tube were removed and placed in 50 ml Erlenmeyer flasks. There were added, in turn, 6.0 ml of 12.5

per cent Na_2CO_3 , and 1.0 ml of M/100 copper sulfate. The flasks were shaken and allowed to stand for 1 hour. At the end of that time, 1.0 ml of a freshly diluted 1:3 mixture of Folin's phenol reagent was added dropwise while the mixture was constantly agitated. The flasks were allowed to stand 30 minutes for the blue color to reach a maximum. Readings were made on the Klett-Summerson photoelectric colorimeter using a red filter, wavelength of 650 m μ , in standard Klett tubes precalibrated with distilled water. A reagent blank was included and also control tubes which had been treated as were the test samples except that these contained saline with the serum dilution in place of antigen. In order to arrive at the final figures the reading of the control was subtracted from readings of the test samples.

The recorded optical densities were converted to micrograms of antibody nitrogen by means of a "Klett Factor". This factor was calculated by comparison of Klett readings with Kjeldahl nitrogen determinations on pooled specific precipitates. In this manner a calibration curve was prepared by the Folin method on solutions containing varying amounts of nitrogen calculated from micro-Kjeldahl analysis. A linear relation was obtained in which the color value of the protein per mg of nitrogen could be calculated and used as a factor in converting optical density to micrograms of nitrogen. With the polysaccharide antigen no correction was needed for the small amount of nitrogen in the antigen because it was not reactive

with the color reagent. However when the sonic lysate was used as antigen appropriate calculations had to be made to correct for the nitrogen in the antigen. Known solutions were analyzed at various intervals as a check on the calibration.

Since the Folin-Ciocalteu method for the determination of nitrogen in protein depends upon measuring the nitrogen content of a fairly constant tyrosine component, a tyrosine stock solution was also used as a reference standard, and suitable dilutions were analyzed for nitrogen by the colorimetric method. The readings derived from the pooled goat serum:SSS precipitates and tyrosine standard solutions gave a "Klett Factor" of 0.1076 μg for converting optical density to antibody nitrogen. However when the sonic extract was used as the precipitating antigen, a recalculation of the "Klett Factor" necessitated the use of 0.2021 μg as the conversion factor.

(b) Interpretation of Results. Calculation of the ratio of antibody, expressed as micrograms of nitrogen precipitated, to antigen, in terms of micrograms incorporated in the precipitate, was made after determining the amount of precipitable nitrogen. The amount of antibody nitrogen in undiluted serums, expressed as mg/ml, also was computed for each antigen increment employed and the maximum value revealed the antibody content of the serum. It was assumed, when no antigen appeared in excess in the supernatant fluid, that all of it was

in the precipitate. This presumption is validated by the work of Heidelberger et al. (36) and Stokinger and Heidelberger (37) who demonstrated that all the antigen is precipitated in the antibody excess and equivalence zones of the precipitin curve.

The micrograms of antigen in the precipitate were used as abscissa and the ratio of antibody precipitated to antigen was used as ordinate, and the best fitting straight line was determined by the method of least squares. Calculations included all the points in which 50 per cent or more of the total antibody was precipitated in the antibody excess zone. Heidelberger showed that a linear relationship existed between the values of antigen and of the antibody:antigen ratio when calculated from the experimental data including only determinations where one third or more of total antibody was precipitated (29). The same author showed that the course of the reaction between a specific polysaccharide of pneumococcus and its antibody could be described in the region of antibody excess by the following equation:

$$N_2 = 2RS - \frac{R^2}{A} S^2 \quad (29)$$

where N_2 is the amount of antibody nitrogen precipitated from the serum by addition of a definite amount of polysaccharide, $S \cdot R$ is the ratio of antibody nitrogen to antigen concentration at a selected point in the equivalence zone of the titration curve, and A is the amount of antibody nitrogen precipi-

tated at that same reference point. The equation was derived from the theoretical consideration that antigen-antibody combination occurs in a series of bimolecular competing reactions between multivalent antigen and antibody obeying the law of mass action. The equation has been shown to be valid for other antigen-antibody systems (16, 36) and Alexander (12), measuring the antibody response of man to B. tularensis with similar polysaccharide antigens, demonstrated excellent agreement between experimental and theoretical values for antibody nitrogen, even when as few as 2 determinations in the antibody excess zone were used in interpolating results.

The Heidelberger equation was applied to a study of the tularensis antigen-antibody system in calculating the amount of antibody nitrogen precipitated by any given amount of antigen in antibody excess. The calculated values were then compared with the experimental values obtained for antibody nitrogen at each antigen increment throughout the antibody excess zone. In contrasting the various tularensis precipitin systems, the values of R, the ratio A/S , A, the amount of antibody precipitated, and S, the amount of antigen combined, at the theoretical reference point of the equivalence zone are used for comparison.

4. Rat Protection Tests with Antitularenss Goat Serums.

Hyperimmune goat serums prepared by I-V inoculations of living strains of (a) maximal virulence (SCHU) and of (b) total

avirulence were adjusted to contain precisely identical precipitable antibody contents. These were studied to ascertain their respective protective powers for the challenged white rat. Foshay et al. (11) had reported differences in protective power between antivirulent and antiavirulent strain serums and these were attributed to their varying precipitable antibody contents. However no study was made for the presence of any antibody component which might be engendered by only one of the antigens. Therefore the present investigation was made to detect any qualitative differences between tularemic protective antibodies produced against the virulent and avirulent strains. The immune serums against strain SCHU and strain 38 were adjusted by dilution so that each would contain precisely the same amount of SSS-precipitable antibody. Evidence for any qualitative differences in protective antibody were then sought by challenge of pretreated groups of white rats with each serum.

At the time the rat protection experiments were performed the precipitable antibody contents were measured by the more simple serologic methods. Since great variation is encountered when performing any biologic experiment in animals, only qualitative measurements were made. Therefore the antibody contents of the serums were calculated in terms of the Marrack-Smith unit, the amount of antibody per ml of serum that is equivalent to 1 microgram of antigen (35). All serums used in the rat

protection test were quantitated with polysaccharides derived from both the SCHU and 38 strains prepared by the Palmer and Gerlough method (22).

Quantitation of Serum Antibody for Rat Protection Tests.

The neutralization method of Culbertson (19) was used with constant antibody solution. This procedure depends upon determination of the maximal amount of antigen which will completely precipitate all antibody as evidenced by absence or minimal traces of antigen and/or antibody in the supernatant. Each serum to be tested was diluted and added to ascending increments of antigen. After appropriate periods of incubation the centrifuged supernatants were tested for excess antigen or antibody with known quantities of SSS and of antibody solution. The fastest reacting mixture was always noted when the reagents were sufficiently concentrated to give visible precipitates within a few hours of mixing. The antibody content was then calculated and compared with estimates obtained by other methods.

The Martin serum dilution method (17) was also applied to all samples of serum used in the rat protection tests. The serum was diluted serially with saline and equal volumes of each serum dilution were mixed with equal volumes of progressively decreasing amounts of antigen. A critical series of selected supernatants were tested for excess antigen and antibody as in the Culbertson test. An approximate zone of equivalence was determined and the appropriate antibody content

calculated for comparison with the Culbertson procedure. Since each serum was quantitated by the 2 techniques it was possible, by a process of dilution, to adjust a serum relatively high in antibody content to one low in antibody content in terms of antibody units per ml.

Technique of Protection Tests. The passive rat protection test described by Foshay et al. (11) was employed. The challenge dose was prepared from the most recent, continuously passaged strain SCHU. The growth from the second serial 16-hour cultures on glucose cystine blood agar was finely emulsified in saline solution and the density was established at 40.0 M.A. (microamperes) in a photometer. From this suspension a 10^{-2} dilution was made in saline. This challenge dose was prepared immediately before rats were to be challenged. The uniform challenge dose per animal was 1.0 ml of the M.A. 40×10^{-2} suspension, administered subcutaneously. This carried a minimum of 25 million viable cells, an average of 25,000 LD₅₀ rat doses. The infecting strain was recovered by heart blood culture from one animal of each group and was used for preparing the challenge dose of the next group.

White rats, weighing 85 to 120 grams, were used as the test animals and death was used as the recorded end-point. Groups of 15 to 20 rats were inoculated with 2.0 ml of the selected serum by intraperitoneal inoculation, followed at once by the 1.0 ml subcutaneous challenge dose. Deaths were

recorded daily for 21 days before release. Owing to individual variability, even among rats of the stated narrow weight range, most antiserum groups were accompanied by a normal serum group as control for the challenge dose used on that day.

Following this procedure a comparison was sought between the protective antibody content of variously paired serums. Examples of such pairs included: (a) serum stored in bulk at 5 C vs serum freshly frozen immediately after removal from the clot, (b) serum from a goat inoculated with a virulent strain vs serum from a goat inoculated with an avirulent strain, and (c) various pairs of serums in which the one of higher antibody content was diluted so that it was equivalent in antibody units per ml to the one of lower antibody content.

In the body of the thesis the following symbols are used to designate the nature of the serum samples: G, goat; H, horse; R, rabbit; L, made with living cells; K, made with killed cells; A, avirulent strain; V, virulent strain; then the abbreviation of the strain used, e.g. SCHU, CHUR, etc., finally -43, -50, for the year in which the antiserum was made, and A for the first bleeding, B for the second bleeding, etc. Thus GLV_{Schu}-43-A means a goat antiserum prepared with living cells of the virulent strain, SCHU, in 1943, and obtained from the first bleeding.

EXPERIMENTAL RESULTS

A. Precipitin Studies on Tularensis Systems

1. Preliminary Orientation: Serum GLV_{Schu-43} with PG Polysaccharides. Goat serum, GLV_{Schu-43}, was adopted as the provisional standard antiserum for the initial determinations and titrations. Numerous studies on the effect of varied serum dilutions and antigen increments were made with this serum for selection of the most applicable procedure. The first analyses were made with antigens in serial 2-fold dilutions added to a constant volume of various serum dilutions. Thereafter, to obtain a more coherent curve, the antigen was added to constant antibody in small ascending increments, usually in steps of 10 to 20 µg, in order to approach the equivalence zone. Examples of orientating studies with the standard goat serum demonstrating certain quantitative antigen:antibody relations for 3 different dilutions of antibody are shown in table 5.

The table gives values for antibody nitrogen precipitated from 1.0 ml of the 3 dilutions of serum by addition of increasing increments of PG SSS-38 antigen. Inspection of the detection tests on supernatants of all reacting mixtures reveals the existence of 3 zones, i.e., a region of antibody excess, an equivalence zone where minimal traces of antigen and antibody were found in the supernatant, and finally a region of antigen excess. For each serum dilution used the amount of precipitable

Table 5

Effect of Adding Increasing Increments of PG SSS-38 to 1.0 ml of
Varied Serum Dilutions

GLV Schu-43 (Final Volume = 2.0 ml)

SSS-38 Added µg/ml	Ab N ₂ Pptd. µg	Ratio Ab N ₂ :SSS	Ab Protein Pptd. µg	Ratio Ab Protein:SSS	Calculated Ab N ₂ in Undil. Serum mg/ml	Supernatant Shows Excess:
A. Serum Dilution 1:25						
1/2	5.25	10.49	32.78	65.57	0.1311	Antibody
1	6.05	6.05	37.81	37.81	0.1513	"
2	8.34	4.17	52.12	26.06	0.2085	"
4	15.47	3.87	106.67	26.50	0.3867	"
8	23.13	2.89	144.59	18.07	0.5784	"
16	36.58	2.16	228.65	14.29	0.9146	"
32	45.99	1.44	287.49	8.98	1.1500	"
64	48.42	0.76	302.63	4.72	1.2105	"
100	48.01	0.48	299.06	2.90	1.2003	Ab + An
B. Serum Dilution 1:50						
1/2	2.55	5.10	15.94	31.87	0.1275	Antibody
1	3.77	3.76	29.56	23.56	0.1885	"
2	6.33	3.16	39.53	19.77	0.3163	"
4	7.53	1.88	47.06	11.77	0.3766	"
8	16.41	2.05	102.56	12.82	0.8205	"
16	21.65	1.35	135.24	8.45	1.0827	"
32	25.54	0.80	159.63	4.98	1.2770	Neither
64	27.57	0.43	172.53	2.69	1.3786	Antigen
100	25.69	0.26	160.56	1.61	1.2845	"
C. Serum Dilution 1:100						
1/2	1.43	2.85	8.91	17.80	0.1425	Antibody
1	1.48	1.48	9.24	9.24	0.1480	"
2	4.44	2.22	27.74	13.87	0.4439	"
4	5.38	1.35	33.63	8.41	0.5380	"
8	9.95	1.24	62.21	7.78	0.9953	"
16	13.05	0.82	81.56	5.09	1.3050	Neither
32	14.26	0.45	89.11	2.78	1.4257	Antigen
64	13.05	0.20	81.56	1.27	1.3050	"
100	12.64	0.13	79.02	0.79	1.2643	"

antibody nitrogen progressively increased throughout the zone of antibody excess as the antigen concentration was increased. The nitrogen rose to a maximum in the first tube in which a definite trace of antigen was first detected in the supernatant. Antibody could not be demonstrated at this point. The combining ratios of antibody nitrogen:SSS showed a progressive decrease as the antigen concentration was increased throughout the entire precipitin curve.

Direct comparison of the combining ratios of antibody nitrogen:SSS with detection tests made on the supernatants revealed a constant ratio near the point of neutrality in each of the 3 serum dilutions. The ratio was between 0.76 and 0.48 near the equivalence zone in the serum diluted 1:25. The antigen concentration ranged from 64 to 100 μ g. In the 1:50 serum dilution the optimal combining ratio was between 0.80 and 0.43 at an antigen concentration ranging from 32 to 64 μ g. Only minimal traces of antigen were detectable in the supernatant in this range. With the 1:100 serum dilution the ratio varied from 0.82 to 0.45 at a concentration of 16 to 32 μ g antigen and the equivalence zone was within this range. By increasing the antigen in smaller increments a more optimal combining ratio may be determined. Data for other relationships calculated from the antibody nitrogen values are also illustrated.

(a) Comparison of Duplicate Quantitative Titrations.

The same serum was diluted 1:15 with saline and a constant

volume of this dilution, 0.5 ml, was mixed with the SSS-38 polysaccharide in ascending 20 μ g increments. This titration was made using smaller antigen units to achieve a more quantitative measurement of antibody. This determination, like all subsequent analyses, was made in duplicate, and the final figures represent averages of the duplicate determinations. The results are tabulated in table 6 to show the applicability of the method and degree of agreement found in the nitrogen analyses and in the detection series tests on the SSS-supernatants. The table shows a comparison of values for antibody nitrogen obtained with each antigen increment and demonstrates excellent correlation between corresponding pairs of analyses, the greatest deviation being only 2.16 μ g nitrogen. The average deviation for the 6 duplicate samples was 0.81 μ g which is within the limits of other sources of error.

As a further study for correlation of duplicate titrations, the same tests were repeated using PG SSS-SCHU as the precipitating antigen instead of the SSS-38. The findings are shown in table 7 which again demonstrates the excellent agreement in these nitrogen determinations and the ability of the method to reproduce results. In this titration the average deviation for the series of 6 pairs of nitrogen determinations was 1.01 μ g.

Even though it was easy to duplicate separate nitrogen determinations all subsequent titrations and nitrogen analyses

Table 6

Addition of Increasing Increments of SSS-38 to 0.5 ml Serum

GLV_SSchu-43 (Diluted 1:15)

Final Volume = 2.0 ml

A.

<u>Comparison of Duplicates for Antibody Nitrogen</u>					
<u>SSS-38 Added</u>	<u>Antibody N Precipitated</u>		<u>Difference in Duplicate Series</u>	<u>Antibody N Precipitated (average)</u>	<u>Antibody N in Undil. Serum</u>
	<u>Series 1</u>	<u>Series 2</u>			
<u>µg</u>	<u>µg</u>	<u>µg</u>	<u>µg</u>	<u>µg</u>	<u>mg/ml</u>
10	15.86	16.29	0.43	16.08	0.4824
20	25.96	27.57	1.61	26.76	0.8028
40	34.97	34.97	0.00	34.97	1.0491
60	38.74	39.44	0.70	39.03	1.1709
80	41.70	41.70	0.00	41.70	1.2510
100	39.54	41.70	2.16	40.62	1.2186

B.

Detection Series on SSS-Supernatants

<u>Series 1</u>			<u>Series 2</u>		
<u>+←PG SSS→+</u>			<u>+←PG SSS→+</u>		
<u>A</u>	<u>µg</u>	<u>Ab</u>	<u>A</u>	<u>µg</u>	<u>Ab</u>
++	10	0	++	10	0
+	20	0	+	20	0
+	40	<u>±</u>	+	40	<u>±</u>
<u>±</u>	60	+	<u>±</u>	60	<u>±</u>
0	80	++	0	80	+
0	100	++	0	100	++

Table 7

Addition of Increasing Increments of SSS-SCHU to 0.5 ml Serum

GLV_{Schu}-43 (Diluted 1:15)

Final Volume = 2.0 ml

A.

Comparison of Duplicates for Antibody Nitrogen

SSS-SCHU Added	Antibody N Precipitated		Difference in Duplicate Series	Antibody N Precipitated (average)	Antibody N in Undil. Serum
	Series 1	Series 2			
µg	µg	µg	µg	µg	mg/ml
10	15.47	14.26	1.21	14.87	0.4459
20	25.02	23.27	1.75	24.14	0.7243
40	32.15	32.28	0.13	32.21	0.9664
60	36.05	38.83	2.78	37.49	1.1232
80	41.02	41.02	0.00	41.02	1.2307
100	42.37	42.64	0.27	42.50	1.2751

B.

Detection Series on SSS-Supernatants

<u>Series 1</u>			<u>Series 2</u>		
+ ← PG SSS → +			+ ← PG SSS → +		
<u>A</u>	<u>µg</u>	<u>Ab</u>	<u>A</u>	<u>µg</u>	<u>Ab</u>
++	10	0	++	10	0
++	20	0	++	20	0
+	40	<u>+</u>	+	40	0
+	60	<u>+</u>	+	60	+
0	80	++	0	80	++
0	100	++	0	100	++

were made in paired series, and the figures given represent the averages. This micro method as described may be used for as little as 0.5 μ g of specific precipitate nitrogen with a fair degree of accuracy. The error in repeated determinations is about ± 2 μ g. Any pair of values which differed by more than 2.0 μ g was discarded and the analysis repeated.

2. Comparative Binding Properties of PG Antigens for Anti-SCHU Goat Serums. The quantitative precipitin reaction curves of the virulent strain antiserums with the 2 tularemic PG antigens were the first to be investigated. The work included a study of the quantitative relationships existing between the 2 polysaccharide antigens and hyperimmune anti-SCHU serums. The course of the reaction curve with each antigen was compared with theoretical curves derived by calculated values from the Heidelberger equation (29).

(a) Serum GLV_{Schu}-43. The course of the precipitin reaction using 0.5 ml of a 1:15 dilution of serum with the SSS-SCHU and SSS-38 polysaccharides is given in paired tables 8 and 9, respectively, and is represented graphically in figure 1. Each antigen was added in ascending 20 μ g increments, and the values for precipitable antibody nitrogen are shown. A comparison of experimental values with calculated values for antibody nitrogen are included for the antibody excess zone of the reaction curve. The calculated combining ratios of antibody nitrogen:SSS are indicated for each antigen

Table 8

Addition of Increasing Amounts of PG SSS-SCHU to 0.5 ml Serum

GLV Schu-43 (Diluted 1:15)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	14.86	13.24	1.49	0.4458	++	0
20	24.14	23.52	1.21	0.7242	++	0
40	32.21	35.28	0.81	0.9663	+	±
60	37.49	23.52	0.62	1.1232	+	+
80	41.02		0.51	1.2306	0	++
100	42.50		0.43	1.2750	0	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.470 S - 0.0147 S^2$$

$$S \text{ max.} = 50 \mu\text{g} \quad \text{Ab max.} = 36.75 \mu\text{g}$$

$$R = 0.735$$

$$\text{Antibody Content} = 1.1025 \text{ mg Ab N/ml}$$

Table 9

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

GLV Schur-43 (Diluted 1:15)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess;	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	16.02	14.75	1.61	0.4810	++	0
20	26.76	26.04	1.34	0.8030	+	0
40	34.97	38.28	0.88	1.0490	+	+
60	39.03	36.72	0.65	1.1700	+	+
80	41.70		0.52	1.2510	0	+
100	40.62		0.41	1.2190	0	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.647 S - 0.0173 S^2$$

$$S \text{ max.} = 47.74 \mu\text{g} \quad \text{Ab Max.} = 39.31 \mu\text{g}$$

$$R = 0.824$$

$$\text{Antibody Content} = 1.1793 \text{ mg Ab N/ml}$$

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Goat Serum GLV-43 and Polysaccharide Antigens

Serum Diluted 1:15; Volume 0.5 ml

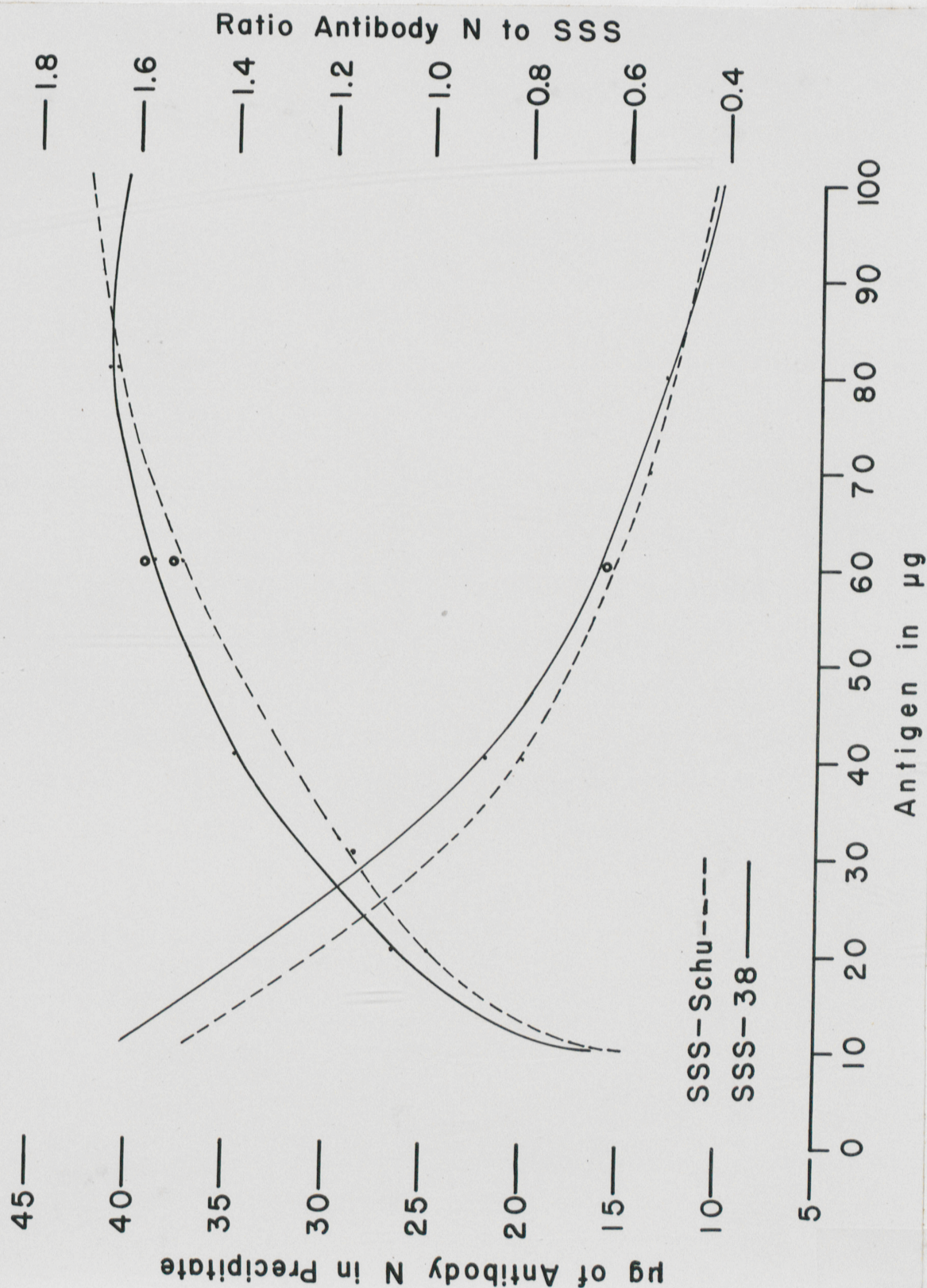


Fig. 1

for SSS-Schu and SSS-38 respectively. These values are in

increment. The plotted values in figure 1 show the comparative courses of the respective precipitin curves with each SSS and the effect of addition of each antigen increment to the constant antiserum. Near the equivalence zone of 50 μ g SSS-SCHU in table 8, as noted by tests for antigen and antibody in the supernatants, the serum contained 1.10 mg Ab N/ml of undiluted serum. At this antigen concentration all the antibody in 0.5 ml of a 1:15 serum dilution was completely precipitated as evidenced by the detection tests on the supernatants. In table 9 the calculated value for antibody nitrogen in undiluted serum was 1.18 mg Ab N/ml near the equivalence zone of 48 μ g SSS-38 where all antibody was also precipitated.

In order to obtain more analytical points on the curve for comparison with theoretical values, the serum was diluted further, 1:20 instead of 1:15, and the antigen was added to constant amounts of antibody in smaller ascending increments. Both SSS-SCHU and SSS-38 were tested in like manner so that it was possible to compare the relative binding properties of each antigen for the same unit of antibody. Each polysaccharide series was run in duplicate and results of averages are tabulated in tables 10 and 11. The corresponding precipitin curves are compared graphically in figure 2. The antibody nitrogen determinations were calculated to the basis of undiluted serum giving values of 1.26 and 1.21 mg/ml for SSS-SCHU and SSS-38 respectively. These values are in

Table 10

Addition of Increasing Amounts of PG SSS-SCHU to 0.5 ml Serum

GLV_Schur-43 (Diluted 1:20)

SSS*SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg		mg/ml		
10	14.66	9.40	1.47	0.5864	++	0
20	17.08	17.14	0.85	0.6832	++	0
30	23.81	23.22	0.79	0.9524	++	0
40	27.03	27.64	0.68	1.0182	±	0
50	30.40	31.40	0.61	1.2160	±	±
60	32.55	31.50	0.54	1.3020	±	+
70	31.07	30.94	0.44	1.2428	±	+
80	32.15		0.40	1.2860	±	++
90	32.01		0.36	1.2804	±	++
100	34.16		0.34	1.3664	±	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.023 S - 0.0083 S^2$$

$$S \text{ max.} = 61.6 \mu\text{g} \quad \text{Ab max.} = 31.52 \mu\text{g}$$

$$R = 0.512$$

$$\text{Antibody Content} = 1.2608 \text{ mg Ab N/ml}$$

Table 11

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

GLV_{Schu}-43 (Diluted 1:20)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	12.78	9.16	1.28	0.5112	++	0
20	16.95	16.58	0.85	0.6780	++	0
30	21.12	22.56	0.71	0.8448	+	0
40	27.98	26.80	0.70	1.1192	±	0
50	29.52	29.40	0.59	1.1808	±	0
60	29.72	30.36	0.50	1.1888	±	0
70	31.87	29.68	0.46	1.2748	±	±
80	30.13		0.38	1.2052	±	++
90	32.28		0.36	1.2912	±	++
100	31.13		0.31	1.2452	±	++

* Values calculated according to equation:
 $\mu\text{g Antibody N Pptd.} = 0.998 S - 0.0082 S^2$
 $S \text{ max.} = 60.85 \mu\text{g} \quad \text{Ab max.} = 30.37 \mu\text{g}$
 $R = 0.499$
 $\text{Antibody Content} = 1.2148 \text{ mg Ab N/ml}$

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Goat Serum GLV-43 and Polysaccharide Antigens

Serum Diluted 1:20; Volume 0.5 ml

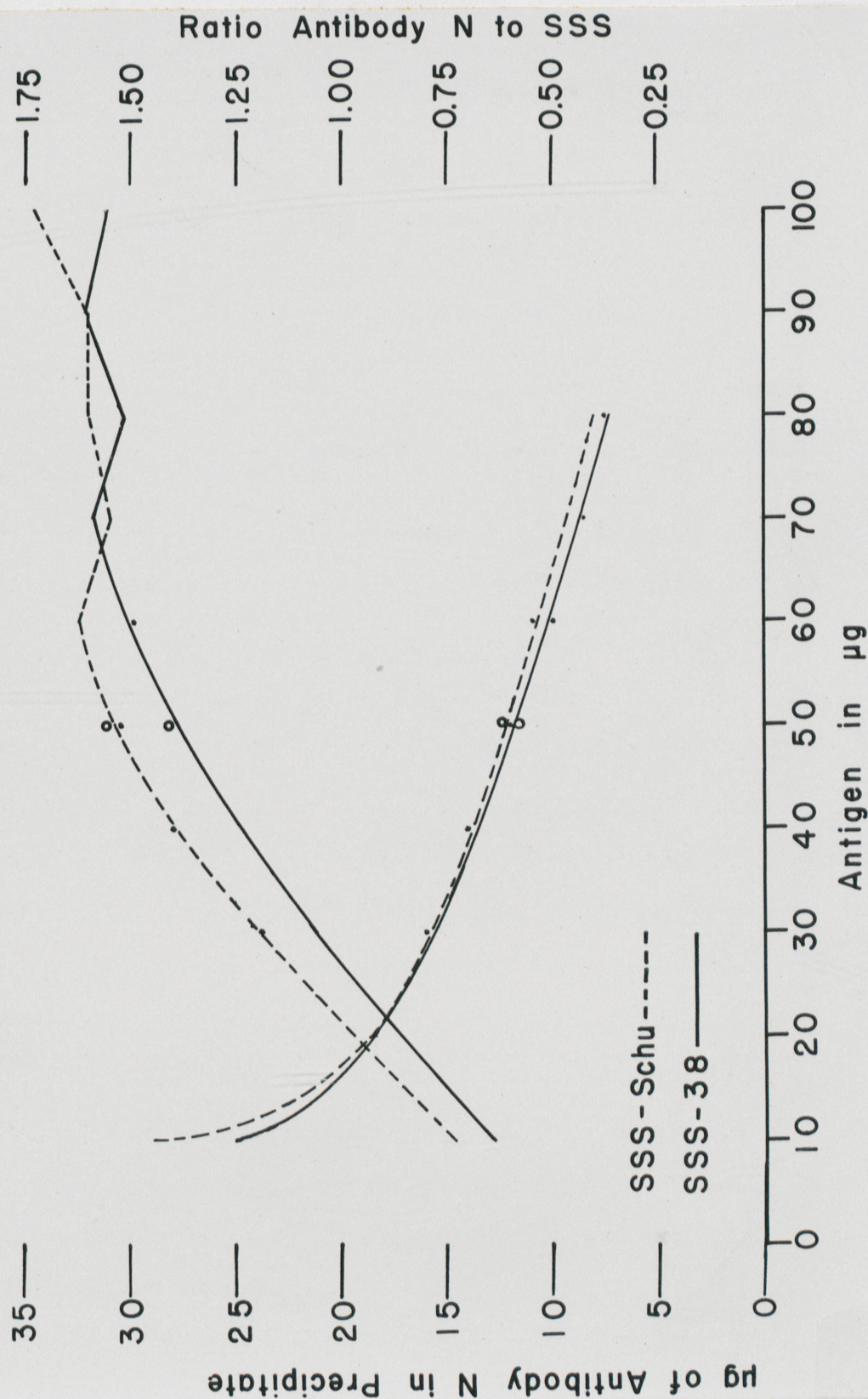


Fig. 2

excellent agreement with observed results for the titration with a 1:15 dilution of the corresponding serum.

(b) Serum GLV_{Schu}-49-A. The quantitative courses of the precipitin reactions between the 1949 anti-SCHU goat serum obtained from the first bleeding and the homologous and heterologous polysaccharides (SSS-SCHU and SSS-38) are set forth in comparative detail in paired tables 12 and 13 for a 1:10 dilution of serum, and in tables 14 and 15 for a 1:15 dilution of serum. These permit further comparative study of the effect of serum dilutions on the course of the precipitin curve as well as analysis of the relative combining powers of the 2 polysaccharide antigens for the same antibody. These titrations have permitted a study of the precipitates over a range of antibody nitrogen to SSS ratios of 1.8 to 0.3 or, from the region of considerable antibody excess to moderately great antigen excess. It is apparent by comparative inspection of the first 2 columns of each pair of tables that the amount of antibody nitrogen precipitated per unit of antigen is approximately the same for each SSS in each of the 2 serum dilutions. Therefore it appears that each SSS, that derived from the virulent as well as that from the avirulent strain, had the same combining power for the anti-SCHU serum.

The courses of these precipitin curves are shown graphically in figures 3 and 4, respectively, for the 1:10 and 1:15 serum dilutions. It is evident that the courses of

Table 12

Addition of Increasing Amounts of PG SSS-SCHU to 0.5 ml Serum

GLV_{Schu}-49-A (Diluted 1:10)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
μg	μg	μg		mg/ml		
10	15.47	11.18	1.55	0.3094	+	0
20	22.59	20.32	1.13	0.4518	+	0
30	25.69	27.41	0.86	0.5138	+	0
40	30.73	32.44	0.77	0.6146	+	±
50	32.21	35.42	0.64	0.6442	+	+
60	34.90	36.36	0.58	0.6980	+	++
70	35.64	34.24	0.51	0.7128	±	++
80	37.32	32.08	0.47	0.7464	±	++

* Values calculated according to equation:

$$\begin{aligned} \mu\text{g Antibody N Pptd.} &= 1.221 S - 0.0103 S^2 \\ S \text{ max.} &= 59.56 \mu\text{g} \quad \text{Ab max.} = 36.36 \mu\text{g} \\ R &= 0.611 \end{aligned}$$

$$\text{Antibody Content} = 0.7272 \text{ mg Ab N/ml}$$

Table 13

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

GLV_{Schu}-49-A (Diluted 1:10)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess;	
	Found	Calc.*			Ab	An
μg	μg	μg				
10	17.89	13.18	1.79	0.3578	++	0
20	24.88	23.24	1.24	0.4976	++	0
30	30.46	30.18	1.02	0.6092	++	0
40	32.95	34.00	0.82	0.6590	++	0
50	33.76	34.00	0.68	0.6752	+	$\pm$
60	35.51	32.28	0.59	0.7102	$\pm$	+
70	34.89		0.50	0.6978	$\pm$	+
80	36.99		0.46	0.7398	$\pm$	+
90	40.75		0.45	0.8150	$\pm$	++
100	42.42		0.42	0.8484	$\pm$	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.474 S - 0.0156 S^2$$

$$S \text{ max.} = 47.2 \mu\text{g} \quad \text{Ab max.} = 34.82 \mu\text{g}$$

$$R = 0.737$$

$$\text{Antibody Content} = 0.6964 \text{ mg Ab N/ml}$$

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Goat Serum GLV-49-A and Polysaccharide Antigens

Serum Diluted 1:10; Volume 0.5 ml

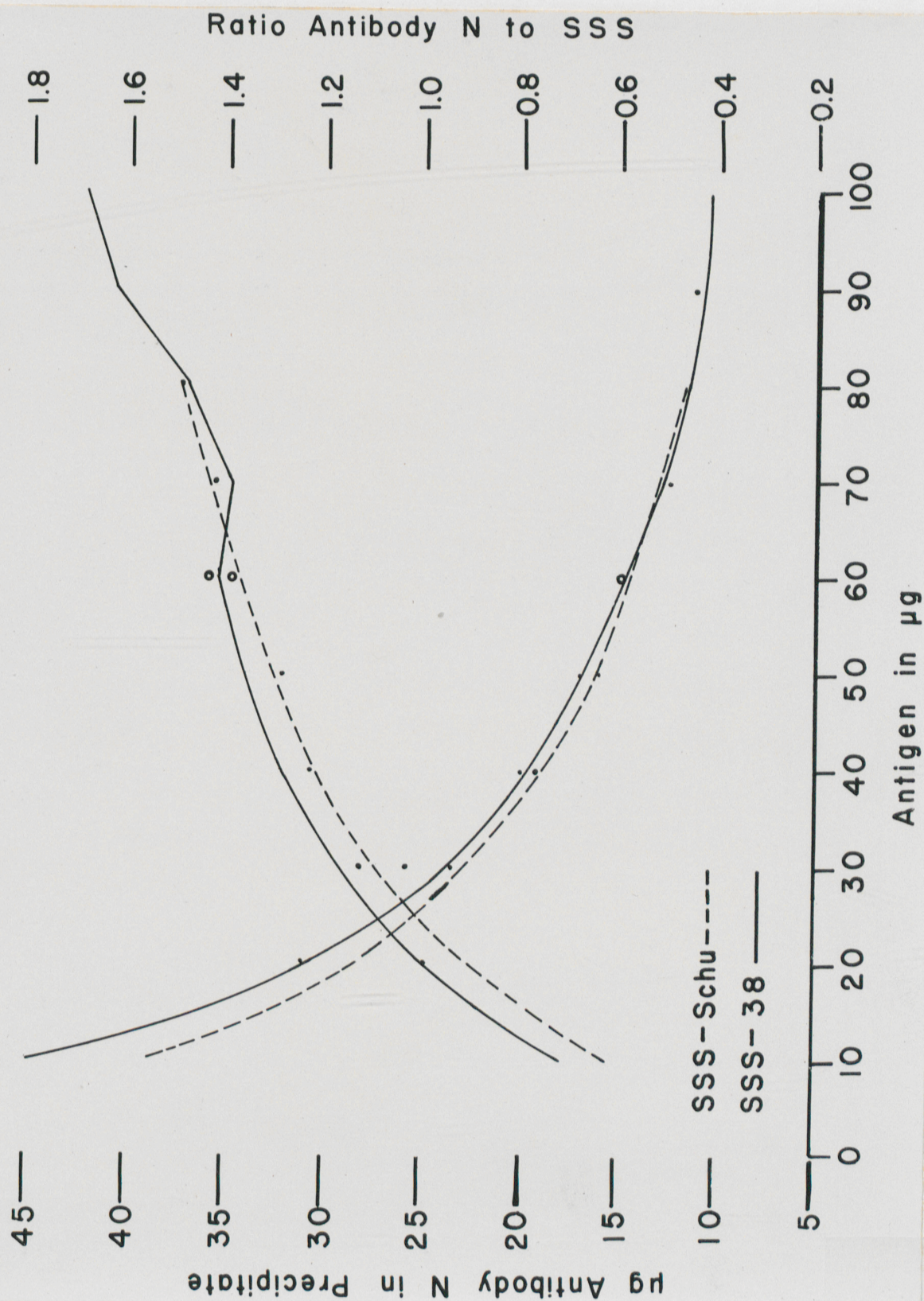


Fig. 2

Table 14

Addition of Increasing Amounts of PG SSS-SCHU to 0.5 ml Serum

GLV_{Schu}-49-A (Diluted 1:15)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	16.27	10.83	1.63	0.4881	++	0
20	20.64	18.06	1.03	0.6192	++	±
30	23.80	23.18	0.79	0.7140	+	±
40	25.55	24.72	0.64	0.7665	+	+
50	27.79	23.15	0.56	0.8337	±	+
60	28.18		0.47	0.8454	±	++
70	27.91		0.40	0.8373	±	++
80	29.59		0.37	0.8877	±	++
90	28.91		0.32	0.8673	±	++
100	29.72		0.30	0.8916	±	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.238 S - 0.0155 S^2$$

$$S \text{ max.} = 39.92 \mu\text{g} \quad \text{Ab max.} = 24.72 \mu\text{g}$$

$$R = 0.619$$

$$\text{Antibody Content} = 0.7416 \text{ mg Ab N/ml}$$

Table 15

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

GLV_{Schu}-49-A (Diluted 1:15)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows	
	Found	Calc.*			Excess:	
μg	μg	μg		mg/ml	Ab	An
10	11.71	9.07	1.17	0.3513	++	<u>+</u>
20	17.48	16.20	0.87	0.5244	++	<u>+</u>
30	20.24	21.39	0.67	0.6082	++	<u>+</u>
40	22.66	24.64	0.57	0.6798	++	<u>+</u>
50	25.09	25.95	0.50	0.7527	+	<u>+</u>
60	28.38	25.32	0.47	0.8514	<u>+</u>	+
70	29.73		0.42	0.8919	<u>+</u>	+
80	29.39		0.37	0.8817	0	++
90	28.92		0.32	0.8676	0	++
100	29.45		0.29	0.8835	0	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.004 S - 0.0097 S^2$$

$$S \text{ max.} = 51.75 \mu\text{g} \quad \text{Ab max.} = 25.98 \mu\text{g}$$

$$R = 0.502$$

$$\text{Antibody Content} = 0.7794 \text{ mg Ab N/ml}$$

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Goat Serum GLV-49-A and Polysaccharide Antigens

Serum Diluted 1:15; Volume 0.5 ml

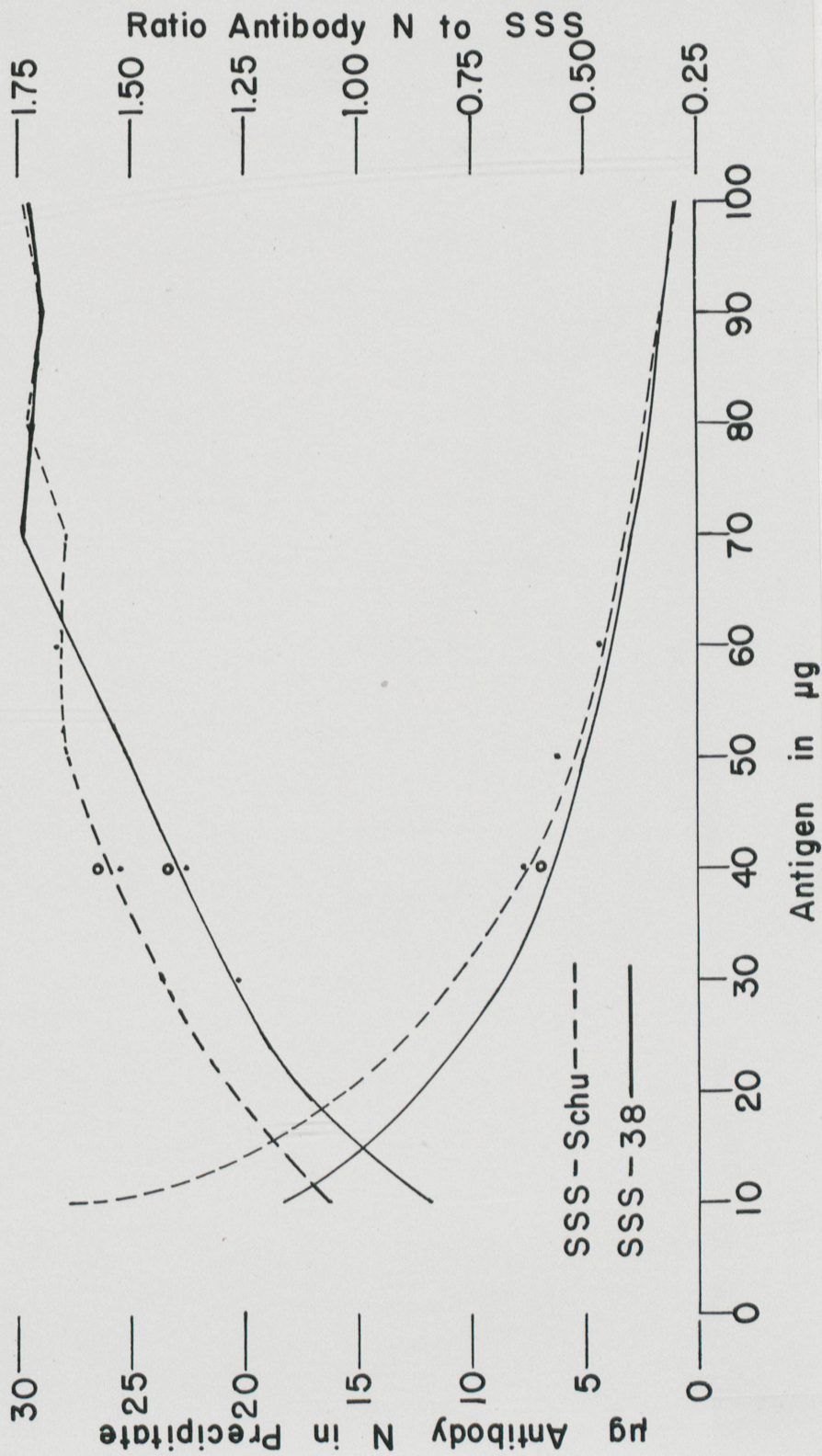


Fig. 4

these precipitin curves, and the courses of the antibody nitrogen to SSS ratios are similar to those obtained by Heidelberg et al. for pneumococcus (21), crystalline egg albumin (16), and other antigen-antibody systems. In one pair of tests the larger amount of antibody nitrogen was brought down by SSS-SCHU; in the other pair the situation was reversed, and all differences appear to be within the limits of error of the method used.

(c) Serum GLV_{Schu}-49-B. The quantitative courses of the precipitin reaction between the 1949 anti-SCHU goat serum and homologous and heterologous polysaccharide antigens for a 1:10 dilution are illustrated in the paired tables 16 and 17. This serum was obtained from a second bleeding of the goats and made about 30 days after the corresponding "A" bleedings. The precipitin curves covered a range of Ab N:SSS ratios from 1.64 to 0.29. The first 2 columns of each table again show that the amount of antibody nitrogen precipitated per unit of antigen was almost identical for each SSS. Likewise each SSS had almost identical combining power for the antibody, a feature previously recognized with the other anti-SCHU serums. A comparison of observed and calculated values for antibody nitrogen reveals good agreement throughout the antibody excess zone except at 10 μ g antigen where the smallest unit was added. The calculated optimal combining ratio, R, at the equivalence zone is practically identical for each SSS. The optimal ratio

Table 16

Addition of Increasing Amounts of PG SSS-SCHU to 0.5 ml Serum

GLV Schu-49-B (Diluted 1:10)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess;	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	16.41	11.32	1.64	0.3282	++	0
20	20.78	19.94	1.04	0.4156	++	0
30	21.65	25.86	0.72	0.4330	+	0
40	25.69	29.08	0.64	0.5138	+	+
50	27.57	29.60	0.55	0.5514	<u>+</u>	+
60	29.72	27.42	0.50	0.5944	<u>+</u>	++
70	28.51		0.41	0.5702	<u>+</u>	++
80	29.59		0.37	0.5918	0	++
90	28.38		0.32	0.5676	0	++
100	30.73		0.31	0.6146	0	++

* Values calculated according to equation:

$$\begin{aligned} \mu\text{g Antibody N Pptd.} &= 1.267 S - 0.0135 S^2 \\ S \text{ max.} &= 46.93 \mu\text{g} \quad \text{Ab max.} = 29.82 \mu\text{g} \\ R &= 0.633 \end{aligned}$$

Antibody Content = 0.5964 mg Ab N/ml

Table 17

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

GLV_{Schu}-49-B (Diluted 1:10)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
μg	μg	μg				
10	15.87	10.88	1.59	0.3174	+	0
20	19.77	19.10	0.99	0.3954	+	0
30	23.33	24.67	0.78	0.4666	+	0
40	24.88	27.60	0.62	0.4976	+	0
50	25.89	27.87	0.52	0.5178	+	+
60	27.37	25.50	0.46	0.5474	$\pm$	+
70	27.37		0.39	0.5474	$\pm$	+
80	28.65		0.36	0.5730	$\pm$	+
90	28.71		0.32	0.5742	$\pm$	+
100	29.25		0.29	0.5850	$\pm$	+

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.220 S - 0.0133 S^2$$

$$S \text{ max.} = 46.04 \mu\text{g} \quad \text{Ab max.} = 28.20 \mu\text{g}$$

$$R = 0.610$$

$$\text{Antibody Content} = 0.5638 \text{ mg Ab N/ml}$$

for SSS-SCHU in table 16 was 0.633, while the corresponding ratio for SSS-38 given in table 17 was 0.610. The difference between these ratios is not sufficiently large to be significant. The antibody content of the serum when titrated with the SSS-SCHU antigen was 0.5964 mg Ab N/ml compared with 0.5638 mg Ab N/ml with the SSS-38 antigen. Again this difference, representing calculations based upon a dilution factor of 20 in each titration, is of no significance. The maximal amount of antigen required to precipitate all the antibody at the equivalence zone was calculated to be 46.93 μ g SSS-SCHU and 46.04 μ g SSS-38. These are identical amounts for each polysaccharide preparation, and the calculated maximal amount of antigen occurred in the middle of the equivalence zone as demonstrated by tests on the supernatants. The range of the equivalence zone in each titration is almost identical and the calculated optimal ratio occurred within this zone.

3. Comparative Binding Properties of PG Antigens for Anti-38 Goat Serums. The quantitative precipitin reaction curves of the avirulent (38 strain) antiserums with the 2 tularemic PG antigens were next investigated. The study consisted of an analysis of quantitative relationships between the 2 polysaccharide antigens and hyperimmune anti-38 serums. The course of these reaction curves was compared with theoretical curves derived from the Heidelberger equation (29). Calculations of combining ratios at the theoretical equivalence zones

are included for comparison with those obtained with the anti-SCHU serums.

(a) Serum G₁₃LA₃₈-49-A. The quantitative courses of the precipitin reactions between the 1949 anti-38 goat serum (first bleeding, goat No. 13, living, avirulent antigen) and the homologous and heterologous polysaccharides are illustrated in paired tables 18 and 19 for 0.5 ml of a 1:5 serum dilution. Examination of these titrations shows that the quantities of antibody nitrogen precipitated by SSS-38 and SSS-SCHU, respectively, were almost the same. It is also apparent that the combining power of each polysaccharide for the 38 strain antibody was identical, just as previously shown for the SCHU strain antibody. However calculation of the optimal combining ratio, R, for the anti-38 serum gave a much larger value than that observed with the anti-SCHU serum. The reaction with the homologous polysaccharide in table 18 gave a combining ratio of 2.120 at the beginning of the equivalence zone and the heterologous reaction with SSS-SCHU in table 19 gave a ratio of 2.125. These ratios are about 3.5 times greater than the corresponding ratios for the SCHU antibody. Therefore both polysaccharides must have identical combining powers for either antibody but the 38 strain antibody has a much higher combining ratio per unit of antigen than the SCHU strain antibody. These titrations showed a serum relatively low in antibody nitrogen. The antibody content determined with the SSS-38 antigen was

Table 18

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

G₁₃LA₃₈-49-A (Diluted 1:5)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	29.79	29.80	2.98	0.2979	+	+
20	34.23	34.20	1.71	0.3423	0	+
30	34.91	13.20	1.16	0.3491	0	+
40	34.36		0.86	0.3436	0	+
50	34.63		0.69	0.3463	0	++
60	34.57		0.58	0.3457	0	++
70	34.98		0.50	0.3498	0	++
80	34.70		0.43	0.3470	0	++
90	34.56		0.38	0.3456	0	++
100	35.51		0.36	0.3551	0	++

* Values calculated according to equation:
 $\mu\text{g Antibody N Pptd.} = 4.250 S - 0.1275 S^2$
 $S \text{ max.} = 16.73 \mu\text{g}$ $Ab \text{ max.} = 35.55 \mu\text{g}$
 $R = 2.120$
Antibody Content = 0.3555 mg Ab N/ml

Table 19

Addition of Increasing Amounts of PG SSS-SCHU to 0.5 ml Serum

G₁₃LA₃₈-49-A (Diluted 1:5)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	29.32	28.30	2.93	0.2932	+	0
20	32.28	28.20	1.61	0.3228	+	±
30	34.38		1.15	0.3438	±	±
40	34.49		0.86	0.3449	±	+
50	34.36		0.69	0.3436	0	+
60	34.16		0.57	0.3416	0	+
70	34.76		0.50	0.3476	0	++
80	34.90		0.44	0.3490	0	++
90	35.51		0.39	0.3551	0	++
100	34.23		0.34	0.3423	0	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 4.250 S - 0.1420 S^2$$

$$S \text{ max.} = 14.95 \mu\text{g} \quad \text{Ab max.} = 31.80 \mu\text{g}$$

$$R = 2.125$$

$$\text{Antibody Content} = 0.3180 \text{ mg Ab N/ml}$$

0.3555 mg Ab N/ml of serum while SSS-SCHU gave a value of 0.3180 mg Ab N/ml undiluted serum. This difference is considered to be of no significance since each estimate was calculated from a dilution factor of 10. Both titrations revealed the existence of a broad zone of antigen excess in which an insoluble, stable complex was formed with either antigen. In this realm of the precipitin curve the maximal amount of antibody nitrogen is precipitated and it is not increased even after addition of more antigen. A very narrow antibody excess zone was observed in both titrations.

Reaction curves for this serum with SSS-38 and SSS-SCHU antigens, respectively, are further characterized by the data in paired tables 20 and 21 for 1.0 ml of a 1:3 serum dilution. The precipitating properties were studied over a range of antibody nitrogen to SSS ratio of 5.34 to 0.99, or from the region of great antibody excess to the beginning of a broad equivalence zone. Calculations of appropriate combining ratios gave a value of 2.084 with the SSS-38 antigen which would occur after addition of 56.30 μ g antigen and a value of 2.506 with the SSS-SCHU antigen. These ratios are in good agreement with those observed for the titration on 0.5 ml of the 1:5 serum dilution, in tables 18 and 19. This offered further evidence for the greater combining property of the anti-38 serum for both polysaccharides. Calculations of antibody nitrogen revealed a value of 0.3516 mg Ab N/ml of serum using the SSS-38

Table 20

Addition of Increasing Amounts of PG SSS-38 to 1.0 ml Serum

G₁₃LA₃₈-49-A (Diluted 1:3)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	53.75	37.96	5.34	0.1613	++	0
20	78.43	68.52	3.92	0.2353	++	0
30	92.80	91.68	3.09	0.2784	++	0
40	96.19	107.44	2.40	0.2886	++	0
50	98.78	115.80	1.98	0.2963	+	±
60	100.23	116.76	1.67	0.3007	+	±
70	103.45	110.32	1.48	0.3104	+	±
80	105.23		1.32	0.3157	+	±
90	106.04		1.18	0.3181	+	±
100	102.49		1.02	0.3075	+	±

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 4.166 S - 0.0370 S^2$$

$$S \text{ max.} = 56.30 \mu\text{g} \quad \text{Ab max.} = 117.21 \mu\text{g}$$

$$R = 2.084$$

$$\text{Antibody Content} = 0.3516 \text{ mg Ab N/ml}$$

Table 21

Addition of Increasing Amounts of PG SSS-SCHU to 1.0 ml Serum

G₁₃LA₃₈-49-A (Diluted 1:3)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	51.65	43.85	5.17	0.1550	++	0
20	78.33	75.16	3.92	0.2350	++	0
30	88.23	93.93	2.94	0.2647	++	0
40	96.08	100.16	2.40	0.2882	++	0
50	100.49	93.85	2.01	0.3015	+	+
60	99.31		1.66	0.2979	+	+
70	102.44		1.46	0.3073	+	+
80	100.11		1.25	0.3003	+	+
90	113.95		1.27	0.3419	+	+
100	98.98		0.99	0.2669	+	+

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 5.012 S - 0.0627 S^2$$

$$S \text{ max.} = 39.97 \mu\text{g} \quad \text{Ab max.} = 100.16 \mu\text{g}$$

$$R = 2.506$$

$$\text{Antibody Content} = 0.3005 \text{ mg Ab N/ml}$$

antigen and 0.3005 mg Ab N/ml of undiluted serum with the SSS-SCHU antigen. Addition of more antigen caused only the precipitation of the maximal amount of antibody nitrogen and the formation of an insoluble stable complex throughout the entire equivalence zone. The precipitable antibody nitrogen gradually rose to a maximum by the addition of each antigen increment and then remained constant denoting the presence of the insoluble complex after passing the optimal ratio.

Since the 4 titrations revealed a much greater combining ratio for the anti-38 serum than for the anti-SCHU serums an additional titration was made on 0.5 ml of a 1:7.5 serum dilution. This was a small titration consisting of only 6 points on the reaction curve with the homologous antigen but it provided further evidence for the larger combining ratio. The data, presented in table 22, represent only 1 point which was clearly in the antibody excess realm of the precipitin curve, but calculation of the constant R gave a value of 1.655 which was still larger than any corresponding ratio found for an anti-SCHU serum. The maximal amount of precipitable antibody nitrogen was 26.33 μ g nitrogen and conversion to undiluted serum gave an antibody content of 0.3950 mg Ab N/ml which was in good agreement with the previous estimates for this serum. The titration again demonstrated the existence of an insoluble, stable complex in the antigen excess realm of the precipitin curve.

Table 22

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

G₁₃LA₃₈-49-A (Diluted 1:7.5)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	22.86	22.70	2.27	0.3429	+	0
20	24.61	24.60	1.23	0.3692	±	±
30	23.60	5.70	0.79	0.3540	±	±
40	24.08		0.60	0.3612	0	+
50	24.61		0.49	0.3692	0	+
60	24.21		0.40	0.3632	0	+

* Values calculated according to equation:
 $\mu\text{g Antibody N Pptd.} = 3.310 S - 0.1040 S^2$
 $S \text{ max.} = 15.91 \mu\text{g} \quad \text{Ab max.} = 26.33 \mu\text{g}$
 $R = 1.655$
 Antibody Content = 0.3950 mg Ab N/ml

(b) Further Precipitin Reaction Studies with Anti-38 Goat Serums and Homologous PG Antigen. It was now apparent that the combining ratio for the avirulent antiserum was consistently higher than those for the virulent strain antisera. This was observed in the 5 previous titrations using both homologous and heterologous reaction systems. Also it is obvious that each polysaccharide had precisely the same combining capacity for either antibody and that a given amount of either SSS precipitated the same quantity of antibody. Therefore further titrations were conducted to confirm the higher combining ratio with other avirulent antisera and to see if this was a constant feature with all goat serums. The SSS-38 antigen was utilized so that all reactions were studied in a strictly homologous system.

(1) Serum G₁₃LA₃₈-49-B. A titration was made on the serum derived from the second bleeding of the goat inoculated with the living 38 strain. Studies of the precipitin reaction were made and the antibody content of the serum was measured to see if there had occurred an in vivo fall in titer during the 25-day interval following the first bleeding. Data for the precipitin curve on 0.5 ml of a 1:5 serum dilution are presented in table 23. This quantitative precipitin reaction revealed a very narrow antibody excess zone which rapidly entered a narrow equivalence zone, and extended well into the antigen excess realm where the maximal amount of antibody nitrogen was

Table 23

Addition of Increasing Amounts of PG SSS-38 to 0.5 ml Serum

G₁₃LA₃₈-49-B (Diluted 1:5)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg		mg/ml		
10	26.16	26.20	2.62	0.2616	+	0
20	29.86	29.80	1.49	0.2986	<u>+</u>	0
30	29.45	10.80	0.98	0.2945	0	<u>+</u>
40	29.79		0.74	0.2979	0	<u>+</u>
50	30.19		0.60	0.3019	0	+
60	31.19		0.52	0.3119	0	+
70	29.05		0.42	0.2905	0	++
80	30.19		0.38	0.3019	0	++
90	28.78		0.32	0.2878	0	++
100	29.85		0.30	0.2985	0	++

* Values calculated according to equation:
 $\mu\text{g Antibody N Pptd.} = 3.750 S - 0.1130 S^2$
 $S \text{ max.} = 16.59 \mu\text{g} \quad \text{Ab max.} = 31.11 \mu\text{g}$
 $R = 1.875$
 Antibody Content = 0.3111 mg Ab N/ml

precipitated. Here the usual insoluble stable complex was observed and the serum contained 31.11 μ g of antibody nitrogen. Appropriate calculation showed an antibody content of 0.31 mg Ab N/ml of undiluted serum which is only slightly lower than the corresponding "A" bleeding. The optimal combining ratio was 1.875, still larger than any corresponding ratio with an anti-SCHU serum. Therefore the interval between bleedings did not cause the combining ratio for the anti-38 serum to be decreased.

(2) Serums G₇LA_{38-46-A} and G₄LA_{38-46-A}. These different combining ratios for the anti-38 serums, as well as their behavior with either polysaccharide, are apparently real and not consequent upon technical factors. Further support for the higher combining ratios is presented in tables 24 and 25, which illustrate similar precipitin curve data obtained with SSS-38 on 2 different anti-38 goat serums that had been prepared in 1946 and kept frozen in the dry-ice chest thereafter. The plotted curves and ratios of antibody N:SSS are compared in figure 5. Each serum came from a different animal, each one having been inoculated intravenously only with living strain 38. It is noted that these serums are considerably lower in antibody content than the 1949 anti-38 serum: serum No. 7 contained 0.0565 mg Ab N/ml of serum and No. 4 had 0.0744 mg Ab N/ml of serum. The calculated optimal combining ratio was 1.120 for serum No. 7 and 1.178 for serum No. 4. These ratios are in good agreement and consistently higher than corresponding ratios

Table 24

Addition of Increasing Amounts of PG SSS-38 to 1.0 ml Serum

G₇LA-46-A (Diluted 1:2.5)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	23.67	21.39	2.37	0.0592	+	0
20	26.23	2.50	1.31	0.0658	+	0
30	26.90		0.90	0.0673	+	0
40	28.24		0.71	0.0706	±	0
50	29.59		0.59	0.0740	0	±
60	29.46		0.49	0.0737	0	+
70	29.86		0.43	0.0747	0	+
80	28.78		0.36	0.0720	0	+

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 2.245 S - 0.1060 S^2$$

$$S \text{ max.} = 10.59 \mu\text{g} \quad \text{Ab max.} = 22.59 \mu\text{g}$$

$$R = 1.120$$

$$\text{Antibody Content} = 0.0565 \text{ mg Ab N/ml}$$

Table 25

Addition of Increasing Amounts of PG SSS-38 to 1.0 ml Serum

G₄LA-46-A (Diluted 1:2.5)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess;	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	21.21	18.89	2.12	0.0530	+	0
20	23.00	28.46	1.15	0.0575	+	0
30	24.21	28.71	0.81	0.0605	+	±
40	27.17		0.68	0.0679	±	±
50	26.70		0.53	0.0668	0	±
60	26.23		0.44	0.0658	0	+
70	26.36		0.38	0.0659	0	+
80	27.03		0.34	0.0676	0	+

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 2.355 S - 0.0466 S^2$$

$$S \text{ max.} = 25.27 \mu\text{g} \quad \text{Ab max.} = 29.75 \mu\text{g}$$

$$R = 1.178$$

$$\text{Antibody Content} = 0.0744 \text{ mg Ab N/ml}$$

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Avirulent Strain Goat Antiserums and SSS-38 Antigen

Serum Diluted 1:2.5; Volume 1.0 ml

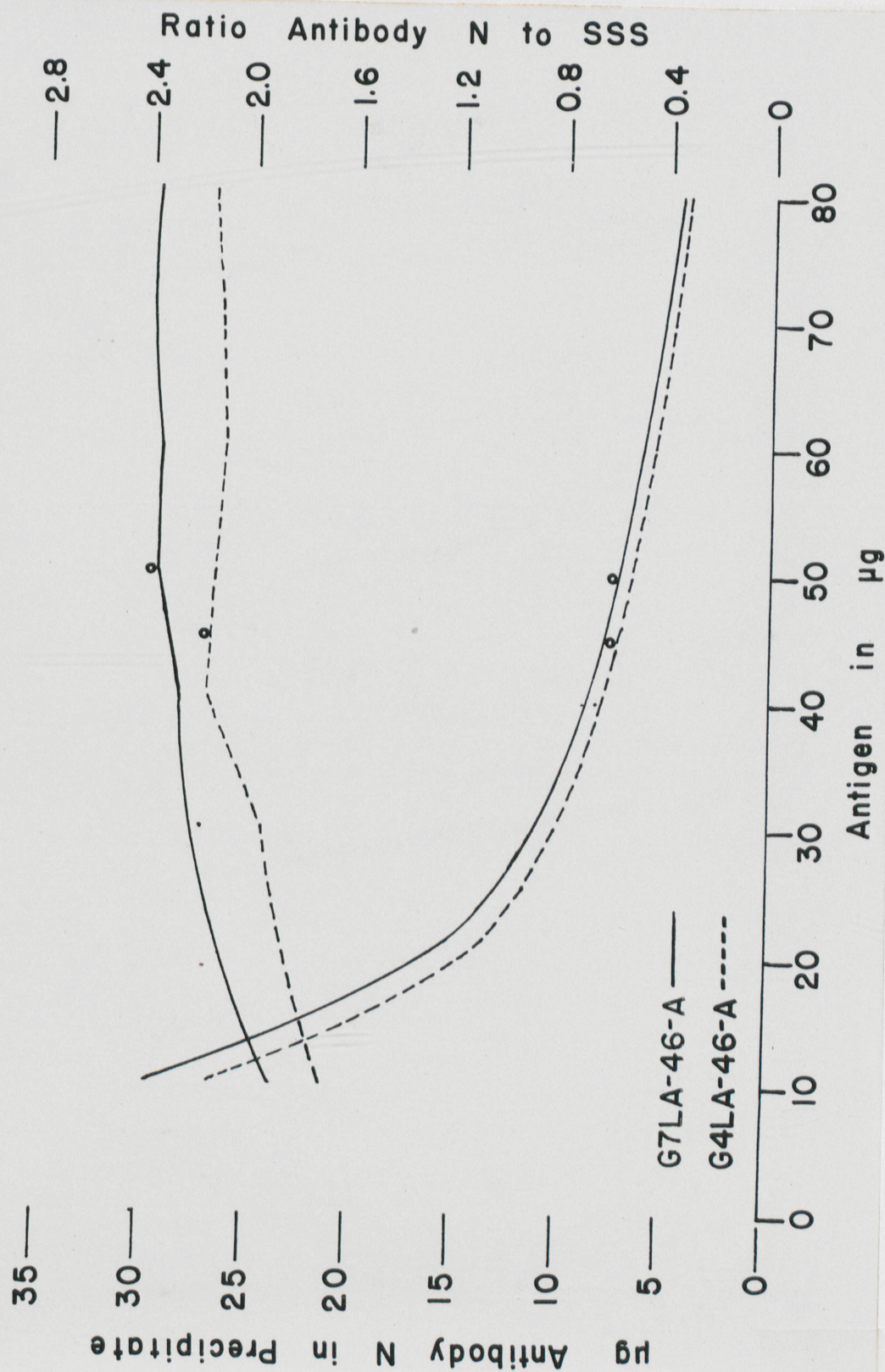


Fig. 5

calculated for any anti-SCHU serum. It will be noted that the data presented in both tables and represented graphically in figure 5 are primarily in the antigen excess realm of the precipitin curve and that the antigen-antibody compound consisted of a stable, insoluble complex in this zone. These titrations offer additional proof for the higher combining ratios of an anti-38 serum even though it is relatively low in antibody content. The ratios are slightly lower than those obtained for the 1949 anti-38 serums but this may be a consequence of the lower antibody titers of these serums. In any case even these low titered anti-38 serums are demonstrated to have higher combining ratios for the homologous antigen than has any anti-SCHU serum.

(c) Precipitin Reaction Studies with Anti-38 Goat Serum and Heterologous PG Antigen. The 2 polysaccharide antigens, derived from either the avirulent or virulent strains, have been shown to **possess** identical combining powers for antibodies engendered by either strain of living bacteria in goats. However the combining power of an anti-38 serum was demonstrated to be considerably larger than that of an anti-SCHU serum. The higher ratio was noted in both homologous and heterologous reaction systems. Special analyses were included for the anti-38 homologous reaction system and additional proof was obtained for the higher ratio with both high titered and low titered antiserums. However an additional titration was made using the

heterologous polysaccharide (SSS-SCHU) to see if the higher ratio continued to be a constant feature.

(1) G₇LA₃₈-50-E. Data for the titration are given in table 26 for 1.0 ml of a 1:5 dilution of serum. This serum represents the fifth bleeding made during the course of immunization in 1950 and it was obtained the day after the last inoculation. Examination of the tests on supernatants demonstrates a difficulty often encountered in selecting any one tube for a "neutral supernatant" as evidenced by absence of both antigen and antibody. However calculation of the optimal combining ratio gave a value of 1.55 and in good agreement with other ratios for an anti-38 serum. Interpolation showed the ratio to occur at 38.44 µg antigen and at this point all of the antibody was precipitated. The antibody content was 0.2978 mg Ab N/ml of undiluted serum, a relatively high titer for an anti-38 serum. The stable insoluble antigen-antibody complex was found in extreme antigen excess. The amount of precipitated antibody nitrogen increased with each additional antigen increment until the stable complex of maximal precipitable nitrogen was obtained.

4. Quantitative Precipitin Reaction Studies with PG SSS-SCHU Antigen. The 2 polysaccharide antigens, SSS-SCHU and SSS-38, now are known to possess identical combining powers for antibodies engendered by either the virulent or avirulent strain of living bacteria. An antiserum can be quantitated accurately

Table 26

Addition of Increasing Amounts of PG SSS-SCHU to 1.0 ml Serum

G7LA38-50-E (Diluted 1:5)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows	
	Found	Calc.*			Excess:	
μg	μg	μg		mg/ml	Ab	An
10	32.18	26.95	3.22	0.1609	++	0
20	47.80	45.84	2.39	0.2390	++	0
30	53.49	56.67	1.78	0.2675	+	0
40	56.54	59.44	1.41	0.2827	+	+
50	58.69	54.15	1.17	0.2935	+	+
60	58.13	40.80	0.97	0.2907	+	+
70	59.80		0.85	0.2990	+	++
80	60.01		0.75	0.3000	+	++
90	61.19		0.68	0.3060	+	++
100	59.31		0.59	0.2966	+	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 3.098 S - 0.0403 S^2$$

$$S \text{ max.} = 38.44 \mu\text{g} \quad \text{Ab max.} = 59.55 \mu\text{g}$$

$$R = 1.55$$

$$\text{Antibody Content} = 0.2978 \text{ mg Ab N/ml}$$

with either SSS so the following precipitin reaction studies were made utilizing only SSS-SCHU as the quantitating antigen. These titrations were made to investigate the characteristics of precipitin reaction curves of various antiserums and to afford a comparison of their respective antibody titers.

(a) Comparative Antibody Titers of Anti-SCHU and Anti-38 Goat Serums One Year after Immunization. In order to measure the relative antibody titers of serums after a lapse of 1 year, the goats inoculated with the living virulent (SCHU) and avirulent (38) strains in 1949 were bled again in 1950. The titers of these serums from bleedings of the previous year have been noted. Determinations for antibody content are shown in tables 27 and 28, respectively, for the anti-SCHU and anti-38 serums. Each table provides data on 2 different units of antibody, both of which are in good agreement. The titration on the anti-SCHU serum in table 27 shows a narrow antibody excess zone progressing into an equivalence zone where both antigen and antibody were detected in the supernatant. However calculation of the combining ratio could be made since there were more than 2 points in the antibody excess realm of this precipitin curve. Interpolation showed the ratio to occur at 39.68 μ g antigen with the 1:5 serum dilution and at 75.63 μ g antigen with the 1:3 serum dilution. Conversion to undiluted serum gave an antibody content of 0.1716 mg Ab N/ml in the first case and 0.1504 mg Ab N/ml in the second. This is precisely

Table 27

Quantitative Titration of Precipitins with PG SSS-SCHU

in Anti-SCHU Serum One Year after Immunization

G₁₄LV_{Schu}-49-C_{50a}

Antigen Used	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess;	
	Found	Calc.*			Ab	An
μg	μg	μg				
Unit of Antibody = 1.0 ml of 1:5 Dilution						
*Equation: $\text{Ab N Pptd.} = 1.730 \text{ S} - 0.0218 \text{ S}^2$						
20	26.36	25.88	1.32	0.1318	+	0
40	30.80	34.32	0.77	0.1540	±	+
50	33.58	32.00	0.67	0.1679	0	+
60	38.85		0.56	0.1693	0	+
80	34.97		0.44	0.1749	0	++
100	35.52		0.36	0.1776	0	++

S max. = 39.68 μg Ab max. = 34.32 μg R = 0.865
 Antibody Content = 0.1716 mg Ab N/ml

Unit of Antibody = 1.0 ml of 1:3 Dilution						
*Equation: $\text{Ab N Pptd.} = 1.332 \text{ S} - 0.0089 \text{ S}^2$						
20	33.72	23.10	1.69	0.1012	+	±
40	41.34	39.12	1.03	0.1240	+	±
60	44.26	48.06	0.74	0.1328	+	±
80	47.45	49.92	0.59	0.1424	+	+
100	49.12	45.70	0.49	0.1474	±	++
120	49.53		0.41	0.1486	±	++
140	50.37		0.36	0.1511	±	++
160	51.20		0.32	0.1532	±	++
180	51.62		0.29	0.1549	±	++
200	52.31		0.26	0.1569	±	++

A max. = 75.63 μg Ab max. = 50.12 μg R = 0.666
 Antibody Content = 0.1504 mg Ab N/ml

Table 28

Quantitative Titration of Precipitins with PG SSS-SCHU

in Anti-38 Serum One Year after Immunization

G₁₃LA₃₈₋₄₉-C_{50a}

Antigen Used	Antibody N Precipitated	Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
µg	µg		mg/ml	Ab	An
Unit of Antibody = 1.0 ml of 1:2 Dilution					
20	11.52	0.58	0.0230	±	+
40	12.90	0.32	0.0258	0	+
60	12.77	0.21	0.0255	0	+
80	14.01	0.18	0.0280	0	++
100	12.49	0.13	0.0250	0	++

Antibody Content = 0.0254 mg Ab N/ml

Unit of Antibody = 1.0 ml Undiluted Serum					
20	14.85	0.74	0.0149	±	+
40	18.87	0.47	0.0189	±	+
60	18.73	0.31	0.0187	0	+
80	18.04	0.23	0.0180	0	+
100	19.98	0.30	0.0200	0	+
120	20.26	0.17	0.0203	0	+
140	20.54	0.15	0.0205	0	++
160	18.87	0.12	0.0189	0	++
180	21.23	0.12	0.0212	0	++
200	19.71	0.10	0.0198	0	++

Antibody Content = 0.0201 mg Ab N/ml

one-half the antibody content of the serum from the second bleeding of the goat in 1949.

The titration of the anti-38 serum in table 28 also gives data for 2 units of antibody. However all determinations were made in the antigen excess realm of the precipitin curve as shown by tests on the supernatants. Therefore calculated values for antibody nitrogen could not be made since the experimental equation does not describe the composition of the precipitate in the antigen excess zone. Previously it was demonstrated that the precipitate consisted of a stable complex of maximal precipitable antibody nitrogen in extreme antigen excess. In this titration the amount of antibody precipitated with every unit of antigen is almost identical. The plotted curve would be represented by a horizontal straight line in antigen excess denoting the presence of the insoluble stable complex of maximal antibody nitrogen. Therefore the antibody content calculated from the 1:2 serum dilution was 0.0254 mg Ab N/ml and 0.0201 mg Ab N/ml on undiluted serum, both results in good agreement. This serum is very low in antibody content and this accounts for the reactions occurring in the antigen excess zone of undiluted serum. The serum was about 8 times lower in antibody nitrogen than the corresponding anti-SCHU serum. Also this anti-38 serum was 15 times lower in antibody content than were samples from the same goat 1 year previously. Accordingly the animal inoculated with the virulent (SCHU) strain had a

much higher precipitin titer which persisted over a longer period of time than had the animal immunized against the avirulent strain.

(b) Changes in Antibody Content of Goat Serum during the Course of Hyperimmunization. The previous studies of precipitin curves were made on hyperimmune serums after the series of immunizations were completed. The changes in the blood serum that occurred during the process of hyperimmunization were not investigated. Therefore to observe changes occurring in a serum during an inoculation schedule, each goat was bled at frequent intervals during ~~the immunization~~ procedure. Quantitative precipitin determinations were made on a serum sample from each bleeding so that the rise in antibody titer could be followed and observations of the respective combining ratios noted.

(1) Serum G₁₀LV_{Schu}-50: The effect of immunization on the antibody titer and changes in the serum of the goat inoculated with the living virulent SCHU strain are set forth in table 29 which includes data for 4 interval bleedings. The first or "A" bleeding determinations are included in part A for 3 titrations on various units of the same antibody. Data on 1.0 ml of a 1:2 serum dilution reveal all determinations to have occurred in the antigen excess zone of the precipitin curve. The maximal precipitable antibody nitrogen throughout this zone was 0.0191 mg Ab N/ml. Additional analyses on 1.0 ml of undiluted serum still did not give a distinct equivalence zone

Table 29

Effect of Immunization on Antibody Titer of Anti-SCHU Goat Serum

Quantitated with PG SSS-SCHU Antigen

G₁₀^{LV}Schu-50

A. "A" Bleeding: Titration of Varied Antibody Units.

Antigen Used	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				

Unit: 1.0 ml of 1:2 Dilution

10	8.81		0.88	0.0176	+	±
20	8.34		0.42	0.0167	±	+
30	9.41		0.31	0.0188	±	+
40	9.28		0.23	0.0186	±	+
50	10.36		0.21	0.0207	0	+
60	11.09		0.18	0.0222	0	+

Antibody Content = 0.0191 mg Ab N/ml

Unit: 1.0 ml Undiluted Serum

10	14.39		1.44	0.0144	±	+
20	14.66		0.73	0.0147	±	+
30	15.60		0.52	0.0156	±	+
40	16.14		0.40	0.0161	±	+
50	17.49		0.35	0.0175	±	+

Antibody Content = 0.0145 mg Ab N/ml

Unit: 2.0 ml Undiluted Serum

*Equation: µg Ab N Pptd. = 3.123 S - 0.0760 S²

10	24.81	23.63	2.48	0.0124	+	0
20	27.37	32.06	1.37	0.0137	+	0
30	28.91	3.30	0.96	0.0146	±	±
40	28.51		0.71	0.0143	±	±
50	30.13		0.60	0.0151	±	±
60	30.39		0.51	0.0152	±	±
70	30.40		0.43	0.0152	±	±
80	31.81		0.40	0.0159	±	±

S max. = 20.55 µg Ab max. = 32.08 µg R = 1.56

Antibody Content = 0.0160 mg Ab N/ml

(Continued)

Table 29 (Continued)

Effect of Immunization on Antibody Titer of Anti-SCHU Goat Serum

Quantitated with PG SSS-SCHU Antigen

G₁₀LV_{Schu}-50

B. "B" Bleeding: Unit of Antibody Used = 0.5 ml of 1:5 Dilution

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	22.32	15.80	2.23	0.2232	++	0
20	28.78	27.72	1.44	0.2878	++	0
30	33.76	35.76	1.13	0.3376	++	0
40	38.20	39.92	0.96	0.3820	++	0
50	42.36	40.20	0.85	0.4236	++	0
60	43.04	36.60	0.72	0.4304	+	±
70	44.10		0.63	0.4410	+	+
80	45.53		0.57	0.4553	+	+
90	45.66		0.51	0.4566	+	++
100	44.72		0.45	0.4472	+	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.774 S - 0.0194 S^2$$

$$S \text{ max.} = 45.72 \mu\text{g} \quad \text{Ab max.} = 40.56 \mu\text{g}$$

$$R = 0.887$$

$$\text{Antibody Content} = 0.4056 \text{ mg Ab N/ml}$$

(Continued)

Table 29 (Continued)

Effect of Immunization on Antibody Titer of Anti-SCHU Goat Serum

Quantitated with PG SSS-SCHU Antigen

G₁₀LV_{Schu}-50

C. "C" Bleeding: Comparison of Antibody Content Calculated from
Two Different Units of Antibody Solution.

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				

Unit of Antibody: 1.0 ml of 1:15 dilution

*Equation: $\mu\text{g Ab N Pptd.} = 1.720 \text{ S} - 0.0199 \text{ S}^2$

10	20.62	15.21	2.06	0.3093	++	0
20	27.24	26.44	1.36	0.4086	++	0
30	32.15	33.69	1.07	0.4823	++	0
40	36.59	36.96	0.91	0.5489	+	0
50	37.39	36.25	0.75	0.5609	±	±
60	37.93	31.56	0.63	0.5690	±	±
70	39.27		0.56	0.5891	±	+
80	41.10		0.51	0.6165	0	++
90	43.23		0.48	0.6485	0	++
100	43.25		0.43	0.6488	0	++

S max. = 43.22 µg Ab max. = 37.16 µg R = 0.860

Antibody Content = 0.5588 mg Ab N/ml

Unit of Antibody: 0.5 ml of 1:10 dilution

*Equation: $\mu\text{g Ab N Pptd.} = 1.550 \text{ S} - 0.0215 \text{ S}^2$

10	17.89	13.35	1.79	0.3550	++	+
20	22.66	22.40	1.13	0.4532	++	+
30	26.83	27.15	0.89	0.5366	+	+
40	28.04	27.60	0.70	0.5608	+	+
50	28.71	23.75	0.57	0.5742	+	++
60	29.79		0.50	0.5958	+	++
70	29.72		0.42	0.5944	+	++
80	30.19		0.30	0.6038	+	++

S max. = 36.05 µg Ab max. = 27.94 µg R = 0.775

Antibody Content = 0.5588 mg Ab N/ml

(Continued)

Table 29 (Continued)

Effect of Immunization on Antibody Titer of Anti-SCHU Goat Serum

Quantitated with PG SSS-SCHU Antigen

G10LV_Schur-50

D. "D" Bleeding: Unit of Antibody Used = 0.5 ml of 1:10 Dilution

Antigen Used	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	21.70	15.23	2.17	0.4340	++	0
20	29.45	27.52	1.47	0.5890	++	0
30	34.23	36.87	1.14	0.6846	++	0
40	39.07	43.28	0.98	0.7814	+	0
50	41.83	46.25	0.84	0.8366	+	0
60	43.98	47.28	0.73	0.8796	±	0
70	45.53	37.87	0.65	0.9106	±	±
80	45.60		0.57	0.9120	±	+
90	45.73		0.51	0.9146	±	+
100	45.96		0.46	0.9192	±	+

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.670 S - 0.0147 S^2$$

$$S \text{ max.} = 56.80 \mu\text{g} \quad \text{Ab max.} = 47.43 \mu\text{g}$$

$$R = 0.835$$

$$\text{Antibody Content} = 0.9486 \text{ mg Ab N/ml}$$

and reactions were predominantly in antigen excess. However the maximal antibody nitrogen value was 0.0145 mg Ab N/ml which is in good agreement with the previous value. Titrations on a unit of 2.0 ml undiluted serum began to show the existence of a narrow antibody excess zone. The calculated optimal ratio was 1.56, the highest recognized for any anti-SCHU serum. The antibody content for this unit of antibody was 0.0160 mg Ab N/ml, in good agreement with the 2 previous estimates. The existence of the antigen excess zone of the precipitin curve for the first 2 units of antibody is easily explained if the optimal ratio of 1.56 represents the equivalence value for this serum. Examination of the column for tests on supernatants demonstrates a difficulty sometimes encountered in selecting any one tube for the neutralization point as evidenced by absence of both antigen and antibody in the supernatant. Attempts to quantitate a serum by increasing the antigen concentrations in such small increments as 10 μ g make the selection of a neutralization zone even more difficult. Agreement among all maximal antibody nitrogen figures is satisfactory in titrations on the 3 units of antibody, varying from 0.0145 to 0.0191 mg Ab N/ml. This disparity is only a fraction greater than the difference between duplicate samples of any 2 representative nitrogen determinations. Averages of the 3 titrations on serum G₁₀LV_SSchu-50-A (first bleeding) reveal the serum to have an antibody content of 0.0165 mg Ab N/ml.

Data for the quantitative precipitin study on the second bleeding from the SCHU goat are shown in part B of table 29 for 0.5 ml of a 1:5 serum dilution. The "ideal" combining ratio was calculated to be 0.887 at 45.72 μ g antigen. This ratio was found in the next tube after a distinct two plus reading for the presence of antibody in the supernatant and just before the detection of antigen was noted. The antibody content of the serum was 0.4056 mg Ab N/ml of undiluted serum. The optimal ratio occurred in the first tube before the plotted precipitin curve reached its peak and leveled out to form the insoluble stable complex characteristic of the antigen excess realm of tularensis systems. The antibody titer of the "B" bleeding was about 25 times greater than the "A" bleeding from the same goat.

Results of 2 titrations on the third bleeding of the anti-SCHU serum are summarized in part C of table 29. The optimal combining ratio was 0.860 with 1.0 ml of a 1:15 dilution of antibody and 0.775 with 0.5 ml of 1:10 dilution. However the antibody content calculated on the basis of undiluted serum was constant for the 2 titrations. A value of 0.5576 mg Ab N/ml calculated from a dilution factor of 15 was obtained in the first titration. This was in good agreement with the value of 0.5588 mg Ab N/ml calculated from a dilution factor of 20 in the second titration. Both values for the optimal combining ratio occurred at the beginning of the antigen excess zone as

indicated by tests on the supernatants.

A summary of a titration on the last bleeding of the SCHU goat is given in part D of table 29. The analysis was made on 0.5 ml of a 1:10 serum dilution and this precipitin curve was characterized throughout the antibody nitrogen:SSS ratio range from 2.17 to 0.46. The optimal combining ratio was 0.835 which is in good agreement with the other ratios calculated for this serum. Interpolation showed the ratio to occur at 56.80 μ g antigen which gave an antibody content equivalent to 0.9486 mg Ab N/ml of undiluted serum. Therefore it is evident that the serum obtained from the fourth bleeding of the SCHU goat was relatively high in antibody nitrogen.

(c) Analyses of Goat Serums Prepared against Selected Virulent Strains. Evidence had accumulated that the antibody engendered by the 38 strain had a higher combining power for the polysaccharide antigens than the antibody produced by the SCHU strain. Therefore studies were made on antibodies formed in animals by inoculating other selected strains of organisms graded in virulence. Analyses of the precipitin curves and comparisons of calculated optimal combining ratios were made on the various serums obtained at interval bleedings during the course of hyperimmunization.

(1) Serum G17LVChur-50. Determinations on 4 bleedings from the CHUR goat are presented in table 30. Calculated values for optimal combining ratios are given for the 4

Table 30

Quantitative Precipitin Determinations with PG SSS-SCHU
on Anti-CHUR Goat Serums during Immunization

G17LVChur-50

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows	
	Found	Calc.*			Excess:	
	µg	µg			Ab	An
"A" Bleeding: 1.0 ml of 1:2 Serum Dilution						
*Equation: µg Ab N Pptd. = 2.77 S - 0.0790 S ²						
10	19.77	19.80	1.98	0.0395	+	0
20	23.74	23.80	1.19	0.0475	±	±
30	24.90	12.00	0.83	0.0498	±	+
40	24.21		0.61	0.0484	±	+
50	26.50		0.53	0.0530	±	+
60	26.47		0.44	0.0529	±	+

S max. = 17.53 µg Ab max. = 24.28 µg R = 1.385
Antibody Content = 0.0486 mg Ab N/ml

"B" Bleeding: 0.5 ml of 1:5 Serum Dilution *Equation: $\mu\text{g Ab N Pptd.} = 1.840 \text{ S} - 0.0205 \text{ S}^2$						
10	20.31	16.35	2.03	0.2031	++	0
20	29.59	28.60	1.48	0.2959	++	0
30	35.24	36.75	1.17	0.3524	++	0
40	39.00	40.80	0.98	0.3900	++	±
50	42.84	40.75	0.86	0.4284	++	+
60	42.90	36.60	0.72	0.4290	+	+
70	42.96		0.61	0.4296	+	++
80	43.85		0.55	0.4385	+	++
90	45.26		0.50	0.4326	+	++
100	47.61		0.48	0.4761	+	++

S max. = 44.88 µg Ab max. = 41.29 µg R = 0.920
Antibody Content = 0.4129 mg Ab N/ml

(Continued)

Table 30 (Continued)

Quantitative Precipitin Determinations with PG SSS-SCNU

on Anti-CHUR Goat Serums during Immunization

G₁₇LV_{Chur}-50

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
"C" Bleeding: 0.5 ml of 1:10 Serum Dilution						
*Equation: µg Ab N Pptd. = 1.890 S - 0.0265 S ²						
10	22.80	16.25	2.28	0.4560	++	0
20	27.77	27.20	1.39	0.5554	++	0
30	30.80	32.85	1.03	0.6160	++	0
40	34.31	33.20	0.86	0.6862	+	±
50	35.71	28.25	0.71	0.7142	+	±
60	35.84		0.60	0.7168	+	±
70	37.53		0.54	0.7506	+	+
80	37.05		0.46	0.7410	+	+
90	37.12		0.40	0.7424	+	+
100	38.40		0.38	0.7680	+	+

S max. = 35.66 µg Ab max. = 33.70 µg R = 0.945
Antibody Content = 0.6740 mg Ab N/ml

"D" Bleeding: 0.5 ml of 1:10 Serum Dilution						
*Equation: µg Ab N Pptd. = 1.880 S - 0.0270 S ²						
10	21.52	16.10	2.15	0.4204	++	0
20	27.10	26.80	1.36	0.5420	++	0
30	30.66	32.10	1.02	0.6132	++	0
40	32.62	32.00	0.82	0.6524	+	±
50	33.63	26.50	0.67	0.6726	+	+
60	35.30		0.59	0.7060	+	++
70	35.37		0.51	0.7074	±	++
80	35.71		0.45	0.7142	±	++
90	35.78		0.40	0.7156	±	++
100	36.31		0.36	0.7262	±	++

S max. = 34.81 µg Ab max. = 33.72 µg R = 0.940
Antibody Content = 0.6744 mg Ab N/ml

titrations. A ratio of 1.385 was observed for the "A" bleeding, while ratios for the "B", "C", and "D" bleedings, respectively, were 0.920, 0.945, 0.940. All of these ratios are in good agreement and within the limits of error except for the elevated ratio with the "A" bleeding. However it was noted that a higher combining ratio also occurred with the corresponding "A" bleeding on the SCHU goat and these ratios from the corresponding bleedings are in close agreement. Therefore the elevated ratio for a virulent strain antiserum may be a function of time after acute infection. The shorter this interval the more closely the ratio approximates that for an avirulent antiserum. The antibody content for the serum from each bleeding is given. It is noted that the first bleeding was low in antibody titer, consisting of only 0.0486 mg Ab N/ml undiluted serum. By the third bleeding the antibody content had attained the maximal titer and thereafter remained at the peak level of 0.6744 mg Ab N/ml of undiluted serum. Agreement between experimental and calculated values for antibody nitrogen was excellent in all titrations throughout the antibody excess realm of the curve and up to the point of maximal antibody nitrogen. Thereafter the calculated values began to decrease and became invalid.

(2) Serum G₁₈LV_{Jap}-50. The next goat antiserum to be quantitated was the anti-JAP serum prepared with a less virulent strain having an LD₅₀ titer of 10^{-4.25}. Analyses were

made on serums obtained from the "C" and "D" bleedings and the results are summarized in table 31. Determinations were made on 1.0 ml of a 1:10 antibody dilution for the "C" bleeding and on 0.5 ml of a 1:10 antibody dilution for the "D" bleeding. The optimal combining ratios were calculated and found to be 0.873 and 0.749, respectively, for the "C" and "D" bleedings. These values are in agreement with the previously determined ratios for goat antiserums prepared against other virulent strains. The antibody content of the "C" bleeding serum was 0.4483 mg Ab N/ml and it had increased to 0.5750 mg Ab N/ml of undiluted serum by the time of the "D" bleeding. Again agreement between observed and calculated values for antibody nitrogen was excellent and it demonstrates the accuracy with which the precipitin curve can be followed by formulas derived from the theoretical equation. Each optimal combining ratio occurred in the first tube before antigen appeared in the supernatant. However the appropriate equivalence zone is better designated by a calculated antibody nitrogen:antigen ratio than by any tests of supernatants for excess of reacting components. The calculated equivalence zone ratios also provide a good reference point for assaying the antibody titer of an immune serum.

5. Quantitative Precipitin Reaction Studies with Rabbit Antiserums. Since the Heidelberger equation (29) was developed from experimental work carried out chiefly with rabbit anti-

Table 31

Quantitative Antibody Determinations with PG SSS-SCHU

on Anti-Jap Goat Serums during Immunization

G₁₈LV_{Jap}-50

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
"C" Bleeding: 1.0 ml of 1:10 dilution						
*Equation: µg Ab N Pptd. = 1.746 S - 0.0170 S ²						
10	21.45	15.76	2.15	0.2145	++	0
20	29.85	28.12	1.49	0.2985	++	0
30	35.31	37.08	1.18	0.3531	++	0
40	40.01	42.64	1.00	0.4001	++	0
50	43.04	44.80	0.86	0.4304	+	±
60	47.95	43.56	0.80	0.4795	+	+
70	48.82		0.70	0.4882	+	+
80	49.90		0.62	0.4990	+	+
90	51.78		0.58	0.5178	+	+

S max. = 51.35 µg Ab max. = 44.83 µg R = 0.873
Antibody Content = 0.4483 mg Ab N/ml

"D" Bleeding: 0.5 ml of 1:10 dilution						
*Equation: µg Ab N Pptd. = 1.498 S - 0.0195 S ²						
10	19.16	13.03	1.92	0.3832	+	0
20	23.47	22.16	1.12	0.4694	+	0
30	26.70	27.39	0.89	0.5340	+	0
40	29.32	28.72	0.73	0.5864	±	±
50	29.99	26.15	0.60	0.5998	±	+
60	31.20		0.52	0.6240	±	+
70	31.69		0.45	0.6338	±	++
80	32.14		0.40	0.6428	±	++
90	33.49		0.37	0.6698	±	++

S max. = 39.42 µg Ab max. = 28.75 µg R = 0.749
Antibody Content = 0.5750 mg Ab N/ml

serums it was anticipated that other rabbit serums would yield data closely approximating the theoretical. Inasmuch as each SSS had been shown to have the same combining power for antibody in goat serum they were regarded as essentially identical precipitating antigens, and only PG SSS-SCHU was used with rabbit antiserums. Determinations were made of the optimal combining ratios with rabbit antiserums and an effort was made to note any differences that might appear between these and the ratios from goat serums. Observations for comparative precipitin curves between anti-SCHU and anti-38 rabbit serums were included for studies of homologous and heterologous reaction systems.

(a) RKV_Schu-48. The quantitative precipitin reaction with a homologous anti-SCHU rabbit serum is summarized in table 32. This antiserum was prepared by only 3 intravenous inoculations given at weekly intervals so that a low titered serum was anticipated. The titration was made on 1.0 ml of a 1:3 serum dilution. This precipitin reaction ranged from the beginning of the antibody excess zone into a broad equivalence zone, and progressed into the extreme antigen excess region of the precipitin curve. The calculated optimal ratio was 0.523 and it occurred when 44.70 μ g antigen was added. This ratio occurred at the beginning of the antigen excess realm of the precipitin curve where maximal precipitable antibody nitrogen was obtained. Good agreement was obtained between calculated

Table 32

Quantitative Precipitin Reaction with Rabbit Antiserum

Addition of Increasing Amounts of PG SSS-SCHU to 1.0 ml Homologous Serum

RKV_{Schu}-48 (Diluted 1:3)

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
μg	μg	μg		mg/ml		
5	7.60	2.30	1.52	0.0228	++	0
10	8.88	9.29	0.89	0.0266	++	0
20	17.01	16.24	0.85	0.0510	++	0
25	18.70	18.84	0.75	0.0561	++	0
30	19.77	20.85	0.65	0.0593	++	0
35	21.70	22.28	0.62	0.0651	++	$\pm$
40	23.06	23.12	0.58	0.0692	++	$\pm$
45	24.54	23.38	0.55	0.0736	+	+
50	24.55	23.05	0.49	0.0737	+	+
60	25.49		0.44	0.0765	+	+
70	27.91		0.40	0.0837	$\pm$	++
80	26.36		0.33	0.0791	$\pm$	++
90	26.63		0.30	0.0799	$\pm$	++
100	26.77		0.27	0.0803	$\pm$	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.046 S - 0.0117 S^2$$

$$S \text{ max.} = 44.70 \mu\text{g} \quad \text{Ab max.} = 23.51 \mu\text{g}$$

$$R = 0.523$$

$$\text{Antibody Content} = 0.0705 \text{ mg Ab N/ml}$$

and observed values for antibody nitrogen throughout the valid range of the reaction. The antibody content was equivalent to 0.0705 mg Ab N/ml of undiluted serum.

(b) RLA₃₈₋₄₈. The quantitative precipitin reaction with a heterologous anti-38 serum is summarized in table 33 and compared graphically in figure 6 with the above homologous 1948 serum. Data are given for titrations on 2 dilutions of the serum. The calculated optimal combining ratio was 0.413 and 0.500, respectively, for the 1:2.5 and 1:3 serum dilutions. These ratios are in good agreement with the value obtained with the homologous anti-SCHU serum. Both ratios occurred at the beginnings of the antigen excess zones of the precipitin curves, and at the points of maximal precipitable antibody nitrogen. The antibody content varied from 0.0395 to 0.0341 mg Ab N/ml of undiluted serum and this represents excellent agreement since it is calculated from 2 different dilution factors. This is precisely one-half the antibody content of the corresponding homologous anti-SCHU serum. The derived equation does describe the course of the reaction curve more closely for rabbit serum than for goat serum, as evidenced by the excellent agreement between observed and calculated values for antibody nitrogen.

(c) Serums: R₁KV_{Schu-50-E} and R₁₁KA_{38-50-E}. The quantitative precipitin reactions with homologous and heterologous hyperimmune rabbit serums prepared in 1950 are pre-

Table 33

Quantitative Precipitin Reaction with Rabbit Antiserum

Addition of Increasing Amounts of PG SSS-SCHU to Heterologous Serum

RLA₃₈-48

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
Unit of Antibody: 1.0 ml of 1:2.5 dilution						
*Equation: µg Ab N Pptd. = 0.825 S - 0.0115 S ²						
10	9.82	7.10	0.98	0.0246	++	0
20	12.28	11.90	0.61	0.0307	+	0
30	13.45	14.40	0.45	0.0336	±	±
40	15.33	14.60	0.38	0.0383	±	+
50	15.20	12.50	0.30	0.0380	±	++
60	14.39		0.23	0.0360	±	++
70	15.06		0.22	0.0377	±	++
80	15.60		0.20	0.0390	0	++
90	16.41		0.18	0.0411	0	++
100	16.27		0.16	0.0407	0	++

S max. = 35.87 µg Ab max. = 15.79 µg R = 0.413
Antibody Content = 0.0395 mg Ab N/ml

Unit of Antibody: 1.0 ml of 1:3 dilution
*Equation: µg Ab N Pptd. = 1.000 S - 0.0220 S²

5	4.57	4.45	0.91	0.0137	+	0
10	7.80	7.80	0.78	0.0234	+	0
15	10.09	10.05	0.67	0.0303	+	0
20	10.34	11.20	0.52	0.0310	+	0
25	11.44	11.21	0.46	0.0343	+	±
30	13.05	10.20	0.44	0.0392	±	±
35	13.45		0.38	0.0435	±	+

S max. = 22.73 µg Ab max. = 11.36 µg R = 0.500
Antibody Content = 0.0341 mg Ab N/ml

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Rabbit Serums with SSS-Schu Antigen
Serums Diluted 1:3; Volume 1.0 ml

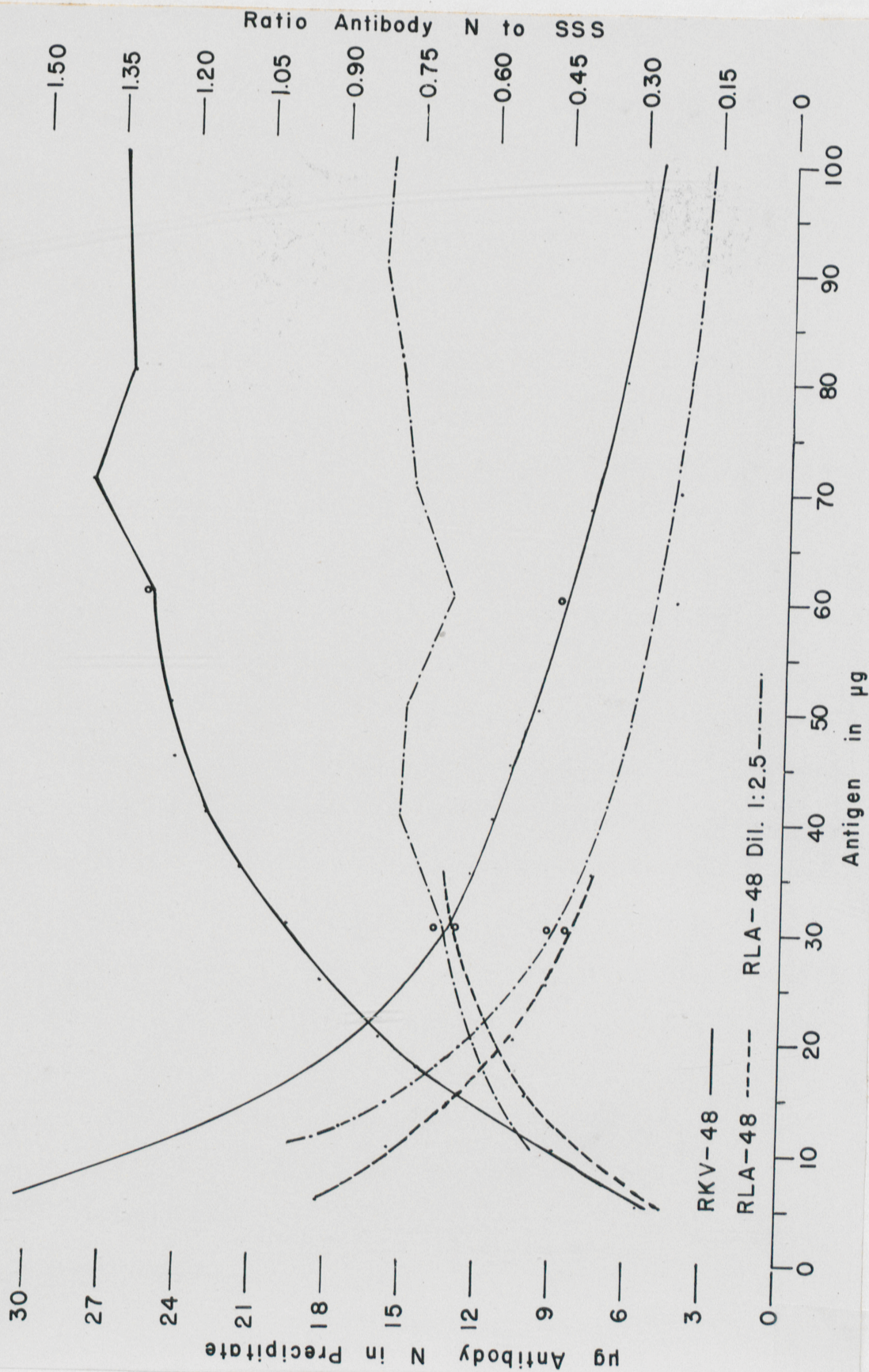


Fig. 6

sented in tables 34 and 35, respectively. These serums resulted from a prolonged immunization schedule and, therefore, should be more comparable with the hyperimmune goat serums. One result of the many inoculations is the higher antibody titers as compared with the rabbit antiserums prepared in 1948. The antibody content of the anti-SCHU serum was 0.2403 mg Ab N/ml undiluted serum while the anti-38 serum contained 0.1101 mg Ab N/ml of serum, or again precisely one-half that of the corresponding SCHU antiserum. The calculated optimal combining ratios were 0.376 and 0.535, respectively, for the homologous and heterologous systems. Both ratios occurred at the beginnings of the antigen excess zones as evidenced by tests on the supernatants. These ratios exhibit fair agreement and the difference is considered to be of no significance. The variation is probably due to the use of different units of antibody in the corresponding titrations. The calculated optimal ratios for rabbit antibody are significantly lower than those for the goat antibody. However the ideal ratios for the anti-SCHU and anti-38 rabbit serums were practically identical, a feature in marked contrast to the findings with the corresponding goat antiserums. The ratio for rabbit antibody is about 1.5 times lower than ratios for anti-SCHU goat serum, and about 3 times lower than corresponding ratios for anti-38 goat serums.

6. Quantitative Precipitin Reaction Studies with Anti-

Table 34

Quantitative Precipitin Reaction with Anti-SCHU Rabbit Serum

and Homologous PG SSS-SCHU Antigen

1.0 ml Serum: R₁KV_{Schu}-50-E: Diluted 1:10

Antigen Added µg	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found µg	Calc.* µg			Ab	An
10	7.56	6.90	0.76	0.0756	+	0
20	12.63	12.58	0.63	0.1263	+	0
30	17.07	17.04	0.57	0.1707	+	+
40	20.05	20.28	0.50	0.2005	+	+
50	22.61	22.30	0.45	0.2261	+	+
60	24.07	23.10	0.40	0.2407	+	+
70	25.46	22.68	0.36	0.2546	+	++
80	26.15		0.33	0.2615	+	++
90	28.13		0.31	0.2813	+	++
100	28.86		0.29	0.2886	+	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 0.751 S^2 - 0.0061 S^2$$

$$S \text{ max.} = 61.56 \mu\text{g} \quad \text{Ab max.} = 24.03 \mu\text{g}$$

$$R = 0.376$$

$$\text{Antibody Content} = 0.2403 \text{ mg Ab N/ml}$$

Table 35

Quantitative Precipitin Reaction with Anti-38 Rabbit Serum

and Heterologous PG SSS-SCHU Antigen

1.0 ml Serum: R11KA38-50-E: Diluted 1:5

Antigen Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg		mg/ml		
20	16.79	16.20	0.84	0.0840	+	0
30	19.49	20.40	0.65	0.0975	+	0
40	20.81	22.00	0.52	0.1041	0	+
50	22.27	21.50	0.45	0.1140	0	+
60	22.90		0.38	0.1145	0	+
70	21.92		0.31	0.1096	0	+
80	22.41		0.28	0.1121	0	+
90	23.51		0.26	0.1176	0	+
100	23.03		0.23	0.1152	0	+

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.070 S - 0.0130 S^2$$

$$S \text{ max.} = 41.15 \mu\text{g} \quad \text{Ab max.} = 22.02 \mu\text{g}$$

$$R = 0.535$$

$$\text{Antibody Content} = 0.1101 \text{ mg Ab N/ml}$$

SCHU Horse Serum. Antitularense horse serum was prepared in 1943 and 1944 by inoculating horses with the living SCHU strain by intravenous injections. After use from bulk storage at 4 C for about 1 year, the serum was lyophilized and stored in a refrigerator until used. Data from 2 series of precipitin reactions are summarized in tables 36 and 37, one with SSS-SCHU and another with SSS-38, and against a 1944 and a 1943 serum, respectively. A plot of the precipitin curve with the homologous SSS-SCHU antigen is shown in figure 7. The antibody contents of the 2 serums were almost identical, varying only from 0.2342 to 0.2668 mg Ab N/ml of undiluted serum. Agreement between observed and calculated values for antibody nitrogen was excellent in both titrations throughout the valid range of antibody excess. A difficulty previously encountered in reading supernatants was again in evidence in both of these titrations. However the calculated optimal combining ratio occurred at the point at which minimal or equivalent amounts of antigen and antibody were detected in the supernatants. The ratio with the 1:4 serum dilution in table 36 was 1.60 whereas the 1:6 serum dilution gave a ratio of 0.956. The difference between these ratios is probably a function of storage of serum rather than of the antigen employed. The anti-SCHU horse serum precipitates showed a relatively large amount of antibody nitrogen precipitated per unit of antigen. The ratio is almost identical for the

Table 36

Quantitative Precipitin Reaction with Horse Antiserum

Addition of Increasing Amounts of PG SSS-SCHU to 1.0 ml Serum

HLV_{Schu-44} (Diluted 1:4)

SSS-SCHU Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum mg/ml	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg				
10	29.80	27.70	2.98	0.1192	++	+
20	46.59	46.80	2.33	0.1864	++	+
30	56.81	56.70	1.89	0.2272	++	+
40	58.21	58.00	1.46	0.2328	+	+
50	61.55	50.50	1.23	0.2462	+	+
60	59.93		1.00	0.2397	+	+
70	61.44		0.88	0.2458	+	++
80	61.22		0.77	0.2449	+	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 3.21 S - 0.0440 S^2$$

$$S \text{ max.} = 36.48 \mu\text{g} \quad \text{Ab max.} = 58.55 \mu\text{g}$$

$$R = 1.60$$

$$\text{Antibody Content} = 0.2342 \text{ mg Ab N/ml}$$

Table 37

Quantitative Precipitin Reaction with Horse Antiserum

Addition of Increasing Amounts of PG SSS-38 to 1.0 ml Serum

HLV_{Schu}-43 (Diluted 1:6)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
μg	μg	μg		mg/ml		
10	17.10	17.07	1.71	0.1026	++	0
20	29.80	30.04	1.49	0.1788	++	0
30	39.49	38.91	1.32	0.2369	+	0
40	43.36	43.68	1.08	0.2602	$\pm$	$\pm$
50	44.01	44.35	0.88	0.2641	0	$\pm$
60	45.94	40.92	0.77	0.2756	0	+
70	45.51		0.65	0.2731	0	+
80	45.35		0.57	0.2721	0	++
90	45.51		0.51	0.2731	0	++
100	45.03		0.45	0.2702	0	++

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 1.91 S - 0.0205 S^2$$

$$S \text{ max.} = 46.63 \mu\text{g} \quad \text{Ab max.} = 44.46 \mu\text{g}$$

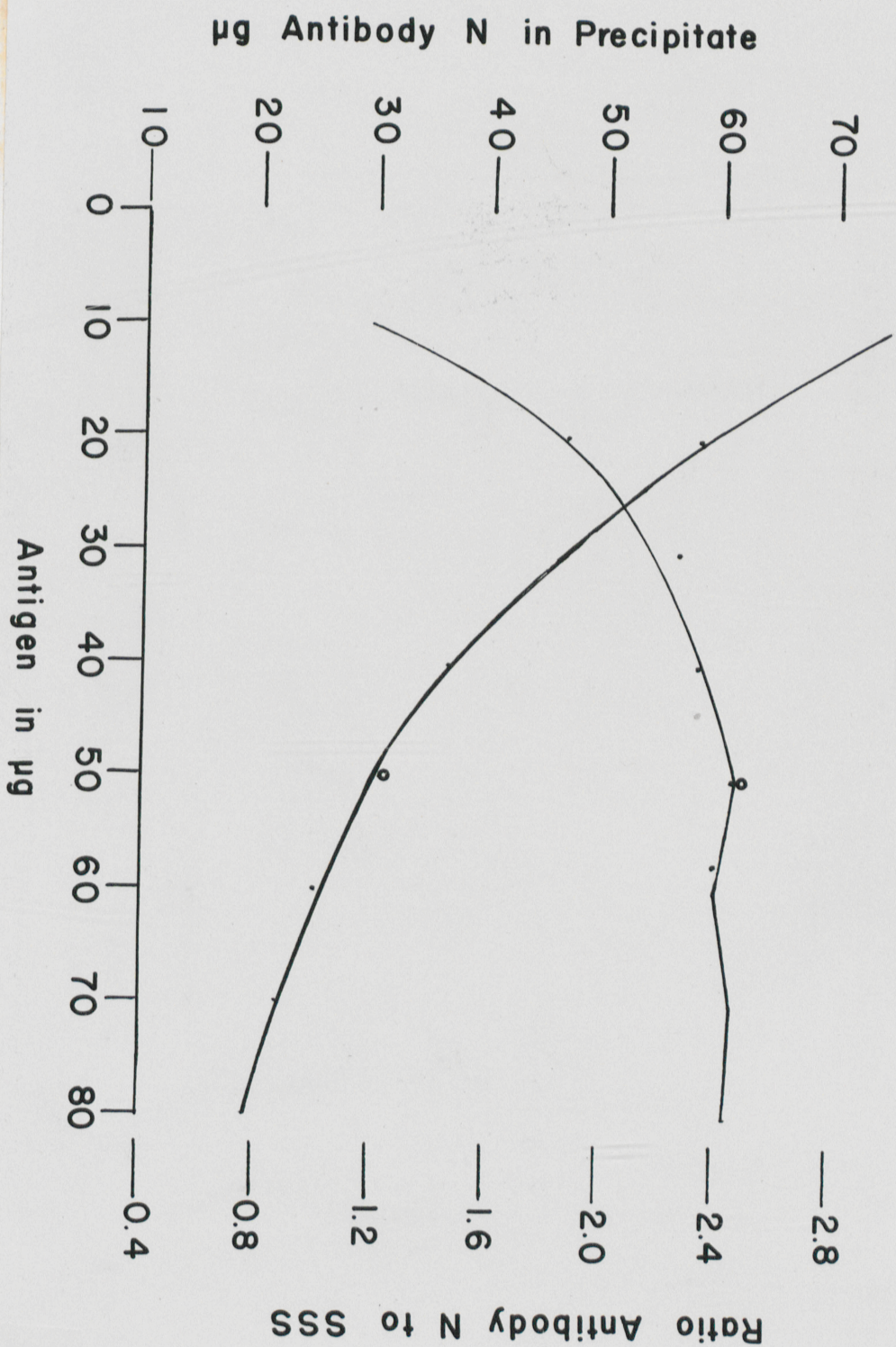
$$R = 0.956$$

$$\text{Antibody Content} = 0.2668 \text{ mg Ab N/ml}$$

QUANTITATIVE COURSE OF PRECIPITIN REACTION

Horse Serum HLV-44 with SSS-Schu Antigen

Serum Diluted 1:4; Volume 1.0 ml



siderably lower than any ratio heretofore noted. The ratio occurred at an antigen concentration of 180.5 µg SSS and addi-

amount of antibody precipitated by each antigen unit with anti-38 goat serums. Again, with this species of animal, the specific precipitates obtained were stable and insoluble in the region of extreme antigen excess.

7. Studies of the Quantitative Precipitin Reaction Employing Boivin SSS Antigen. The quantitative precipitin technique affords an accurate method for comparing the relative combining powers of 2 antigens for the same antibody. Therefore the method was used to compare the Boivin SSS-38 antigen with the polysaccharides extracted by the phenol method of Palmer and Gerlough (22). Serums, prepared against the SCHU and 38 strains, were selected which had previously been quantitated for antibody nitrogen with the phenol extracted SSS; the courses of their respective precipitin curves and the various antigen-antibody relationships were already known.

(a) Serum G₁₀LV_{Schu}-50-D. Previous titrations with the phenol extracted polysaccharides had revealed an antibody content equivalent to 0.9114 mg Ab N/ml for this serum. Two titrations on different units of antibody with Boivin SSS are summarized in tables 38 and 39. A study of the various antigen-antibody relations in table 38 shows that all determinations were made in distinct antibody excess. Calculation of the optimal combining ratio gave a value of 0.157 which is considerably lower than any ratio heretofore noted. The ratio occurred at an antigen concentration of 180.5 μ g SSS and addi-

Table 38

A Study of Antigen-Antibody Relations Using Boivin SSS-38

and Anti-SCHU Goat Serum

G₁₀LV_{Schu}-50-D

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg		mg/ml		
Unit of Antibody = 1.0 ml of 1:20 dilution						
10	4.58	3.04	0.458	0.0916	++	0
20	7.01	5.91	0.351	0.1402	++	0
30	10.27	8.62	0.342	0.2054	++	0
40	11.69	11.14	0.292	0.2338	++	0
50	14.36	13.45	0.287	0.2872	+	0
60	16.30	15.62	0.271	0.3260	+	0
70	18.60	17.61	0.265	0.3720	+	0
80	19.70	19.43	0.246	0.3940	+	0
90	20.54	21.08	0.228	0.4108	+	0
100	20.95	22.64	0.210	0.4190	+	0
120	24.49	24.99	0.204	0.4898	+	0
140	27.54	26.83	0.196	0.5508	+	0
160	29.00	27.76	0.181	0.5800	+	0
180	32.33	28.10	0.179	0.6466	+	0
200	34.27	27.76	0.121	0.6854	+	±

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 0.3132 S - .0008676 S^2$$

$$S \text{ max.} = 180.50 \mu\text{g} \quad \text{Ab max.} = 28.27 \mu\text{g}$$

$$R = 0.157$$

$$\text{Antibody Content} = 0.5654 \text{ mg Ab N/ml}$$

Table 39

Quantitative Precipitin Reaction with Boivin SSS-38 Antigen

and 0.5 ml Anti-SCHU Goat Serum

G₁₀LV_SSchu-50-D (Diluted 1:15)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess;	
	Found	Calc.*			Ab	An
µg	µg	µg		mg/ml		
20	6.66	4.97	0.333	0.1998	+	0
40	11.31	9.45	0.283	0.3393	+	0
60	14.92	13.43	0.249	0.4476	+	0
80	17.55	16.92	0.219	0.5265	+	0
100	19.49	19.90	0.195	0.5847	+	0
120	21.85	22.40	0.182	0.6555	+	0
140	24.28	24.40	0.173	0.7284	+	0
160	25.53	25.90	0.160	0.7659	+	0
180	27.26	26.91	0.151	0.8178	+	0
200	27.96	27.42	0.140	0.8388	+	0

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 0.2610 S - .0006196 S^2$$

$$S \text{ max.} = 210.62 \mu\text{g} \quad Ab \text{ max.} = 27.49 \mu\text{g}$$

$$R = 0.131$$

$$\text{Antibody Content} = 0.8247 \text{ mg Ab N/ml}$$

tion of the next increment of antigen caused it to appear in excess in the supernatant. Agreement between experimental and calculated values for antibody nitrogen was good, the disparities actually less than similar values calculated for the PG antigen.

Since the combining ratios of antibody N:SSS were consistently low, and since all reactions were made in antibody excess, the titration was repeated on a smaller unit of antibody, 0.5 ml of a 1:15 serum dilution. Antigen likewise was increased in 20 μ g increments and the results are included in table 39. The titration is representative of characteristic precipitin curves in that each additional antigen increment precipitated more antibody when reactions occurred in antibody excess. Calculation of the optimal combining ratio gave a value of 0.131 which is quite low but in good agreement with the previous ratio shown in table 38. Interpolation showed that the maximal amount of antibody nitrogen would be precipitated after addition of 210.60 μ g of antigen. Since the largest amount of antigen used in this titration was only 200 μ g it is obvious that all reactions occurred in antibody excess, as confirmed by tests on the supernatants. Calculation of the antibody content revealed a concentration of 0.8247 mg Ab N/ml. This is only 0.0867 mg of antibody nitrogen lower than the estimate made previously with the PG antigen, and the difference is probably of no significance since such large

dilution factors were involved. Therefore the Boivin antigen also may be used to quantitate accurately an antiserum. However it takes more of this antigen to exhaust the antibody from an equivalent unit of serum.

(b) Serum G₁₀LV_{Schu}-50-B. In order to study the reaction of a lower titered serum with the Boivin antigen the 1950 anti-SCHU serum, obtained from a second bleeding, was quantitated. Previous determinations with the PG antigen showed this serum to contain 0.4056 mg Ab N/ml. The Boivin SSS was added in ascending 20 μ g increments to a constant unit of antibody consisting of 1.0 ml of a 1:20 serum dilution. Results of the test are given in table 40 and show that the amount of precipitable antibody nitrogen increased with addition of each ascending antigen increment. The calculated optimal ratio was again quite low, yielding a value of only 0.114 but in good agreement with the 2 previous estimates. Interpolation showed that the maximal amount of antibody nitrogen would be precipitated after the addition of 212 μ g antigen. However the largest antigen increment added was 200 μ g so all mixtures were still in antibody excess. The antibody content of the serum was equivalent to 0.4814 mg Ab N/ml which approximates the titer determined with the PG antigen. Agreement between observed and calculated values for antibody nitrogen was excellent throughout the entire range, the greatest disparity being less than 1 μ g nitrogen. Therefore the course of the reaction curve with the

Table 40

Quantitative Precipitin Reaction with Boivin SSS-38 Antigen

and 1.0 ml Anti-SCHU Goat Serum

$G_{10}LV_{Schu-50-B}$ (Diluted 1:20)

SSS-38 Added	Antibody N Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
μg	μg	μg		mg/ml		
20	5.13	4.33	0.257	0.1026	+	0
40	8.92	8.23	0.223	0.1784	+	0
60	12.41	11.70	0.207	0.2482	+	0
80	14.77	14.74	0.185	0.2954	+	0
100	17.55	17.35	0.176	0.3510	+	0
120	18.94	19.54	0.158	0.3788	+	0
140	21.51	21.29	0.154	0.4302	$\pm$	0
160	22.20	22.85	0.139	0.4440	$\pm$	0
180	23.95	23.52	0.133	0.4790	$\pm$	0
200	24.56	23.99	0.123	0.4912	$\pm$	0

* Values calculated according to equation:

$$\mu g \text{ Antibody N Pptd.} = 0.2271 S - .0005357 S^2$$

$$S \text{ max.} = 211.97 \mu g \quad Ab \text{ max.} = 24.07 \mu g$$

$$R = 0.114$$

$$\text{Antibody Content} = 0.4814 \text{ mg Ab N/ml}$$

Boivin antigen can be described quite well by a theoretical equation derived from experimental values included in the table.

(c) Serum G₁₃LA₃₈-49-A. The course of the precipitin reaction for an anti-38 goat serum with the homologous Boivin antigen was investigated. Differences in combining ratios of the PG antigens for anti-38 and anti-SCHU goat serum have been demonstrated. This study was made to see if the higher combining ratio of the anti-38 serum was a consequence of the antigenic fraction employed or perhaps the result of some other or unknown factor. Previous determinations for antibody content with the PG antigens showed this serum to contain 0.3500 mg Ab N/ml. A similar quantitative precipitin reaction with the Boivin SSS-38 was made on a unit of 1.0 ml of a 1:10 serum dilution, results of which are shown in table 41. This afforded a study of the reaction curve throughout the antibody excess zone and into the equivalence and antigen excess zones. The amount of antibody nitrogen precipitated with each additional antigen increment increased until the optimal combining ratio was achieved. This occurred after addition of 116.31 µg antigen and gave a value of 0.284 for the "ideal ratio". This is 2 times larger than the corresponding ratio obtained for an anti-SCHU serum with the same antigen. Likewise the ratio was 2 to 3 times larger for the anti-38 goat serum than for the anti-SCHU goat serum when the PG antigens were employed. The calcu-

Table 41

Quantitative Precipitin Reaction with Boivin SSS-38 Antigen
and 1.0 ml Anti-38 Goat Serum
G₁₃LA₃₈-49-A (Diluted 1:10)

SSS-38 Added	Antibody Precipitated		Ratio Antibody N to SSS	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
	Found	Calc.*			Ab	An
µg	µg	µg		mg/ml		
20	11.66	10.35	0.583	0.1166	++	0
40	19.06	18.66	0.479	0.1905	++	0
60	24.42	24.93	0.407	0.2442	++	0
80	28.40	29.17	0.355	0.2840	+	0
100	30.76	31.37	0.308	0.3076	±	0
120	32.83	31.54	0.274	0.3283	0	0
140	31.91	29.67	0.228	0.3191	0	±
160	34.30		0.214	0.3420	0	±
180	34.34		0.191	0.3434	0	±
200	34.41		0.172	0.3441	0	±

* Values calculated according to equation:

$$\mu\text{g Antibody N Pptd.} = 0.5682 S - .002545 S^2$$

$$S \text{ max.} = 116.31 \mu\text{g} \quad \text{Ab max.} = 34.20 \mu\text{g}$$

$$R = 0.284$$

$$\text{Antibody Content} = 0.3420 \text{ mg Ab N/ml}$$

lated ratio occurred in the middle of the equivalence zone as evidenced by tests on supernatants. The antibody content was 0.3420 mg Ab N/ml of undiluted serum which is precisely the value previously obtained by determinations with the PG antigen. Agreement between experimental and calculated values for antibody nitrogen was excellent and the course of the reaction curve was adequately described by the theoretical equation. Hence it is shown that an anti-38 serum can be quantitated with the Boivin antigen, and that determinations for antibody content were in good agreement with results obtained by use of the PG antigens. However it took more of the Boivin antigen to exhaust all antibody from an equivalent unit of serum.

8. Quantitative Precipitin Reaction Studies Employing Sonic Lysate Antigen (Son-SCHU). A serum with the antibody content previously determined with polysaccharide antigen was used as a standard antiserum in analyses employing the crude sonic extract as a quantitating antigen. The serum selected was GLV_{Schu}-49-A. This serum contained 0.6798 mg Ab N/ml when titrated with the nitrogen-free polysaccharides. The type of data obtained with the predominantly protein antigen and antitularense goat serum is set forth in tables 42 and 43, respectively, for 1.0 ml units of a 1:15 and 1:10 dilution of serum. Increasing amounts of antigen in terms of nitrogen were added in 1.0 ml volumes to a series of tubes containing 1.0 ml of the respective serum dilutions. Constituents were

Table 42

Studies Using Sonic Antigen in Quantitative Precipitin Reaction

Addition of Increasing Amounts of SON-SCHU to 1.0 ml Anti-SCHU Serum

GLV_{Schu}-49-A (Diluted 1:15)

Antigen Nitrogen Added	Total Nitrogen Pptd.	Antibody N by Difference		Ratio Antibody N to Antigen N	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
		Found	Calc.*			Ab	An
mg	mg	mg	mg		mg/ml		
0.01	0.0840	0.074	0.078	7.4	1.110	++	±
0.02	0.1113	0.091	0.104	4.6	1.365	++	+
0.03	0.1231	0.093	0.108	3.1	1.395	+	+
0.04	0.1385	0.099	0.079	2.5	1.485	+	+
0.05	0.1482	0.098		2.0	1.470	+	+
0.06	0.1552	0.095		1.6	1.425	+	+
0.08	0.1743	0.094		1.2	1.410	±	++
0.10	0.1770					±	++
0.20	0.1931					0	++
0.40	0.1861					0	++
0.50	0.1631					0	++
0.75	0.1431					0	++
1.00	0.1325					0	++

* Values calculated according to equation:

$$\text{mg Antibody N Pptd.} = 8.45 \text{ SON N} - 162 (\text{SON N})^2$$

$$\text{SON N max.} = 0.026 \text{ mg} \quad \text{Ab max.} = 0.110 \text{ mg}$$

$$R = 4.2$$

$$\text{Antibody Content} = 1.645 \text{ mg Ab N/ml}$$

Table 43

Studies Using Sonic Antigen In Quantitative Precipitin Reaction

Addition of Increasing Amounts of SON-SCHU to 1.0 ml Anti-SCHU Serum

GLV_{Schu}-49-A (Diluted 1:10)

Antigen Nitrogen Added	Total Nitrogen Pptd.	Antibody N by Difference		Ratio Antibody N to Antigen N	Antibody N in Undiluted Serum	Supernatant Shows Excess:	
		Found	Calc.*			Ab	An
mg	mg	mg	mg		mg/ml		
0.0005	0.0085	0.008	0.056	16.0	0.080	++	±
0.005	0.0817	0.076	0.093	15.2	0.760	++	±
0.01	0.1077	0.098	0.148	9.8	0.980	++	±
0.02	0.1545	0.135	0.164	6.8	1.350	++	±
0.03	0.1822	0.152	0.142	5.1	1.520	+	+
0.04	0.1984	0.158		4.0	1.580	+	+
0.05	0.2168	0.167		3.3	1.670	+	+
0.06	0.2290	0.169		2.8	1.690	+	+
0.08	0.2495	0.170		2.1	1.700	+	+
0.10	0.2701	0.170		1.7	1.700	±	++
0.20	0.3124	0.112			1.120	±	++
0.40	0.3101					±	++
0.50	0.3079					±	++
1.00	0.2601					0	++

* Values calculated according to equation:

$$\text{mg Antibody N Pptd.} = 11.2 \text{ SON N} - 191 (\text{SON N})^2$$

$$\text{SON N max.} = 0.020 \text{ mg} \quad \text{Ab max.} = 0.164 \text{ mg}$$

$$R = 4.2$$

$$\text{Antibody Content} = 1.640 \text{ mg Ab N/ml}$$

mixed and treated as for the other precipitin tests. The first 2 columns in each table show the amount of antigen nitrogen added and the total nitrogen (antigen + antibody) precipitated. When the supernatant showed no antigen, but only antibody, all the antigen nitrogen was assumed to be in the precipitate. The antibody nitrogen was calculated by subtracting antigen nitrogen from the total nitrogen precipitated. Ratios of antibody nitrogen:antigen nitrogen are illustrated. Inspection of values for antibody nitrogen precipitated with successive increases in antigen nitrogen added demonstrated increased solubility of the precipitate when passing into the realm of antigen excess, a feature not previously observed with either of the polysaccharide antigens. The SSS precipitates were demonstrated to be a stable, insoluble complex of maximal precipitable antibody nitrogen in the region of antigen excess. The amount of precipitable antibody nitrogen progressively increased throughout the zone of extreme antibody excess as antigen nitrogen was increased and until the optimal combining ratio of antibody nitrogen:antigen nitrogen was achieved. Calculation of the optimal combining ratios gave values of 4.2 and 5.4, respectively, for a 1:15 serum dilution in table 42 and a 1:10 dilution in table 43. Both of these ratios occurred on the antibody excess side of the precipitin curve. These ratios are considerably larger than values obtained with the polysaccharide preparations. Calculations for antibody

content from the values with the sonic antigen in each titration showed good agreement. The titrations revealed an antibody content of 1.645 and 1.640 mg Ab N/ml of undiluted serum, respectively, when calculated from a 1:15 and a 1:10 serum dilution. This is approximately 2.5 times the antibody content as determined by the PG polysaccharide. The "ideal" combining ratio of this protein reactant system was about 5 times higher than similar ratios with the PG antigen and about 40 times larger than corresponding ratios with the Boivin antigen.

B. Rat Protection Studies Employing Antitularensis Goat Serums

A study of antitularensis goat serums prepared by intravenous inoculations of living strains of maximal virulence (SCHU) and of total avirulence (38) was made for their respective protective powers for the challenged white rat after both serums were adjusted to contain precisely identical precipitable antibody contents. This work is in part a continuation and in part an outgrowth of a previous report by Foshay et al. (11) from which it was not possible to judge whether their observed differences in protective power between antivirulent and anti-avirulent strain serums were attributed to their differing precipitable antibody contents, as seemed likely, or to the presence of an antibody component which may have been engendered by only one of the antigens.

At the time the rat protection experiments were performed the precipitable antibody contents, in terms of $\mu\text{g/ml}$ of specific polysaccharide necessary to exhaust antibody without a resultant antigen excess, were:

<u>Serum</u>	<u>Ab U/ml</u>
Anti-SCHU No. 1	700
Anti-SCHU No. 1-b	700
Anti-38 No. 7	112
Anti-38 No. 7-b	40
Anti-38 No. 4	140

Therefore, to have paired serum samples of equivalent antibody content, serum No. 1 was diluted 1:6.25 to make it equivalent to serum No. 7, and diluted 1:5 to equal serum No. 4. In addi-

tion serum No. 4 was diluted 1:1.25 to equal serum No. 7, and to furnish a comparative test between 2 anti-38 serums. The antiserums were also selected with reference to continuous storage frozen in dry ice or bulk storage at 4 C.

1. Quantitation of Serum Antibody. The antibody content of each antiserum was determined by the neutralization method of Culbertson (19), and the antibody titer was calculated in terms of the Marrack-Smith unit (35). In addition, each serum also was quantitated by the Martin serum dilution method (12) for comparative determinations. The antigens employed in both methods were the polysaccharides from strains SCHU and 38 prepared by the Palmer and Gerlough method (22). There follows a protocol of antibody titrations of anti-38 serum No. 4 by means of the Culbertson and Martin methods.

(a) Culbertson Method. Each stock solution of SSS from SCHU and 38, containing 100 $\mu\text{g/ml}$ was diluted 1:10 with saline, and of each 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 ml amounts were placed in 2 series of 6 tubes each. Serum No. 4 (anti-38) was diluted 1:5 with sterile saline and 0.5 ml added to each tube, then sufficient saline was added to make all volumes 2.5 ml. The tubes were incubated at 40 C for 3 hours, then at 4 C for 48 hours. Thereafter the tubes were centrifuged and supernatants from each reactant mixture were divided so that 0.5 ml was placed into each of 2 new series of tubes. To one series was added 2 μg SSS-SCHU in 0.5 ml for the detection of

excess antibody. To the other series was added 0.5 ml of a 1:5 dilution of serum L-44-F containing 200 Ab U/ml. The final concentrations of test materials were 1 μ g of SSS and 20 units of antibody per ml of mixture. These tubes were incubated at 40 C for 3 hours, then were refrigerated at 4 C for 48 hours, and then examined for evidence of any precipitate, and the readings recorded. Results were expressed as follows:

0.5 ml SSS-SCHU Supernatants Plus:			0.5 ml SSS-38 Supernatants Plus:		
<u>An</u>	<u>μg</u>	<u>Ab</u>	<u>An</u>	<u>μg</u>	<u>Ab</u>
+	10	0	+	10	0
+	12	0	+	12	0
<u>0</u>	<u>14</u>	<u>0</u>	<u>0</u>	<u>14</u>	<u>0</u>
0	16	+	0	16	+
0	18	+	0	18	+
0	20	+	0	20	+

The supernatants from original tubes containing 0.5 ml of 1:5 diluted No. 4 serum and 14 μ g of either SSS showed excesses of neither antigen nor antibody. Therefore within the limits of this method it required 140 μ g of either SSS to "neutralize" 1 ml of serum No. 4. Hence the serum contained 140 Ab U/ml and no differences in the combining powers of the 2 SSS preparations were noted for this anti-38 serum since all antibody was exhausted by the same quantity of SSS.

(b) Martin Method. Since the antibody content of serum No. 4 had been determined as 140 Ab U/ml by the Culbertson

method, it was decided to test the Martin procedure for agreement by diluting the serum initially in such manner that a 1:140 dilution would appear in the series after equal volume mixtures were made with the antigen. This is illustrated as follows:

Direct Precipitin Tests

No. 4 Serum Dilutions	Antigen in $\mu\text{g/ml}$								
	16	8	4	2	1	0.5	0.25	0.125	0.0625
8.75	+	+	+	+	+	+	+	-	-
17.5	+	+	+	+	+	+	+	-	-
35	+	+	+	+	+	+	+	-	-
70	+	+	+	+	+	+	+	-	-
140	+	+	+	+	-	-	-	-	-
280	-	-	-	-	-	-	-	-	-
560	-	-	-	-	-	-	-	-	-

+ = Precipitation.

- = No precipitation.

+ = Faintest detectable precipitation.

The reactant mixtures enclosed within the dotted lines were selected for testing the supernatants for excess of antigen and antibody.

Two such serum dilution series were set up. To one was added SSS-SCHU dilutions as shown; SSS-38 in 2-fold dilution was added to the other series.

The table represents the results obtained after 2

hours at room temperature and 48 hours at 4 C. The selected supernatants were tested, as previously, using 5 µg per tube of SSS. Since the results were identical using SSS-SCHU and SSS-38 either antigen is illustrated by the data in the previous chart.

The results of the supernatant tests are shown by the accompanying diagram to follow:

Tests on Supernatants

No. 4 Serum Dilutions	Antigen in µg/ml							
	16	8	4	2	1	0.5	0.25	0.125
8.75	<u>A-Ab</u>	A-Ab	A-Ab	Ab	Ab	Ab	Ab	Ab
17.5	A	A	<u>A-Ab</u>	Ab	Ab	Ab	Ab	Ab
35	A	A	-	Ab	Ab	Ab	Ab	Ab
70	A	A	A	A	A-Ab	<u>A-Ab</u>	Ab	-
140	A	A	A	-	-	-	-	-

A = Antigen excess; if underlined in relative excess.
 Ab = Antibody excess; if underlined in relative excess.
 - = Neutral supernatants; neither antigen nor antibody detectable.

The approximate zone of equivalence is indicated by the staircase underlined. Of the 5 mixtures within this zone, two were neutral, two showed slight antigen excess, and one, containing the most antibody showed traces of both antigen and antibody, but antigen in slight excess. This result indicated an antibody content of 140 Ab U/ml, in agreement with the results on the same serum by the Culbertson method.

Repeated titrations of anti-SCHU serum No. 1 by both methods revealed a content of 700 Ab U/ml, exactly 5 times the antibody content of serum No. 4. Since it was desired to know if dilution of a serum of higher antibody content to theoretical equivalence with one of lower content would actually give that value in antibody units upon measurement, serum No. 1 was diluted initially 1:5 with saline and then direct precipitin test series, one for each SSS, were set up as before with this diluted serum, in such manner that 1 μ g of each antigen would react with the 1:140 dilution of serum. Results for this test are demonstrated as follows:

Direct Precipitin Tests

No. 1 Serum (1:5) Dilutions	Antigen in μ g/ml									
	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125
8.75	(+)	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>
17.5	+	+	+	+	+	+	<u>+</u>	<u>+</u>	<u>+</u>	-
35	+	+	+	+	+	+	<u>+</u>	-	-	-
70	+	+	+	+	+	+	<u>+</u>	-	-	-
140	-	-	<u>+</u>	<u>+</u>	-	-	-	-	-	-
280	-	-	-	-	-	-	-	-	-	-
560	-	-	-	-	-	-	-	-	-	-

() = Optimal ratio by fastest reacting mixture.

+ = Trace of precipitation.

The procedures used were exactly as those described for serum No. 4. In this case the most rapid visible pre-

cipitation occurred in the tubes containing a 1:8.75 dilution of serum and 16 μ g of either SSS, indicating an optimal ratio of 1:140, in agreement with the undiluted No. 4 serum. The direct tests indicated an antibody content of 140 Ab U/ml, confirmed by examination of appropriate supernatants indicated as follows:

Tests on Supernatants

No. 4 Serum (1:5) Dilutions	Antigen in μ g/ml						
	16	8	4	2	1	0.5	0.25
1:							
8.75	(A-Ab)	Ab	Ab	Ab	Ab	Ab	Ab
17.5	A	A-Ab	Ab	Ab	Ab	Ab	Ab
35	A	A	-	Ab	Ab	Ab	Ab
70	A	A	A	-	A-Ab	Ab	Ab
140	A	A	A	A	-	-	-

Hence it appeared that the antibody content of anti-tularensis goat serums could be quantitated by either the neutralization method of Culbertson or the serum dilution method of Martin; that these methods gave essentially identical results when applied simultaneously or at different times to the same serum. There was also good agreement with the method based upon the fastest reacting mixture whenever it could be detected.

Since this study also was made for any evidence of a possible labile antibody, both methods were used to titrate the antibody in all samples of fresh bulk serums, of serum stored

in bulk at 4 C for 1 year, and in samples of every lot which had been held either lyoprocessed or continuously frozen in the deep freeze for 1 year. Results of final determinations follow:

Serum Designations	Antibody Content in Ab U/ml	
	Frozen in dry ice	Storage 4 C for 1 year
Anti-SCHU No. 1	700	700
Anti-SCHU No. 1-b	700	700
Anti-38 No. 4	140	140
Anti-38 No. 7	112	112
Anti-38 No. 7-b	40	45
L-44-F		200

Examination shows that serums No. 1 and No. 1-b had identical titers when freshly removed from the clot, and that these titers were unchanged after either continuous freezing or bulk storage for 12 months. The same was true of the lower titering anti-38 serums No. 4 and No. 7. However the 25-day interval between the 2 bleedings permitted the anti-38 serum of goat No. 7 to fall to almost one-third of its initial titer. This result might have been anticipated since the avirulent strain 38 does not produce active infection. The higher titer of bulk stored serum No. 7-b was probably caused by antibody concentration due to evaporation since the flask containing this serum was not covered with any seal.

It was observed that the Martin method did not behave with these tularensis antigen-antibody systems as reported for pneumococcus systems in the original paper (17),

notably in that the end points of serial antigen dilution titrations were not the least quantity of antigen which will give a visible precipitate with the highest antibody dilution, but a different quantity of antigen for each serum dilution used. In fact, with these polysaccharides and hyperimmune goat serums, the critical limiting factor for precipitation was actually the optimal antigen-antibody combining ratio, as measured by either the Culbertson neutralization technique or by the method of determining the fastest reacting mixture. A further dilution of either antigen or antibody beyond this optimal ratio caused failure of visible precipitation. This unusual behavior has been verified repeatedly with several immune goat serums but the phenomenon in no way invalidates the Martin procedure as a measure of antibody content.

2. Quantitation of Protective Antibody. The cumulative results of several serum protection experiments are collected in table 44. Data in this table show that antiserums of equivalent antibody content as measured by Ab U/ml protected white rats equally well regardless of the virulence of the antigen used to make them. All challenge doses were identical throughout. A comparison of the survival rates in the comparable group 1A, 1B, 3A shows a variation only between 57 and 75 per cent, and that the paired survival rates of tests performed simultaneously varied among themselves only from 0 to 10 per cent. These figures represent relatively good agreement

Table 44

Cumulative Record of Rat Protection Tests

2 ml I-P of each respective serum. Simultaneous challenge with 25,000 LD₅₀ rat doses (minimum of 25 million virulent, viable cells).

Group	Goat Serums Kind	Dilutions	Storage	No. of Rats	Mortality Ratios	Survival per cent	Ab Content Ab U/ml
1A	Normal-4	1:6.25	Bulk	20	13/20	35	0
	1 (Schu)	1:6.25	"	21	9/20	57	112
	7 (38)	0	"	21	7/21	67	112
1B	1 (Schu)	1:6.25	Bulk	20	5/20	75	112
	7 (38)	0	"	20	5/20	75	112
2A	Normal-6	1:5	Bulk	20	16/20	20	0
	1 (Schu)	1:5	Frozen	20	6/20	70	140
	4 (38)	0	"	20	5/20	75	140
2B	Normal-6	1:5	Bulk	15	14/15	7	0
	1 (Schu)	1:5	Frozen	15	9/15	40	140
	4 (38)	0	"	15	5/15	67	140
3A	Normal-4	1:6.25	Bulk	16	9/16	44	0
	1 (Schu)	1:6.25	Frozen	16	5/16	69	112
	7 (38)	0	"	16	6/16	63	112
4A	Normal-6	1:1.25	Bulk	15	15/15	0	0
	4 (38)	1:1.25	Frozen	16	11/16	31	112
	7 (38)	0	"	15	6/15	60	112

for use of the white rat as the test animal. A comparison of groups 1A and 1B with group 3A shows no significant difference in mortality (or survival) with respect to the freshness or age of the antiserums. Groups 2A and 2B show a somewhat greater difference in mortality between serums No. 1 and No. 4 than the first mentioned groups show between serums No. 1 and No. 7. The variation occurred in rats pretreated with diluted serum No. 1 (66 per cent as against 51 per cent survival). The survival rates for the No. 7 and No. 4 serum groups are identical, at 71 per cent. However a direct comparison of serums No. 7 and No. 4 in group 4A showed an almost 2-fold difference in survival when approximately 15 rats were tested with each serum.

Groups 2B and 4A show the greatest disparity between mortality ratios or survival rates.

Groups 1A and 3A indicate an unusually high apparent protective capacity of a normal serum, taken from goat No. 4 before it was inoculated with the 38 strain. Old animal records show no previous use of this animal but recollections of Dr. Foshay and associates who were engaged in earlier animal work are that this animal did receive some intravenous inoculations of several chemically isolated fractions of Bacterium tularensis which provoked no antibody formation that could be detected by precipitin or agglutination tests. It is the belief that some "nonantigenic" inoculations were not entered into the permanent records of the animals. The same

phenomenon was previously observed with 2 other series of challenged rats for which this "normal" No. 4 serum was used as a control.

Summarized data for all rat protection tests are set forth in table 45.

Table 45

Summary of Results on Rat Protection Tests

Serum Used	Dilution	Mortality Ratios	Survival per cent	Antibody Content Ab U/ml
No. 1 (Anti-SCHU)	1:6.25	19/57	67	112
No. 7 (Anti-38)	Undiluted	24/72	67	112
No. 1 (Anti-SCHU)	1:5	15/35	57	140
No. 4 (Anti-38)	Undiluted	10/35	71	140
No. 4 (Anti-38)	1:1.25	11/16	31	112
No. 6 (Normal)	1:1.25	15/15	0	0
No. 6 (Normal)	1:5	30/35	14	0
No. 4 (Normal)	1:6.25	22/36	39	0

DISCUSSION

A resume of the salient characteristics of the various tularenses precipitin systems is presented in table 46. This study has permitted a comparison of the relative combining powers of various polysaccharides derived from an avirulent (38) strain and a typical virulent (SCHU) strain of B. tularensis. This was followed by an investigation of the quantitative precipitin reaction curves of virulent and avirulent strain antiserums. Each quantitative precipitin reaction was compared with a corresponding theoretical curve derived from an equation to express relationships in the antibody excess realm:

$$\mu\text{g N}_2 \text{ Pptd.} = 2 RS - \frac{R^2}{A} S^2 \quad (29)$$

It is evident that, in general, the various tularenses precipitin systems proceed in the same manner as those studied by Heidelberger et al. The calculated values obtained from it are everywhere in good or close agreement with those determined experimentally throughout the region of antibody excess. Hence it may be assumed that the characteristics of these tularenses systems are quite analogous to those previously defined for crystalline egg albumin, pneumococcus SSS, and others. Thus, by plotting the experimental combining ratios of antibody nitrogen:antigen against micrograms of SSS precipitated, it is possible in each case to derive equations

Table 46

SUMMARY OF PRECIPITIN STUDIES ON TULARENSE SYSTEMS

Determination of Antibody Content and Serological Comparison

of Equivalence Zone Constants

Serum		Ml Used	Antigen Used		Antibody Content		Equivalence Values		
Designation	Dil'n.		PG	SSS	mg Ab	N/ml	S(µg)	R	A(µg)
A. Comparative Analyses of Anti-SCHU Goat Serums with PG Antigens.									
GLV Schu-43	1:15	0.5	SCHU		1.1025		50.00	0.735	36.75
"	"	"	38		1.1793		47.74	0.824	39.31
"	1:20	"	SCHU		1.2608		61.60	0.512	31.52
"	"	"	38		1.2148		60.85	0.499	30.37
GLV Schu-49-A	1:10	0.5	SCHU		0.7272		59.56	0.611	36.36
"	"	"	38		0.6964		47.20	0.737	34.82
"	1:15	"	SCHU		0.7416		39.92	0.619	24.72
"	"	"	38		0.7794		51.75	0.502	25.98
GLV Schu-49-B	1:10	0.5	SCHU		0.5964		46.93	0.633	29.82
"	"	"	38		0.5638		46.04	0.610	28.20

B. Comparative Analyses of Anti-38 Goat Serums with PG Antigens.

G ₁₃ LA ₃₈ -49-A	1:5	0.5	38		0.3555	16.73	2.120	35.55
"	"	"	SCHU		0.3180	14.95	2.125	31.80
"	1:3	1.0	38		0.3516	56.30	2.084	117.21
"	"	"	SCHU		0.3005	39.97	2.506	100.16
"	1:7.5	0.5	38		0.3950	15.91	1.655	26.33
G ₁₃ LA ₃₈ -49-B	1:5	0.5	38		0.3111	16.59	1.875	31.11
G ₇ LA-46-A	1:2.5	1.0	38		0.0565	10.59	1.120	22.59
G ₄ LA-46-A	1:2.5	1.0	38		0.0744	25.27	1.178	29.75
G ₇ LA-50-E	1:5	1.0	SCHU		0.2978	38.44	1.550	59.55

C. Comparative Values on Goat Serums One Year after Immunization.

G ₁₄ LV Schu-49-C _{50a}	1:5	1.0	SCHU		0.1716	39.68	0.865	34.32
"	1:3	"	"		0.1504	75.63	0.666	50.12
G ₁₃ LA ₃₈ -49-C _{50a}	1:2	1.0	SCHU		0.0225	Not calculated.		An XS.

Table 46 (Continued)

SUMMARY OF PRECIPITIN STUDIES ON TULARENSE SYSTEMS

Determination of Antibody Content and Serological Comparison
of Equivalence Zone Constants

Serum		Ml Used	Antigen	Antibody	Equivalence Values		
Designation	Dil'n.		PG SSS	Content mg Ab N/ml	S(μg)	R	A(μg)
D. Changes in Values on Goat Serums during Immunization.							
G ₁₀ LV _{Schu} -50:	A 1:5	2.0	SCHU	0.0160	20.55	1.560	32.08
"	B 1:5	0.5	"	0.4056	45.72	0.887	40.56
"	C 1:15	1.0	"	0.5588	43.22	0.860	37.16
"	C 1:10	0.5	"	0.5588	36.05	0.775	27.94
"	D 1:10	0.5	"	0.9486	56.80	0.835	47.43
G ₁₇ LV _{Chur} -50:	A 1:2	1.0	SCHU	0.0486	17.53	1.385	24.28
"	B 1:5	0.5	"	0.4129	44.88	0.920	41.29
"	C 1:10	0.5	"	0.6740	35.66	0.945	33.70
"	D 1:10	0.5	"	0.6744	34.81	0.940	32.72
G ₁₉ LV _{Jap} -50:	C 1:10	1.0	SCHU	0.4483	51.35	0.874	44.83
"	D 1:10	0.5	"	0.5750	39.42	0.749	28.75

E. Results of Values on Rabbit Serums with PG Antigen.

RKV _{Schu} -48	1:3	1.0	SCHU		0.0705	44.70	0.523	23.51
RLA ₃₈ -48	1:2.5	1.0	"		0.0395	35.87	0.413	15.79
"	1:3	"	"		0.0341	22.73	0.500	11.36
R ₁ KV _{Schu} -50-E	1:10	1.0	"		0.2403	61.56	0.376	24.03
R ₁₁ KA ₃₈ -50-E	1:5	1.0	"		0.1101	41.15	0.535	22.02

F. Results of Values on Horse Serum with PG Antigens.

HLV _{Schu} -44	1:4	1.0	SCHU		0.2342	36.48	1.600	58.55
"	1:6	1.0	38		0.2668	46.63	0.950	46.63

Table 46 (Continued)

SUMMARY OF PRECIPITIN STUDIES ON TULARENSE SYSTEMS

Determination of Antibody Content and Serological Comparison of Equivalence Zone Constants

Serum		Antigen	Antibody	Equivalence Values			
Designation	Dil'n.	Used	Used	Content	S(μ g)	R	A(μ g)
		Used	SSS	mg Ab N/ml			
G. Comparative Analyses of Goat Antiserums with Boivin Antigen.							
G ₁₀ LV Schu-50-D	1:20	1.0	38	0.5654	180.50	0.157	28.27
	1:15	0.5	38	0.8247	210.62	0.131	27.49
G ₁₀ LV Schu-50-B	1:20	1.0	38	0.4814	211.97	0.114	24.07
G ₁₃ LA 38-49-A	1:10	1.0	38	0.3420	116.31	0.284	34.20

H. Studies of Values on Goat Serum with Sonic Lysate Antigen.

			<u>Son</u>	<u>mg Ab N/ml</u>	<u>mg An N</u>	<u>R</u>	<u>mg Ab N</u>
GLV Schu-49-A	1:15	1.0	SCHU	1.645	0.026	4.2	0.110
	1:10	1.0	SCHU	1.640	0.029	5.6	0.164

which describe the behavior of each reactant system.

The polysaccharides prepared by the Palmer and Gerlough method (22) were studied for their comparative binding properties for virulent and avirulent strain antiserums. The precipitin reaction curves for both SSS preparations obtained from simultaneous reaction mixtures with the same serum under precisely the same conditions were almost identical. A comparative analysis of anti-SCHU goat serums with the PG antigens revealed that differences between determinations for specific precipitate nitrogen were only slightly larger than the observed experimental error in duplicate analyses. Furthermore, the higher figure was given by SSS-SCHU in 1 duplicate series with serum GLV_{Schu}-43 and by SSS-38 in another series. The same phenomenon was observed with serum GLV_{Schu}-49-A. Therefore it is concluded that the combining powers of the PG SSS-38 and PG SSS-SCHU for the SCHU antibody were identical. The data in part A of table 46 indicate a variation in the antibody nitrogen:SSS ratios, given by the constant R, within the equivalence zone for the serums studied. These ratios ranged from 0.499 to 0.824 for the anti-SCHU goat serums. However the majority of the values for the ratio, R, are fairly constant and in good agreement. The average value for the optimal combining ratio of either SSS with the SCHU antibody was 0.630. The observed variation among the ratios is believed to be a consequence of the different dilution factors

employed. Slightly higher ratios were observed when smaller volumes of undiluted serums were studied. However the values did not vary sufficiently to make the average of 0.630 for the optimal combining ratio of each SSS for the SCHU antibody invalid. It is significant that the combining property of the anti-SCHU serum obtained from the second bleeding is not altered and that the value for R remained remarkably constant.

A distinct difference in the calculated equivalence values for R was noted when avirulent strain antiserums were studied for precipitin content by the same methods. The disparity is observed by examining part B of table 46. Here the SCHU and 38 polysaccharides had identical combining properties for any anti-38 goat serum. However the equivalence value for R is considerably higher than those observed with the anti-SCHU goat serums. The larger values were noted with both high titered and low titered anti-38 serums. The ratios with the low titered serums from goat Nos. 4 and 7 were smaller than the corresponding values for R with the higher titered anti-38 serum. Nevertheless all the R values were sufficiently larger than those for the anti-SCHU serums and, consequently, are considered to be of real significance. The larger ratios may be the result of a higher molecular weight antibody produced in goats by massive doses of strain 38. It is obvious that the animals used to prepare the respective anti-SCHU and anti-38 serums suffered different experiences in the process of

antibody formation; those which received the nonintoxicating strain 38 exhibited no loss of weight and, of course, were not infected. Tissue cells were not invaded and necrosis of cells did not occur. Therefore these animals could be given large inoculations of massive doses of living cells which could stimulate the formation of antibodies of higher molecular weight. Another possible interpretation is that the antibody in anti-38 serums has a greater valence for antigens and may contain more specific reactive groups for either SSS. It is not difficult to conceive the possibility of many more reactive groups becoming oriented owing to the injection of massive doses of antigen. Consequently the combining ratio of the anti-38 serum for either polysaccharide antigen could be greater. All the values for R are greater than 1.0 and most of them approach a ratio of 2.0. Averages of the values revealed an antibody nitrogen:SSS ratio of 1.80 for anti-38 goat serum. This is 3 times larger than the corresponding ratio for anti-SCHU serum, indicating that the anti-38 goat serum contained 3 times as many reactive units for either SSS or that the antibody was proportionately 3 times heavier in terms of molecular weight. It is realized that antiserums actually contain a complex mixture of antibodies differing possibly in both the number and kinds of groupings reactive with the antigen. Massive doses of strain 38 may possess the ability to form antibody capable of reacting with an increasing number of group-

ings on the antigen molecule. Also a different component may be produced consisting of protein molecules of higher molecular weight.

Analyses of values for calculated ratios on goat serum obtained 1 year after immunization showed no decrease in combining power for the antibody against the PG polysaccharide. The antibody titer of the serum from the SCHU goat, expressed as mg Ab N/ml of undiluted serum, was 4.5-fold lower than the serum obtained from a bleeding of the same goat 1 year previously. However the antibody does possess the same binding properties for antigen as that obtained following the acute stage of infection. In contrast the antibody titer of the anti-38 goat serum was 15- to 16-fold lower in antibody nitrogen/ml of undiluted serum than the one obtained following the inoculation schedule 1 year previously. In fact, this animal retained very little of its original antibody 1 year after immunization with the living 38 strain. Alexander (12) similarly showed a higher antibody content in serum from individuals recovered from tularemia than in serum from individuals who had been vaccinated.

A comparison of antibody nitrogen values for the serum bleedings from the JAP goat and those for the SCHU and CHUR goats, tabulated in part D of table 46, showed a slight correlation with homologous strain virulence. The hyperimmune serum prepared against the most virulent strain, SCHU, gave

higher antibody nitrogen values than either the CHUR or JAP strains. Likewise the anti-CHUR serum gave higher antibody nitrogen values for the corresponding bleeding than the anti-JAP serum. The evidence indicates that the CHUR antigen produced antibody more rapidly, and that it attained a maximal level in a shorter time than did the antibody produced by the SCHU strain. However the SCHU strain continued to stimulate antibody production for a longer time, and ultimately its antibody exceeded that of the CHUR strain. This may be due to individual variation among goats or it may be a characteristic feature of the individual strains of B. tularensis. A comparison of the respective combining ratios of antibody nitrogen:SSS showed them to be relatively constant for each strain of antiserum. However the ratio for determinations on the "A" bleedings are characteristically greater than the ratios for the later bleedings during the course of immunization. This is opposed to results of Heidelberger et al. (38) who presented data demonstrating that successive courses of immunization were accompanied by a rise in the initial combining ratio of antibody to antigen. On the other hand, he observed that the properties of antibodies engendered in another animal remained relatively constant under the same treatment for the same time. It is difficult to postulate experimentally founded reasons for such differing behavior. The elevated ratio is 1.5 and approximates the usual ratios consistently observed

for an anti-38 serum. It might, however, be assumed that a low grade or incomplete antibody was formed in the early stages of immunization in response to the virulent strains of the organism.

Studies on tularensen rabbit serums with the PG SSS-SCHU antigen showed an equivalence value of antibody nitrogen:SSS significantly lower than that found with either anti-SCHU or anti-38 goat serums. The lower ratio was observed with both high titered and low titered rabbit serums. It is apparent from part E of table 46 that the calculated ratios at the equivalence zone for virulent and avirulent strain antiserums are almost identical. The disparity noted in the combining ratios with the virulent and avirulent strain goat serums was not observed in the rabbit serums. It is also evident that the antibody content of the avirulent strain antiserum is precisely one-half that of a corresponding anti-SCHU serum prepared by inoculations of the same number of bacterial cells. This was a constant feature regardless of whether living or killed organisms were used to prepare the anti-38 serum. The lower combining ratios for the rabbit antiserums may be a function of the molecular weight of the antibody molecule. Therefore when antiserums are prepared by the inoculation of equal amounts of the virulent and avirulent strains, the same molecular weight antibodies are produced. However since living organisms were used in preparing goat antiserums, it

was possible to administer much larger doses of the avirulent 38 antigen. Consequently an antibody of higher molecular weight may have been produced. It may, however, be assumed that an antibody of equivalent molecular weight would be produced in the goat if equal numbers of organisms were used to immunize the animal.

Corresponding studies on results obtained with horse serum prepared against a virulent strain show an antibody nitrogen:SSS ratio approximating that of the anti-38 goat serum. A study of the comparative ratios in table 46 reveals a value for horse serum higher than similar ratios for rabbit serum and antivirulent goat serum, but almost identical with those for the antiavirulent goat serum. Kabat (39) showed that the molecular weight of pneumococcus antiscarbohydrate in the horse is probably 5 to 6 times that of the principal globulin component. This antibody was indicated to have a molecular weight of 990,000. Therefore it is postulated that the antibody produced in the goat against the 38 strain may fall in the class of high molecular weight antibodies. Similar classes of antibodies have also been demonstrated in the cow and pig (39). These heavy antibodies do not appear to be so firmly constituted as antibodies of lower molecular weight, and they may show evidence of breakdown into inhomogeneous materials. Therefore this may explain the marked decrease in antibody content of the anti-38 goat serums ob-

tained 1 year after immunization. The significance of these large antibodies is not clear, but it is evident that the response to immunization on the part of certain animals is more than the production of normal globulins modified only in configuration; a new type of globulin may in fact be formed.

From studies of the antipneumococcus rabbit serums, the mean value of 2R, the maximum calculated antibody nitrogen to SSS ratio, was 0.940. It is observed that this number is one-third the maximum calculated ratio for horse serums under corresponding conditions (about 3.2 in table 46). If these figures be multiplied by 6.3, the values 5.9 and 20.2 were obtained for 2R expressed as the antibody protein:SSS ratio. Dividing these members into 150,000 and 500,000, the tentative rough estimates of the molecular weights of anticarbohydrate antibody produced in the rabbit and in the horse (40), respectively, a value of 25,400 and 24,700 is obtained as the weight of the minimum chain lengths of each SSS reactive with an antibody molecule in each serum. These theoretical values for molecular weights of minimal reactive chain lengths are in excellent agreement. This may explain, in part, the low combining ratios repeatedly observed in all analyses made with either polysaccharide antigen. These low ratios may result from some contaminating material that is antigenically inert in vitro, but gives the high molecular weight ratio to the antigen. Alexander (12) postulated the presence of a con-

taminating substance in similar antigenic fractions but she did not calculate it on a molecular weight basis. In fact such weight values are wholly out of range for any pure polysaccharide known and the large values could only be accounted for by some inert material present in the antigen.

With the data now available it is possible to make rough calculations for the molecular weight of the antibody in virulent strain goat serums. Molecular weights of 150,000 and 500,000 for the rabbit and horse, respectively, could be assumed, and experimental ratios of antibody nitrogen:antigen were 0.49 and 1.6. The ratio for the anti-SCHU goat serum was 0.630. Since these ratios are proportional to the molecular weights of the antibody in the known animal serums, the weight of the antibody in the anti-SCHU goat serum was calculated to be 192,000 and 197,000, respectively, from equivalent values for the rabbit and horse. These estimates fall in good agreement and they probably represent good approximations of the true molecular weight of the antibody in the anti-SCHU goat serum.

The data in part G of table 46 afford a comparative study of the precipitin reaction when a different antigenic fraction is employed. Results are shown for the quantitative relationships between a Boivin fraction extracted from strain 38 and its homologous and heterologous goat antiserums. Inspection of the antibody contents of each of these serums shows that

the Boivin antigen precipitated precisely the same amount of antibody as did the PG antigens. The maximal value for antibody nitrogen on serum G₁₀LV_{Schu}-50-D was 0.9486 mg Ab N/ml with the PG antigen and 0.8247 mg Ab N/ml with the Boivin SSS. However this was a high titered serum, and since the combining ratio of this antigen for antibody was so small, insufficient amounts of antigen were added to precipitate all the antibody. With a lower titered serum from the "B" bleeding of the same goat, the maximal amount of antibody nitrogen precipitated with each antigen was practically identical. There was a difference of only 0.06 mg antibody nitrogen between determinations made with the 2 antigenic fractions. This disparity is attributed to dilution factors involved in the calculations. In similar fashion the amount of antibody nitrogen precipitated from the anti-38 serum, G₁₃LA₃₈-49-A, was alike and maximal in quantitative determinations with each antigen.

A study of calculated ratios of antibody nitrogen:SSS, shown by the factor R, revealed very low values, smaller than those observed with the PG antigen. The average combining ratio for the 3 values obtained from the anti-SCHU serum was 0.134 and this ratio was constant regardless of the order of bleeding for the serum samples. However the ratio for the anti-38 goat serum was 0.284 which was more than 2 times larger than the corresponding value for the anti-SCHU serum. This disparity is in agreement with the one derived from values obtained with

the PG antigens. Therefore this Boivin antigen also appears to combine with an antibody of greater molecular weight in anti-38 goat serums. The difference between the combining ratios for the anti-SCHU and anti-38 goat serums is not large, but it is in the same direction as the ones previously observed with the PG antigen, and it is regarded as a true relationship and not one consequent upon any technical factors.

Calculations of the weight of the minimum chain length reactive with the antibody molecule in each antiserum gave a molecular weight of approximately 120,000 for the Boivin antigen. This is arrived at by the same method previously used in calculations of weights for the PG antigens. Thus it is observed that the molecular weight of the Boivin antigen is about 4.5 to 5 times greater than that of the PG antigen. This is in agreement with their respective combining ratios since it is observed that the antibody nitrogen:SSS ratio for the Boivin antigen is 4.7 lower than the corresponding ratio for the PG antigen. These ratios are consistently low but have been repeatedly observed in all analyses made with either antigen. The low ratios are curious findings in comparison with various results reported by Heidelberger and Kendall (29), Scherp (41), Pennell and Huddleson (42), and Culbertson (19) in comparable studies on pneumococcus, meningococcus, brucella, and crystalline egg albumin antigen-antibody systems. However Kabat (32) observed similar low ratios in the case of antiserum to blood

group A substances and he also postulated the presence of a contaminating material that was antigenically inert in vitro, thus accounting for the high weight ratio of the antigen. If this be the explanation, the Boivin antigenic fraction must contain more of the contaminating material to account for the lower combining ratio. However either antigen may be equally useful in estimating the antibody content of an antiserum. Both antigens ultimately give the same value in terms of mg/ml even though it requires 4 to 5 times more of the Boivin antigen to exhaust an equivalent amount of antibody from the same serum. This results from the lower combining power of the Boivin antigen, and the amount is in direct proportion to the difference between the quantities of the respective antigens required to attain the equivalence zone of a specific antiserum. However the precipitin reaction with either antigen may be described by the equation developed from the law of mass action. The explanation for this combination of antibody with either antigen is to assume that a multivalent antibody and antigen unite by a series of competing bimolecular reactions before visible precipitation occurs. In this respect the tularensis system is quite analogous to other systems for which this equation has been found to be descriptive.

Extension of these studies to protein-antiprotein systems is illustrated in part H of table 46 in which a sonic lysate was used as antigen. This system was more complicated since it was necessary to distinguish between antigen nitrogen and

antibody nitrogen. However appropriate calculations revealed a much higher antibody nitrogen:antigen nitrogen ratio, designated by R, than similar ratios achieved by use of the SSS antigens. This greater combining ratio is explained by the predominantly protein antigen whereas the SSS fraction might be considered a haptene antigen. The difference in values obtained with the sonic antigen and SSS antigen on the same serum represent the amount of antibody precipitated by the protein fraction of the organism. However the calculated ratios, R, at the equivalence zone are not comparable values since the ratios for the SSS preparations are based upon a gravimetric standard whereas the ratio derived for the sonic antigen is based upon a standard derived by chemical analysis. The total antibody content of the 1949 anti-SCHU goat serum of the first bleeding was 1.645 mg Ab N/ml when determined with the sonic lysate antigen. This is almost 2.5 times greater than the corresponding titer determined with the SSS antigen. However the difference of 0.9 mg is accounted for by the protein portion of the organisms. The relations of this predominately protein system are also described by the same equation so characteristic of the polysaccharide system. Similar relations also were found to cover the reactions of anti-Brucella goat serums with the appropriate antigens by Pennell and Huddleson (42). In the protein system successive increases in antigen-nitrogen showed increased solubility of the precipitate

when passing into the realm of antigen excess. In contrast the SSS precipitate was demonstrated to be a stable, insoluble complex of maximal precipitable antibody nitrogen in the region of excess antigen. However studies with the sonic antigen demonstrated the presence of an antibody capable of reacting with protein antigen in excess of that precipitated by the polysaccharide antigen. Further evidence for this excess antibody is revealed by the observation that an SSS equivalence zone "supernatant" will agglutinate whole bacteria whereas a similar supernatant from the sonic antigen will not agglutinate bacteria. This is explained by the presence of antibody capable of reacting with surficial protein portions of the whole cell antigen.

A survey of the rat protection tests illustrates that the paired mortality ratios for groups of rats pretreated with serums betray variability in resistance to experimental tularemia despite careful selection on the basis of weight. In the experiments no evidence was secured to indicate that there existed a labile fraction of protective antibody. The serums held in bulk at 5 C were just as protective as those which had been removed from the clot within 24 hours and then maintained continuously in a dry ice box. No evidence was found to indicate that the antibody formed in response to a virulent strain was qualitatively different from one formed by a totally avirulent strain as judged by their protective capacities for the

challenged white rat. The summarized data for serums No. 1 and No. 7 in table 45 permit no other inference. Ample evidence was found to show that much more antibody was formed after I-V inoculation with living virulent organisms than with living avirulent organisms, even though the latter were administered in far greater numbers.

The titration of antibody in an antitularense goat serum prepared from either a virulent or an avirulent antigen, by means of the Culbertson, the Martin, or the fastest reaction mixture methods always yielded the same titer. When a serum made from a virulent strain was diluted to theoretical antibody equivalence with a serum made from an avirulent strain the antibody of each serum combined with exactly the same amount of polysaccharide, regardless of the source of the SSS, virulent or avirulent strain. Hence it appears that the combining powers of the SSS preparations for antitularense serums are properties wholly unrelated to virulence.

SUMMARY

The immunological properties of selected antigenic fractions prepared from a virulent and from an avirulent strain of B. tularensis were studied by means of the quantitative precipitin reaction. The antigens were phenol-extracted and trichloroacetic acid-extracted polysaccharides from each strain, and a crude sonic lysate from the virulent strain.

A study was made of the quantitative relationships existing between the 2 tularemic antigens and antiserums made from and produced by various strains of B. tularensis ranging in degree of virulence from zero to about maximal for the white mouse. The antiserums were prepared in 3 animal species, namely, goats, rabbits and a horse. The courses of the reaction curves, from great antibody excess to considerable antigen excess, were followed by nitrogen determinations on specific precipitates. It was shown that the course of the precipitin reaction may be described by an equation developed from the law of mass action.

The polysaccharides, derived from the virulent and avirulent strains, were compared serologically and there was no indication of any variation dependent upon virulence of the strain. This was true for the polysaccharide fractions derived from each strain when extracted by 2 different methods. Results show the quantitative precipitin reaction curves for

both SSS preparations obtained from simultaneous reaction mixtures with the same serum under precisely the same conditions to be almost identical.

Analyses of goat antiserums show that the combining power of an avirulent strain antibody for either antigenic fraction is greater than a similar ratio for the corresponding virulent strain antibody. However serums from rabbits did not exhibit this disparity. Comparison was not possible with horse serum since antiserum was prepared against only the virulent strain. Significance of the relative combining ratios is discussed.

At the equivalence zone the combining ratios of antibody nitrogen:SSS are fairly constant and characteristic for each animal species and each antigenic fraction. The molecular weight of each polysaccharide preparation is postulated from calculations based upon assumed molecular weights of rabbit and horse antibody and experimental antibody nitrogen:SSS ratios. A similar analysis of the weight of antibody in goat serum also was presented.

A study was made to detect qualitative differences between tularemic protective antibodies that were produced by stimulus with a virulent and an avirulent strain in goats. Evidence for such qualitative differences were sought by challenge of pretreated groups of white rats with each serum. Analyses of results show that there is no evidence for a labile antibody

in antitularensis goat serums. The antibody engendered by an avirulent strain appears to be identical in its protective qualities with the antibody prepared with a highly virulent strain and, therefore, protection of rats depends only upon the quantity of antibody present.

REFERENCES

1. McCoy, G. W. 1911 A plague-like disease of rodents.
U. S. Pub. Health Service, Pub. Health Bull., 43,
53-71.
2. McCoy, G. W., and Chapin, C. W. 1912 Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, Bacterium tularense. J. Infectious Diseases, 10, 61-72.
3. Wherry, W. B., and Lamb, B. H. 1914 Infection of man with Bacterium tularense. J. Infectious Diseases, 15, 331-340.
4. Vail, D. T. 1914 A case of "squirrel-plague" conjunctivitis in man. Ophthalmic Record, 23, 487-497.
5. Foshay, L., Hesselbrock, W. H., Wittenberg, H. J., and Rodenberg, A. H. 1942 Vaccine prophylaxis against tularemia in man. Am. J. Pub. Health, 32, 1131-1145.
6. Kadull, P. J., Reames, H. R., Coriell, L. L., and Foshay, L. 1950 Studies on tularemia. V. Immunization of man. J. Immunol., 65, 425-435.
7. Ruchman, I., and Foshay, L. 1949 Immune response in mice after vaccination with Bacterium tularense. J. Immunol., 61, 229-234.
8. Downs, C. M., and Woodward, J. M. 1944 Studies on pathogenesis and immunity in tularemia. III. Immuno-

genic properties for the white mouse of various strains of *Bacterium tularensis*. *J. Immunol.*, 63, 147-163.

9. Foshay, L. 1940 1940 Tularemia: A summary of certain aspects of the disease including methods for early diagnosis and the results of serum treatment in 600 patients. *Medicine*, 19, 1-83.
10. Larson, C. L. 1947 A serum protection test in tularemia infections in white rats. U. S. Pub. Health Service, Pub. Health Repts., 62, 1793-1799.
11. Foshay, L., Ruchman, I., and Nicholes, P. S. 1947 Anti-tularensis serum: Correlation between protective capacity for white rats and precipitable antibody content. *J. Clin. Invest.*, 26, 756-760.
12. Alexander, M. M. 1950 A quantitative antibody response of man to infection or vaccination with *Pasteurella tularensis*. *J. Exptl. Med.*, 92, 51-57.
13. Nicholes, P. S. 1946 On the antigenic structure of *Bacterium tularensis*. Unpublished Ph.D. thesis, Univ. of Cincinnati.
14. Francis, E., and Evans, A. C. 1926 Agglutination, cross-agglutination and agglutinin absorption in tularemia. U. S. Pub. Health Service, Pub. Health Repts., 41, 1273-1295.
15. Haurowitz, F., and Schwerin, P. 1943 The specificity of antibodies to antigens containing two different

determinant groups. J. Immunol., 47, 111-119.

16. Heidelberger, M., and Kendall, F. E. 1935 A quantitative theory of the precipitin reaction, III. The reaction between crystalline egg albumin and its homologous antibody. J. Exptl. Med., 62, 697-720.
17. Martin, D. S. 1943 A simplified serum dilution method for the quantitative titration of precipitins in a pure antigen-antibody system. J. Lab. Clin. Med., 28, 1477-1482.
18. Dean, H. R., and Webb, R. A. 1926 The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. J. Path. Bact., 29, 473-492.
19. Culbertson, J. T. 1932 A quantitative study of the precipitin reaction with special reference to crystalline egg albumin and its antibody. J. Immunol., 23, 439-453.
20. Heidelberger, M., and Kendall, F. E. 1929 A quantitative study of the precipitin reaction between type III pneumococcus polysaccharide and purified homologous antibody. J. Exptl. Med., 50, 809-823.
21. Heidelberger, M., and Kendall, F. E. 1937 A quantitative theory of the precipitin reaction, IV. The reaction of pneumococcus specific polysaccharides with homologous rabbit antisera. J. Exptl. Med., 65, 647-660.

22. Palmer, J. W., and Gerlough, T. D. 1940 A simple method for preparing antigenic substances from the typhoid bacillus. *Science*, 92, 155-156.
23. Gibby, I. W., Nicholes, P. S., Tamura, J. T., and Foshay, L. 1946 The effect of an extract of blood cells upon the cultivation of *Bacterium tularensis* in liquid media. *J. Bact.*, 55, 855-863.
24. Boivin, A., Mesrobianu, I., and Mesrobianu, L. 1933 Technique pour la préparation des polysaccharides microbiens spécifiques. *Compt. Rend. Soc. Biol.*, 113, 490-492.
25. Boivin, A., and Mesrobianu, L. 1934 Remarques concernant la technique d'extraction du complexe polysaccharidique antigénique renfermé dans le bacille d'aertrycke. *Compt. Rend. Soc. Biol.*, 115, 304-306.
26. Freeman, G. G., Challinor, S. W., and Wilson, J. 1940 The use of a synthetic medium in the isolation of the somatic antigens of *Bact. typhi-murium* and *Bact. typhosum*. *Biochem. J.* 34 I, 307-324.
27. Folin, O., and Ciocalteu, V. 1927 On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.*, 73, 627-650.
28. Tamura, J. 1950 Personal communication.
29. Heidelberger, M., and Kendall, F. E. 1935 The precipitin reaction between type III *Pneumococcus* polysaccharide and homologous antibody, III. A quantitative study

and a theory of the reaction mechanism. J. Exptl. Med., 61, 563-591.

30. Heidelberger, M., and MacPherson, C. F. W. 1943 Quantitative micro-estimation of antibodies in the sera of man and other animals. Science, 97, 405-406.
31. Kabat, E. A., Kaiser, H., Sikorski, H. 1944 Preparation of the type-specific polysaccharide of the type I meningococcus and a study of its effectiveness as an antigen in human beings. J. Exptl. Med., 80, 299-307.
32. Kabat, E. A., and Bezer, A. E. 1945 Immunochemical studies on blood groups, I. Estimation of A and B isoantibodies in human serum by the quantitative precipitin method. J. Exptl. Med., 82, 207-215.
33. Herriot, R. M. 1941 Reaction of Folin's reagent with proteins and biuret compounds in presence of cupric ion. Proc. Soc. Exptl. Biol. Med., 46, 642-644.
34. Summerson, Wm. H. 1939 A simplified test-tube photoelectric colorimeter and the use of the photoelectric colorimeter in colorimetric analysis. J. Biol. Chem., 130, 149-166.
35. Marrack, J., and Smith, F. C. 1931 Quantitative aspects of immunity reactions: The precipitin reaction. Brit. J. Exptl. Path., 12, 30-35.
36. Heidelberger, M., and Kendall, F. E. 1935 A quantitative theory of the precipitin reaction, II. A study of

an azoprotein-antibody system. J. Exptl. Med., 62, 467-483.

37. Stokinger, H. E., and Heidelberger, M. 1937 A quantitative theory of the precipitin reaction, VI. The reaction between mammalian thyroglobulins and antibodies to homologous and heterologous preparations. J. Exptl. Med., 66, 251-272.
38. Heidelberger, M., Treffers, H. P., and Mayer, M. 1940 A quantitative theory of the precipitin reaction, VII. The egg albumin-antibody reaction in antisera from the rabbit and horse. J. Exptl. Med., 71, 271-282.
39. Kabat, E. A. 1939 The molecular weight of antibodies. J. Exptl. Med., 69, 103-118.
40. Heidelberger, M., and Pedersen, K. O. 1937 The molecular weight of antibodies. J. Exptl. Med., 65, 393-414.
41. Scherp, H. W. 1939 Studies on meningococcal infection. XI.- A quantitative study of the precipitative reaction between type I polysaccharide and anti-meningococcal horse sera. J. Immunol., 37, 469-487.
42. Pennell, R. B., and Huddleson, I. F. 1938 Quantitative studies of Brucella precipitin systems. I. Precipitation of homologous antisera by Brucella endoantigens. J. Exptl. Med., 68, 73-81.