

## THE FORMATION AND PROPERTIES OF POLIOVIRUS- NEUTRALIZING ANTIBODY\*

### IV. NORMAL ANTIBODY AND EARLY IMMUNE ANTIBODY OF RABBIT ORIGIN: A COMPARISON OF BIOLOGICAL AND PHYSICOCHEMICAL PROPERTIES

By SVEN-ERIC SVEHAG,† D.V.M.

(From the Department of Virology, The Public Health Research Institute of  
The City of New York, Inc., New York)

(Received for publication, December 7, 1963)

A previous communication (1) in this series reported that transient, very rapid macroglobulin antibody responses could be induced in rabbits by small doses of poliovirus (PV) antigen. 19S antibody appeared 8 to 16 hours following immunization in such responses. It was also observed that almost all normal rabbit sera (NRS) contained low background neutralizing activity to several viral antigens, among them PV (2). In the present study the physicochemical nature, cross-reactivity, and avidity of the serum factor responsible for this background neutralization of PV and its relationship to the rapid 19S antibody response, were explored.

Normal antibody has been reported to differ from immune antibody in specificity (3) and thermostability (3-6). While in those studies normal antibody was compared with immune antibody collected at a fairly late stage in the course of immunization, in the present study, normal antibody was compared with the earliest detectable induced antibody. Under these circumstances it was found that the physicochemical properties, cross-reactivity, and avidity of normal and *early* immune antibody to PV were similar, if not identical. The thermodynamic data for the neutralization of PV by normal antibody were the same as those of serological reactions employing immune antibody.

#### *Materials and Methods*

*Virus.*—Type 1 (Brunhilde), type 2 (MEF-1), and type 3 (Saukett) of poliovirus and Coxsackie viruses B-4 and B-5<sup>1</sup> were used. Poliovirus, types 1 and 2, were cloned on HeLa monolayers. All viruses were grown on HeLa monolayers, and virus stocks were partly purified and concentrated by differential centrifugation prior to their storage at  $-20^{\circ}\text{C}$ . The virus stocks contained about 1  $\mu\text{g}$  protein per  $10^{6.4}$  plaque-forming units (PFU).

\* This investigation was supported in part by research grant E-4360 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

† Fellow of The American-Scandinavian Foundation.

<sup>1</sup> The Coxsackie viruses were kindly supplied by Dr. John P. Fox.

*Immunization Procedure.*—Healthy, 3½ to 5 months old female, randomly inbred Chinchilla-Flemish rabbits weighing 2½ to 3½ kg were given a single intravenous injection of 2 ml diluted stock virus ( $8 \times 10^8$  PFU) into the marginal ear vein. At various intervals thereafter the rabbits were bled by cardiac puncture, the blood allowed to clot, and the sera collected and stored at  $-70^\circ$  or  $3^\circ\text{C}$  without preservatives.

*Assay of Virus-Neutralizing Antibody, Zone Density Gradient Ultracentrifugation, Zone Electrophoresis, Reduction of Serum Macroglobulins with Mercaptans, Ultraviolet Light Inactivation of Virus, and Protein Determination.*—These methods were the same as described in previous papers (2, 7) in this series.

*Acid Reactivation of Neutralized Virus.*—Virus-serum mixtures were diluted tenfold in a buffered salt solution of the desired pH and left at room temperature for 40 minutes. The pH was then adjusted to neutrality by the addition of 24 volumes of ice-cold neutral-buffered salt solution. When dissociation of virus-antibody complexes was carried out at pH levels between 2 and 3 further adjustment with minute amounts of 0.1 M NaOH was required. The virus-serum mixtures were then immediately assayed on HeLa monolayer cultures.

## RESULTS

*Occurrence and Cross-Reactivity of the Virus-Neutralizing Activity in Normal Rabbit Sera.*—Twenty-eight randomly collected NRS were tested simultaneously against Coxsackie B-4 virus and poliovirus type 1 (PV-1). It can be seen in Fig. 1 that titers of neutralizing activity against PV-1 were generally higher and more widely distributed than titers against Coxsackie B-4. The fact that the ratio of the neutralizing titers to these two viruses varied greatly from one serum to another, suggested that the activity had some specificity.

In order to test the cross-reactivity of the background antiviral activity further, five NRS with moderate to high activity against PV-1 were tested against poliovirus type 2 (PV-2), poliovirus type 3 (PV-3), Coxsackie B-4, Coxsackie B-5, and phage T-2<sup>2</sup> (Fig. 2). The neutralization of these viruses, except PV-2, was only slight and independent of the activity against PV-1. When electrophoretically purified PV-neutralizing factor from NRS was used, the results were similar. The activity of whole normal sera or normal serum fractions against the closely serologically related PV-2 however, was quantitatively related to the same sera's activity against PV-1 (Fig. 2). Similarly, anti-PV-1 immune sera cross-reacted with PV-2 but showed no or only very low background neutralization to Coxsackie B-4, Coxsackie B-5, or T-2.

The specificity of the PV-neutralizing factor in NRS was further established by absorption with antigen. In one absorption experiment an undiluted NRS with moderate activity against PV-1 was divided into five aliquots, two of which were absorbed with ultraviolet light-irradiated PV-1. Two others were absorbed with similarly irradiated Coxsackie B-4 virus, and the fifth aliquot served as an unabsorbed control. Two virus concentrations were employed and the absorption was carried out at  $4^\circ\text{C}$  for 4 days, with virus added once daily. Virus and virus-antibody complexes were then pelleted by centrifuga-

<sup>2</sup> Kindly assayed for phage-neutralizing activity by Dr. Marvin Fishman.

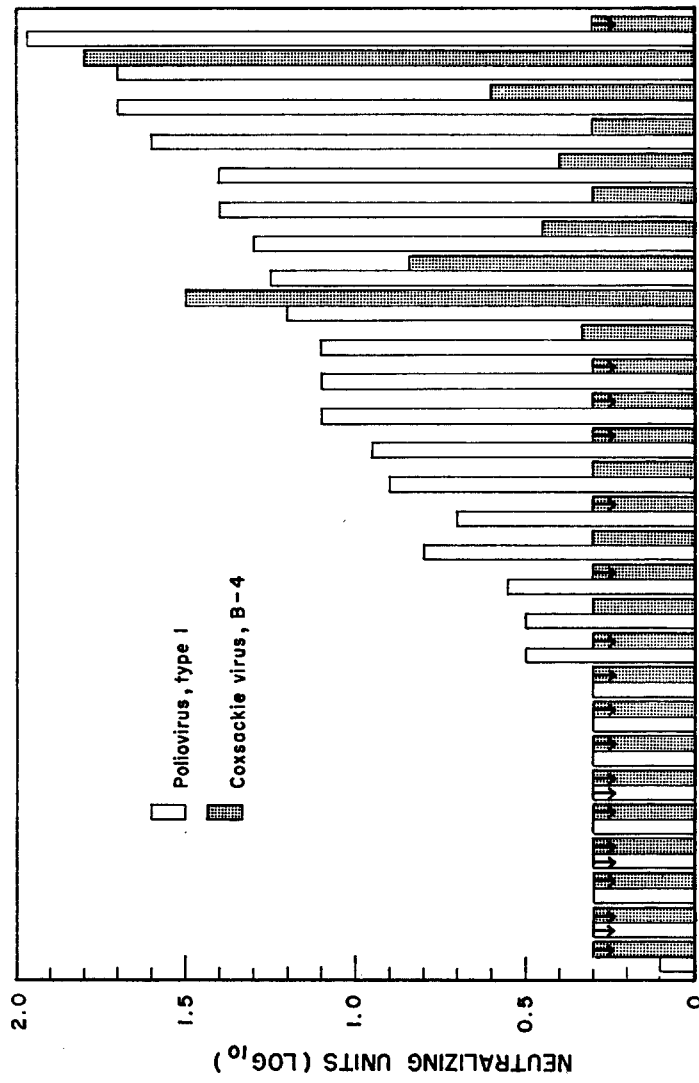


FIG. 1. Distribution of background-neutralizing activity to poliovirus, type 1 and Coxsackie virus, B-4 among twenty-eight randomly collected normal rabbit sera.

tion at 46,000 RPM for 2 hours and the supernatants tested for activity against PV-1. It can be seen in Table I that both concentrations of PV-1 absorbed out virtually all neutralizing activity against this virus, while absorption with heterologous Coxsackie virus resulted in only a very slight reduction of the same activity.

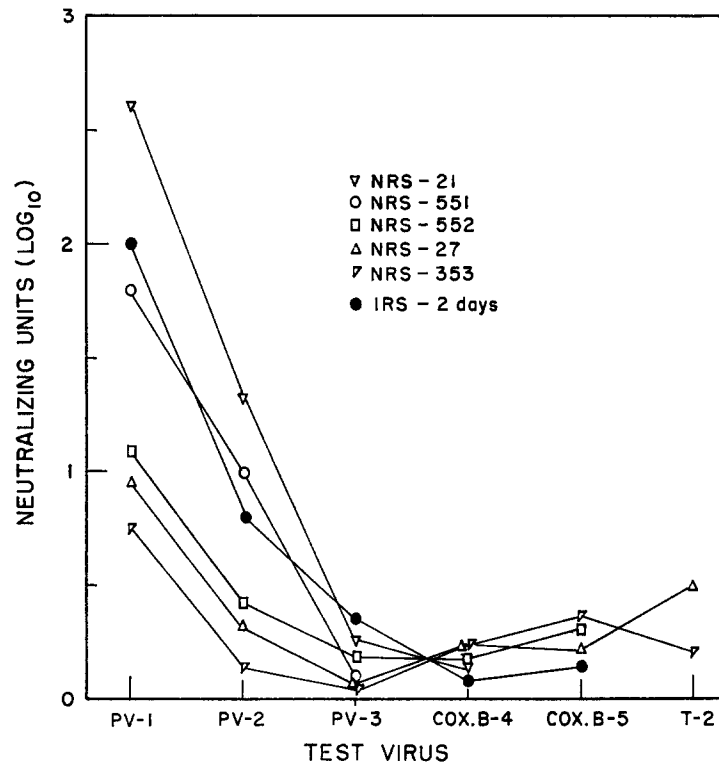


FIG. 2. Cross-reactivity of normal and early immune poliovirus (type 1)-neutralizing antibody to five other viral antigens. The immune serum was collected 2 days after a single intravenous injection of  $8 \times 10^8$  PFU of virus. NRS, normal rabbit serum. IRS, immune rabbit serum.

An attempt was made to recover the activity against PV-1 by acid dissociation of the sedimented PV-normal antibody complexes. All pellets were washed with buffer of neutral pH, resuspended in a buffer of pH 2.2, left at room temperature for 40 minutes, and recentrifuged at 46,000 RPM for 2 hours. The supernatants were then collected, adjusted to neutral pH, and assayed for activity against PV-1. Table I shows that efficient recovery of activity was obtained only from those pellets which contained PV-normal antibody complexes.

*Cross-Reactivity of the Poliovirus-Neutralizing Activity in Early Immune*

*Rabbit Sera.*—In further experiments it was determined whether the activity of normal and early immune antibody to PV-1 could be reduced after absorption of the sera on HeLa monolayers or HeLa cell debris. The possibility was considered that some early immune antibody was produced against antigenic components of HeLa cell origin which were present in the partly purified virus preparation used for immunization. In the assay for early antibody to PV-1 the absorption of such antibody to HeLa monolayers might interfere with subsequent virus absorption (8) thus simulating the presence of neutralizing antibody to PV-1. To investigate this, the kinetics of the reaction at 37°C between virus and a 2 day immune rabbit serum (IRS) was studied. One of the determinations was made with serum previously absorbed on HeLa monolayers. A

TABLE I  
*Specific Absorption of Normal Rabbit Antibody to Poliovirus*

	Absorption with				
	Poliovirus	Poliovirus	Coxsackie virus	Coxsackie virus	Buffer
PFU prior to UV irradiation* . . . . .	$2.2 \times 10^9$	$7.0 \times 10^8$	$2.2 \times 10^9$	$7.0 \times 10^8$	—
Neutralizing units ( $\log_{10}$ ) in absorbed sera . . . . .	<0.5	<0.5	1.4	1.3	1.6
Neutralizing activity, ‡ recovered by acid dissociation of virus-antibody complexes . . . . .	100	95	5	17	0

\* Both viruses were irradiated to a survival of  $\approx 10$  PFU/0.5 ml.

‡ The activity is expressed in per cent of the highest neutralization obtained.

NRS was similarly absorbed and tested. Fig. 3 shows that the kinetic curves for unabsorbed and absorbed (40 minutes at 37°C) aliquots of the same sera were indistinguishable, indicating that no appreciable activity had been absorbed by the cells. Neither did the sensitivity of the monolayers to PV-1 infection (plaque count and plaque size) change after their incubation with these early IRS or NRS, again suggesting that the antibody did not absorb to the monolayer. Two-day IRS were also incubated for 40 minutes at 37°C with HeLa cell debris, obtained by three cycles of freezing and thawing of  $2 \times 10^7$  freshly harvested cells. Such absorbed sera, clarified by centrifugation, caused as much virus neutralization as untreated serum aliquots.

*Physicochemical Properties of the Virus-Neutralizing Factor in Normal and Early Immune Rabbit Sera.*—

*Electrophoretic mobility:* The neutralizing factor in NRS with high activity and in early IRS was recovered in the  $\gamma_1$ - $\beta$ -region when these sera were subjected to starch block zone electrophoresis (Fig. 4). In comparing the mobility of

normal and early immune antibody, only immune antibody from rabbits devoid of background activity was used. Thus, the results in Fig. 4 were obtained with sera from two different animals. The electrophoretic distribution of the early (2-day) immune antibody to PV-1 was broader, and this antibody tended to migrate predominantly to the  $\beta$ -region, that is, slightly faster than the normal antibody. In contrast, most of the immune 19S antibody synthesized from day 3

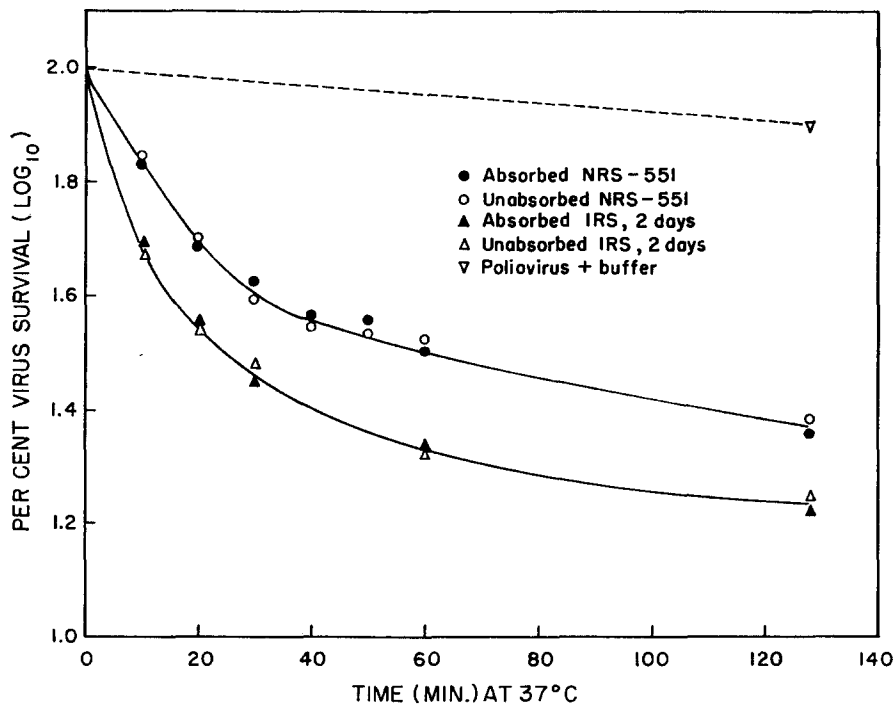


FIG. 3. Kinetics of neutralization of type 1 poliovirus by unabsorbed and absorbed (with HeLa cells) normal and early immune rabbit sera. The immune serum was collected 2 days after a single intravenous injection of  $8 \times 10^8$  PFU of virus. NRS, normal rabbit serum. IRS, immune rabbit serum.

on was associated with  $\gamma_1$ -globulins (9). The sequential changes which occur in the electrophoretic mobility of immune 7S and 19S antibodies after immunization were previously described (9).

*Sedimentation coefficient.* When NRS with high activity to both Coxsackie B-4 virus and PV-1 were subjected to zonal density gradient centrifugation in sucrose the activity against the two viruses was associated with the same rapidly sedimenting fractions. Typical results are shown in Fig. 4 (top figure) and Table II (left column). In less than 10 per cent of NRS traces of activity against PV-1

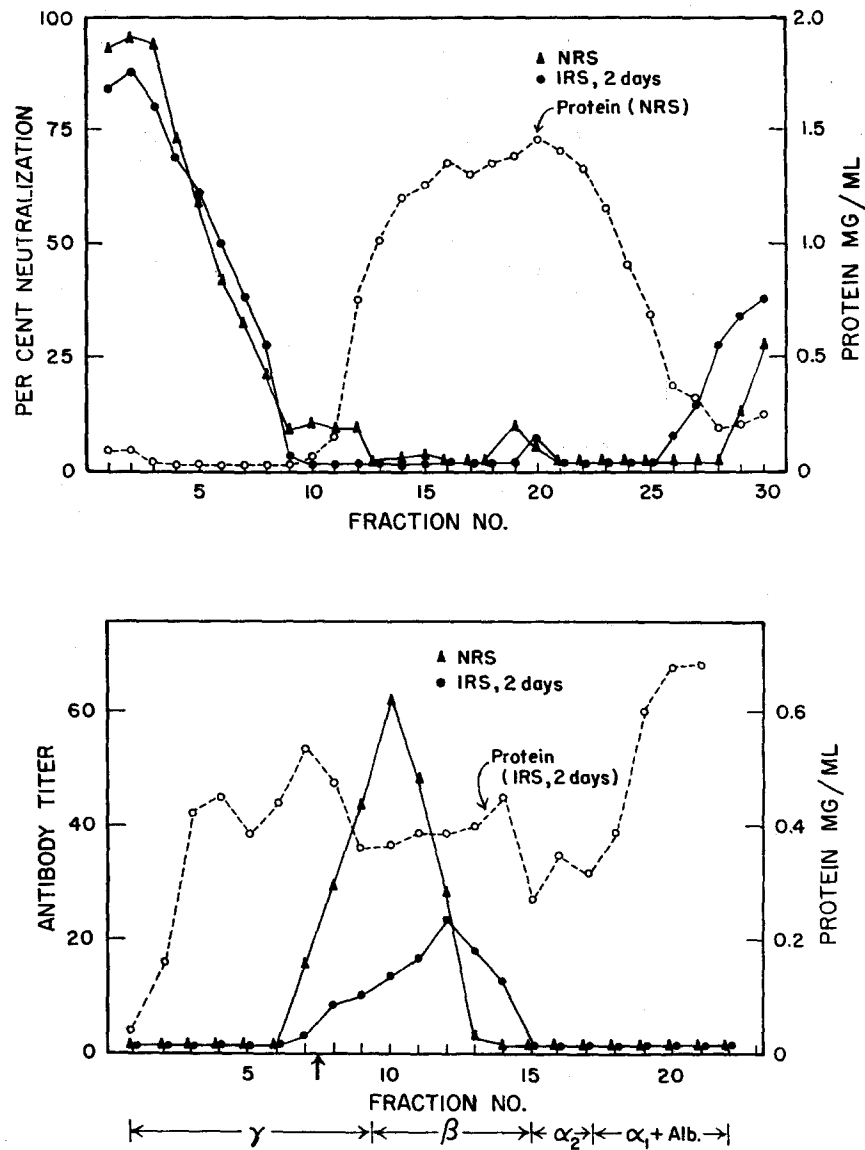


FIG. 4. Distribution of normal and early immune poliovirus-neutralizing antibody in two rabbit sera when subjected to starch block zone electrophoresis (bottom figure) and zonal density gradient centrifugation in sucrose (top figure). The immune serum was collected 2 days after a single intravenous injection of  $8 \times 10^8$  PFU of virus. NRS, normal rabbit serum. IRS, immune rabbit serum.

were obtained also in fractions sedimenting slightly faster ( $\approx 7S$ ) than the bromphenol blue-labeled 4.5S albumin. This is illustrated in serum 2 in Table II.

In one instance a NRS with weak activity to PV-1 was concentrated by dialysis against carbowax 20-M (Union Carbide Chemicals Co., New York) and subjected to density gradient centrifugation. Here too, neutralizing activity

TABLE II  
*Distribution of Normal Antibodies to Poliovirus and Coxsackie B-4 Virus in Two Normal Rabbit Sera after Fractionation by Density Gradient Ultracentrifugation in Sucrose\**

Fraction No.	Neutralization			
	Serum 1		Serum 2	
	Poliovirus type 1	Coxsackie B-4 virus	Poliovirus type 1	Coxsackie B-4 virus
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	82	69	40	31
2	88	83	45	77
3	76	25	56	96
4	12	3	26	46
5	0	2	3	20
6	0	0	0	0
7	0	0	13	0
8	0	0	5	0
9	0	0	0	0
10	0	0	0	0
11	0	0	30	34
12	0	12	44	46
13	22	15		
14	34	30		

Areas in *italics* indicate albumin bands.

\* Gradient was collected by puncture of the bottom of the tube.

was found in the most rapidly sedimenting fractions of the sucrose gradient. In respect to specificity, electrophoretic and sedimentation properties the PV-neutralizing substance in NRS thus conforms to the definition of antibody.

*Thermostability:* Inactivation curves were obtained when NRS and IRS were heated for 30 minutes at temperatures ranging from 53° to 86°C (Fig. 5). Only immune sera from animals devoid of background activity were used so that the results would not be obscured by the presence of normal antibody.

The activity of NRS and immune sera collected prior to day 4 was lost in a complex manner. A trace of the more labile component appeared to remain in 4-day sera but was not detectable in subsequently collected sera. Since all sera were diluted to the same extent in buffered-salt solution prior to heating in



order to maintain similar protein concentrations, sera collected 7 days post-immunization or later had much higher antibody levels than early IRS or NRS. The possibility was considered that the heat-labile component was also present in the former sera but obscured by their higher antibody titers. Potent 120-day IRS were diluted in NRS (devoid of activity) so that their antibody titers would approach those of 3-day IRS. When such sera, as well as very weak, late

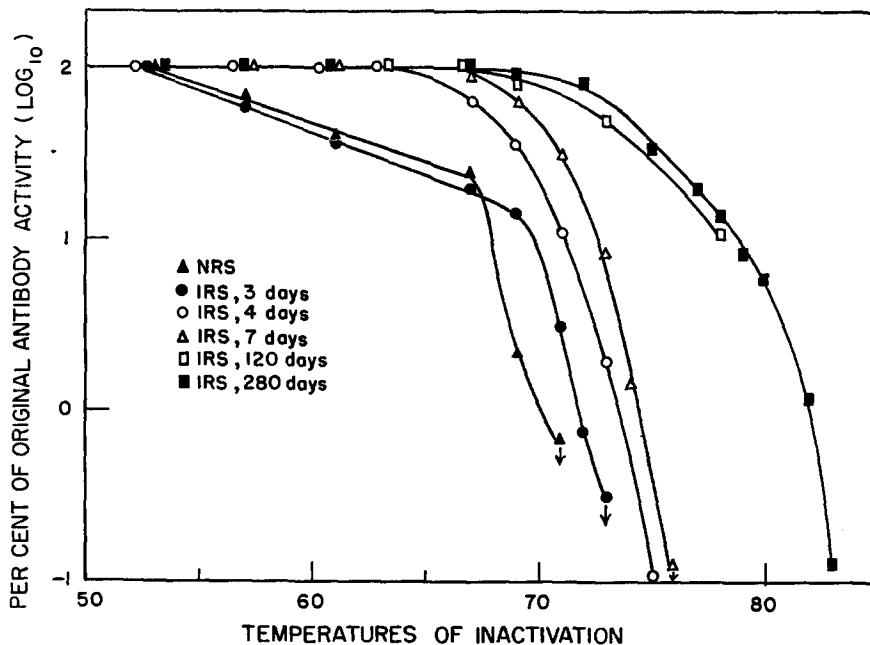


FIG. 5. Thermal inactivation curves for normal and immune poliovirus-neutralizing antibody. Diluted sera, of similar protein levels, were heated for 30 minutes at each indicated temperature. The 3, 4, 7 and 120 day immune sera were obtained from the same rabbit following a single intravenous injection of  $8 \times 10^8$  PFU of virus. NRS, normal rabbit serum. IRS, immune rabbit serum.

(280-day) IRS were heated, only one temperature-dependent component was observed (Fig. 5). This is compatible with the denaturation of a single type of molecule.

The part of the inactivation curves which represented the loss of the first 80 per cent of the activity differed distinctly when NRS or 2- to 3-day IRS were compared with IRS collected later. The inactivation of the remaining 20 per cent displayed similar slopes in all sera. These results suggested the inactivation in NRS and very early IRS of two entities both participating in the neutralization of PV, with the more thermostable one also present in late IRS. The steeper slopes, shown in Fig. 5, most likely represent antibody denaturation, while the

more labile component may reflect inactivation of one of the components of complement (C'). The steeper part of the inactivation curves showed a gradual displacement towards higher temperatures for IRS collected later in the course of immunization. Since this could be related to neither the protein content nor

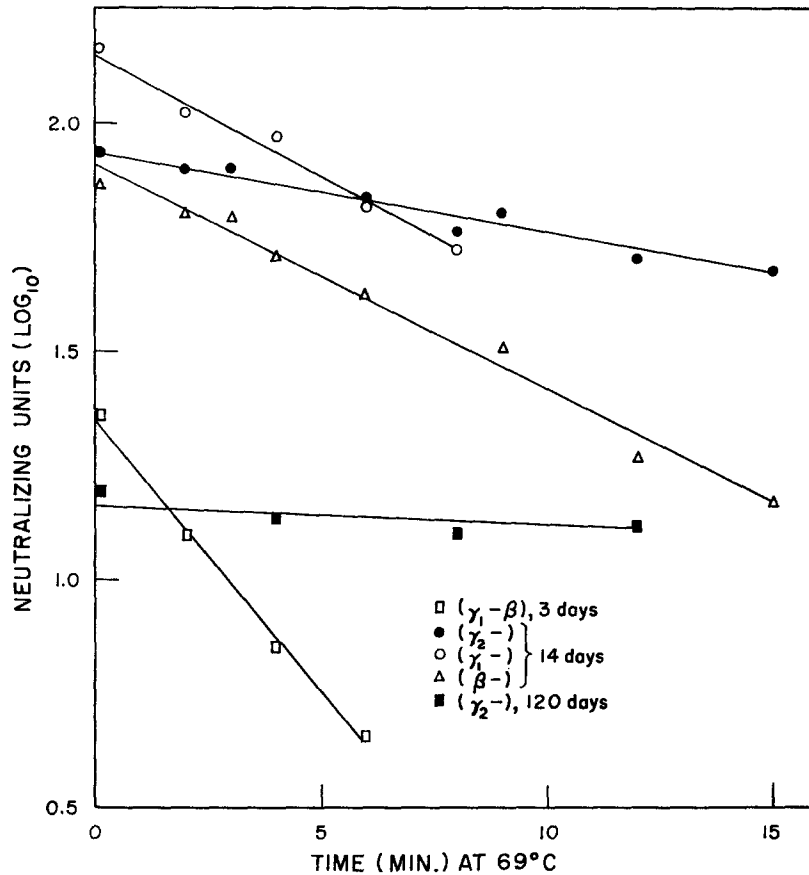


FIG. 6. Kinetics of thermal inactivation of poliovirus-neutralizing antibody in electrophoretically isolated serum fractions. All 3 sera were collected after stimulation with  $8 \times 10^8$  PFU of virus; the 3 and 14 day sera were obtained from the same animal.

the antibody titer of such sera, it indicated a progressive increase in the thermostability of the antibody with time after immunization.

When NRS or early IRS were heated at  $69^\circ\text{C}$  the labile serum component was inactivated within about 15 minutes. Thereafter the neutralizing activity was lost at a very slow rate. The inactivation data for the labile component were compatible with a destruction of C'-3 and/or C'-4. However, the addition of

freshly collected NRS (diluted 1:4 or 1:16) or normal guinea pig serum (diluted 1:4), failed to restore the activity.

Fig. 6 illustrates results obtained when electrophoretically isolated serum fractions with antibody activity were heated at 69°C. The 3- and 14-day sera were collected from the same animal. Electrophoretic fractions representing the antibody peaks in 3- (□) and 120-day sera (■) differed strikingly in their thermostability. Because the antibodies in these fractions were associated with 19S  $\gamma_1$ - $\beta$ - and 7S  $\gamma_2$ -globulins respectively, their differences in stability could be due entirely to these differences in molecular properties. But it could also be

TABLE III  
*Effect of 2-Mercaptoethanol on Normal and Early Immune 19S Antibodies to Poliovirus*

2-Mercaptoethanol concentration	Incubation with 2-mercaptoethanol	Type of serum	Zonal density centrifugation results	Material treated	Antibody titer	
					Un-treated	Treated
M			S			
0.05	1 day—3°C	NRS*	19	Whole serum	60	40
0.05	1 day—3°C	NRS	19	Whole serum	110	70
0.05	1 day—3°C	4-day IRS*	19	Isolated 19S‡	24	12
0.10	5 days—3°C	NRS	19	Whole serum	50	<4
0.10	5 days—3°C	2-day IRS	19	Whole serum	150	<4
0.10	5 days—3°C	4-day IRS	19	Whole serum	400	<4
0.20	1 day—3°C	4-day IRS	19	Whole serum	150	<4
0.20	1 day—3°C	9-month IRS	7	Isolated 7S‡	250	350
0.20	1 day—3°C	NRS	19	Whole serum	75	<4

\* NRS, normal rabbit serum; IRS, immune rabbit serum.

‡ Isolated by zonal density gradient ultracentrifugation.

the result of other structural changes in antibodies occurring with time after immunization. To examine this, the stability of antibodies of different mobilities (and molecular weight) from the same 14 day serum, was ascertained (Fig. 6). It was found that the antibodies associated with  $\beta$ -globulins (19S) and  $\gamma_1$ -globulins (mainly 19S) were inactivated only slightly faster than the 7S  $\gamma_2$ -antibodies. Since these results could not explain the pronounced difference in thermostability between the two molecular types of antibody in 3- and 120-day sera, it appeared, that the thermostability of both 19S and 7S antibody increased with time after immunization. That the thermostability of antibody molecules was largely unrelated to their net charge was further supported by the finding that 19S  $\gamma_1$ - and 19S  $\beta$ -antibodies from the same 7 day serum were inactivated at the same rate at 69°C.

*Effect of 2-Mercaptoethanol on Normal and Early Immune 19S Antibodies.*—The sensitivity of the two macroglobulin antibodies to 2-mercaptoethanol was studied. Table III shows that incubation with 0.05 M 2-mercaptoethanol for

1 day at 3°C resulted in incomplete inactivation of the neutralizing activity, while treatment with 0.1 M for 5 days at 3°C effectively abolished the activity of both normal and early immune 19S antibodies. The lower borderline concentration (0.05 M) was used primarily because it could be expected to detect minor differences between the two macroglobulin antibodies in mercaptan sensitivity. When the final concentration of mercaptan was 0.2 M only 1 day's

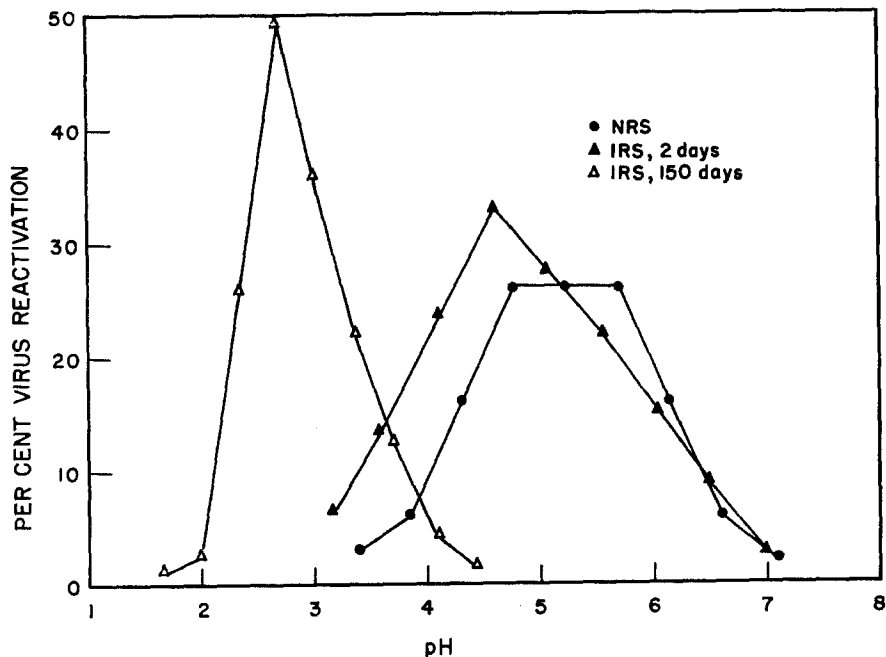


FIG. 7. Per cent reactivation at acid pH of poliovirus neutralized by normal, early immune, and late immune antibodies. The results are plotted as frequency polygons and the means ( $\bar{x}$ ) and standard deviations ( $s_x$ ) of the three distributions are: for normal rabbit serum (NRS)  $\bar{x} = 5.05$ ,  $s_x = 0.94$ ; for 2-day immune rabbit serum (IRS, 2 days)  $\bar{x} = 5.24$ ,  $s_x = 0.97$ ; and for 150-day immune serum (IRS, 150 days)  $\bar{x} = 3.05$ ,  $s_x = 0.70$ . The sera were obtained from 3 different animals.

incubation at 3°C was needed for complete destruction of the antibody activity. Immune 7S antibodies, in contrast, were unaffected by incubation with as much as 0.8 M mercaptan (9). Thus, the macroglobulin antibody in normal rabbit sera has similar sensitivity to reduction by mercaptan as the early immune antibody.

*The Avidity<sup>3</sup> of Normal and Early Immune 19S Antibodies.*—It was reported

<sup>3</sup> The term avidity as used here refers to the firmness of the virus-antibody complex as measured by the efficiency with which infectious virus can be recovered at acid pH. Thus, avidity is not expressed by any particular thermodynamic constant.

(10) that the early immune 19S antibody to PV formed easily dissociable complexes with the virus. In the present study, acid dissociation (11, 12) of virus-antibody complexes was employed in a comparison of the avidity of normal and early immune 19S antibodies. Two thousand to 4000 PFU of PV-1 were allowed to react overnight at 4°C with serum dilutions which neutralized 80 to 90 per cent of the virus. Acid reactivation was then carried out on samples of the reaction mixture at pH levels ranging from 2.0-6.6. This method of

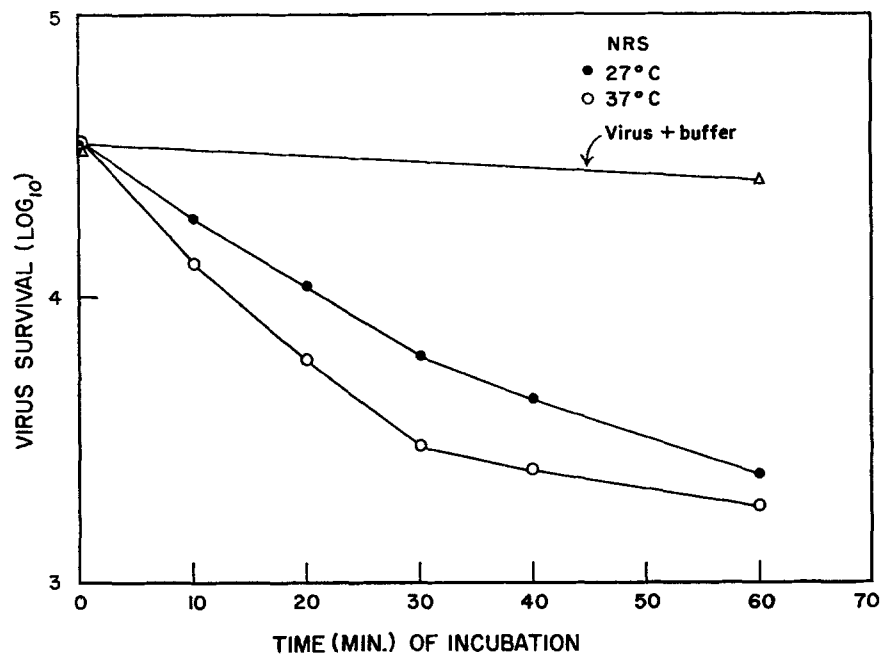


FIG. 8. Kinetics of neutralization of poliovirus, type 1 by normal macroglobulin antibody NRS, normal rabbit serum.

dissociation resulted in no loss of virus and only slight inactivation of antibody between pH 2.0 and 3.5.

Fig. 7 shows results obtained when the avidity of normal 19S antibody was compared with that of early (2-day) and late (150-day) immune antibodies. The percentage virus reactivation at different pH levels was plotted as frequency polygons (13) in order to allow an estimation of antibody homogeneity (the width of the base of the distributions) and separation of antibody populations of differing avidity. The reduction in virus recovery on the more acid side of the curves is not due to a decrease in the efficiency of dissociation of virus-antibody complexes but to the fact that at these pH values most of the virus has already been recovered.

For NRS the percentage virus reactivation at different pH levels followed a smooth symmetric curve, indicative of an approximately normal distribution of antibody of low avidity. The 2-day IRS contained antibody of similar low avidity but the reactivation curve showed a slight positive skewness, suggesting a beginning trend towards synthesis of more avid antibody. It was subsequently found that 19S antibodies of higher avidity had already started to appear on the 3rd and 4th day following immunization (14). Using NRS or 2-day IRS, most virus and antibody was recovered at about pH 4.0, while virtually no virus reactivation occurred at this pH with the use of late IRS which contained predominantly 7S  $\gamma_2$ -antibody. The 150-day IRS also gave a positively skewed distribution which may indicate that the trend towards synthesis of more avid antibody still continues.

*Kinetics and Thermodynamic Parameters of Neutralization of Poliovirus by Normal 19S  $\gamma_1$ - $\beta$ -antibody.*—The neutralization of PV by normal 19S  $\gamma_1$ - $\beta$ -antibody appears to be a pseudo first order reaction, without any apparent lag phase (Fig. 8). When the kinetics of the reaction were studied at 27 and 37°C the temperature coefficient ( $Q_{10}$ ) was 1.6. In reactions where the reaction rate is governed mainly by the diffusion (collision frequency) of the two reactants,  $Q_{10}$  has a value of 1.4 to 1.5.

The free energy change ( $E_{act.}$ ) for the reaction was about  $-9$  kcal/mole, which is similar to or somewhat higher than  $E_{act.}$  values reported for serological reactions employing immune antibody (15, 16). Using the Eyring equation (17), the enthalpy change ( $\Delta H$ ) was calculated to be  $-8$  kcal/mole, in excellent agreement with data for immune antibody-hapten interactions obtained by equilibrium dialysis (16). The entropy change ( $\Delta S$ ) was 36 cal/mole/degree.

#### DISCUSSION

A previous paper in this series (2) reported that background neutralizing activity against several viral antigens, among them PV-1, could be detected in most normal rabbit sera, provided sufficiently sensitive assay procedures were employed. The origin and specificity of such background or normal antibodies has long been a matter of controversy. The distinction between normal and immune antibody has been based on reported differences in specificity and thermostability (3-6).

In recent studies (18-21) normal antibody was found however to possess considerable specificity. This was observed as a lack of correlation between the activities of a given serum to various antigens and the corresponding activities of another serum (18, 21). Similar results were obtained in the present study when NRS were tested simultaneously against PV-1, PV-3, Coxsackie B-4 and B-5 viruses, and phage T-2. In contrast, sera which neutralized PV-1 also had low, but proportional activity against the serologically related PV-2. The specificity of the normal antibody to PV-1 was further established by the

finding that this antibody could not be absorbed by serologically unrelated antigens, while absorption of normal rabbit sera with ultraviolet-irradiated PV-1 caused virtually complete loss of the antibody activity.

The background neutralization of PV-1 and Coxsackie B-4 virus was found to be associated with relatively heat-stable, mercaptan-sensitive,  $\gamma_1$ - $\beta$ -macroglobulins of the 19S type. Normal antibodies have usually been described as more heat-labile than immune antibodies (3-6). In these studies normal antibodies were compared with immune antibodies collected fairly late in the course of immunization. Since the present findings, and those of Michael and Rosen (22), have shown that normal and late immune antibodies differ distinctly in their physical properties the above results probably represent a difference in heat stability of antibodies of different physicochemical properties rather than a difference between normal and immune antibodies *per se*. This notion is supported by the observation that normal and *early* immune antibody to PV-1, both of which are associated with 19S  $\gamma_1$ - $\beta$ -globulins, showed nearly identical, 2-component thermal inactivation curves, while the late immune 7S  $\gamma_2$ -globulin antibody was more thermostable. The dissimilarity in heat stability of these two antibodies appeared to be unrelated to differences in their net charge ( $\gamma_1$ - $\beta$  vs.  $\gamma_2$ ). Heat inactivation data obtained with mixed protein preparations must be interpreted with caution. Non-antibody molecules, particularly serum albumins, may stick to antibody molecules during the heating (23), and cause loss of activity, thus simulating antibody denaturation. But since the protein levels were similar in heated sera, and the same results were obtained with electrophoretically purified antibodies to PV-1, interactions with other serum proteins could not explain the marked difference in thermostability between normal and early immune antibody on the one hand and late immune antibody on the other.

The 2-component inactivation curves obtained with normal and early immune rabbit sera suggested the presence of two molecular species participating in the neutralization of PV. This finding was probably related to another described difference between normal and immune antibodies, namely in their dependence on complement (4, 19, 24-26). But recent studies by Muschel and Toussaint (27) have indicated that both normal and early immune antibodies, in contrast to late immune antibodies, are dependent on or potentiated by complement. Thus, the distinction is made again between antibodies of different physical properties rather than between normal and immune antibodies.

In the present study normal rabbit antibody to PV was found to be of uniformly low avidity. The dependence of antigen-normal antibody complexes on complement as a stabilizer (26, 27) is in agreement with this finding. It was shown that normal and early immune antibodies to PV, having similar physicochemical properties, also do not differ in avidity. Both these antibodies form loose complexes with the viral antigen.

The normal 19S  $\gamma_1$ - $\beta$ -antibody appeared to neutralize PV principally in accordance with first order kinetics, and the thermodynamic parameters of this reaction did not differ significantly from those of serological reactions employing immune antibody (15, 16). A gain in entropy was observed for the antigen-normal antibody complex molecules; this has been reported also for antigen-immune antibody systems (16). This unexpected finding was attributed to the liberation of ion-bound water from the reagent molecules (28).

Only rarely was a NRS encountered which contained, besides normal antibody of the 19S  $\gamma_1$ - $\beta$ -type, also minor amounts of background neutralization due to 7S  $\gamma_2$ -globulins. It is of interest, that Michael and Rosen (22) found bactericidal antibody to Gram-negative bacteria to be associated with  $\gamma_2$ -globulins in only 10 per cent of normal human sera, all of which contained normal antibody of the  $\gamma_1$ -macroglobulin type. Properdin, which appears to be an antibody system with specificity to a large number of naturally occurring antigenic determinants of lipopolysaccharide nature (29) has also been reported to be a  $\gamma_1$ -macroglobulin (30). Several lines of evidence suggest that the properdin system is identical with the bactericidal effect of normal antibody and complement (26, 29, 31).

It is proposed that the macroglobulin antibodies to viral antigens found in normal sera are associated with an immunological response to repeated stimulation with minute amounts of cross-reacting or identical antigenic determinants. This proposal is consistent with the finding (1, 7) that only 19S antibody was produced following single as well as repeated stimulation with very small doses of PV antigen. Further, since repeated antigenic stimulation is necessary to maintain a certain level of macroglobulin antibody in normal sera, it is postulated that this antibody response, like the immune 19S antibody response to PV (1, 7) lacks or has a very short "immunological memory." This theory is also supported by the observation that no booster effect is demonstrable in the 19S antibody response to PV-1 or Coxsackie B-4 virus in rabbits with high background (2). Normal antibody levels to PV appeared to be higher in older animals which may also support the "immune" theory since the chance of encountering a particular antigen would increase with the age of the animal. Preliminary data suggest that the formation of normal 19S antibody to PV in the rabbit is suppressed by whole body x-irradiation (700 roentgens). Similar findings have been made for the background-neutralizing activity to T-2 in rats (32). Finally, when serum levels of normal antibody to PV-1 were followed over a period of 1½ years, they were found to fluctuate in a manner compatible with the concept that they represented immune 19S antibody responses. The reduction in properdin (normal antibody) levels in germ-free animals (33) is also in agreement with this notion. The formation of immune 19S antibody was also reported to precede 7S antibody in studies with other antigens (34-38). Since the successful demonstration of background activity appears to depend



more on the sensitivity of the assay procedure than on the antigen used one may consider the possibility that in these systems too low specific activity was associated with a macroglobulin in the preimmunization sera.

The absence of a demonstrable "booster effect" in the immune 19S antibody response of rabbits with normal 19S antibody was surprising, since a memory effect was observed in immune 19S antibody formation when antigenic restimulation occurred within the 1st week of the response, at a time when antibody synthesis proceeded at a moderate or high rate (7). However, since anamnestic 19S antibody responses were obtained only when background levels of immune 19S antibody were about ten times as high as observed backgrounds of normal antibody, the background of cells producing normal antibody may be too low to allow the demonstration of a booster effect.

#### SUMMARY

Rabbit sera were found to possess neutralizing activity (normal antibody) to polioviruses and Coxsackie B viruses. This normal antibody showed high specificity in cross-neutralization and absorption tests. It was associated with heat-stable, mercaptan-sensitive, 19S  $\gamma_1$ - $\beta$ -macroglobulins, which formed weak complexes with the viral antigen. In rare instances, sera with normal macroglobulin antibody, also contained very low activity which was due to 7S  $\gamma_2$ -globulins. The neutralization of poliovirus by normal 19S  $\gamma_1$ - $\beta$ -antibody appeared to follow first order kinetics, and the thermodynamic parameters of this reaction were the same as those of serological reactions employing immune antibody.

The electrophoretic mobility, sedimentation properties, sensitivity to mercaptan, thermostability, and avidity of normal and *early* (up to day 3) immune antibodies to poliovirus were similar, but differed in several respects from those of late immune antibodies. Thus, the available evidence suggested, that earlier reported differences between normal and immune antibodies reflected differences between antibodies of diverse physicochemical properties rather than between normal and immune antibodies *per se*.

It is proposed that the normal macroglobulin antibody is associated with an immunological response to repeated stimulation with minute amounts of antigen.

The excellent technical assistance of Miss Joan Reinstein is gratefully acknowledged.

#### BIBLIOGRAPHY

1. Svehag, S.-E., and Mandel, B., The production and properties of poliovirus neutralizing antibody of rabbit origin, *Virology*, 1962, **18**, 508.
2. Svehag, S.-E., and Mandel, B., The formation and properties of poliovirus-neutralizing antibody. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction, *J. Exp. Med.*, 1964, **119**, 1.

3. Skarnes, R. C., and Watson, D. W., Antimicrobial factors of normal tissues and fluids, *Bact. Rev.*, 1957, **21**, 273.
4. Van Vunakis, H., Barlow, J. L., and Levine, L., Neutralization of bacteriophage by the properdin system, *Proc. Nat. Acad. Sc.*, 1956, **42**, 391.
5. Landy, M., Trapani, R.-J., and Shear, M. J., Interference of endotoxic polysaccharides with inactivation of coliphage by human serum, *Brit. J. Exp. Path.*, 1958, **39**, 618.
6. Michael, J. G., Whitby, J. L., and Landy, M., Studies on natural antibodies to Gram-negative bacteria, *J. Exp. Med.*, 1962, **115**, 131.
7. Svehag, S.-E., and Mandel, B., The formation and properties of poliovirus-neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, anamnesis, and sensitivity to x-irradiation, *J. Exp. Med.*, 1964, **119**, 21.
8. Habel, K., Hornibrook, J. W., Gregg, N. C., Silverberg, R. J., and Takemoto, K. K., The effect of anticellular sera on virus multiplication in tissue culture, *Virology*, 1958, **5**, 7.
9. Svehag, S.-E., The formation and properties of poliovirus-neutralizing antibody. III. Sequential changes in electrophoretic mobility of 19S and 7S antibodies synthesized by rabbits after a single virus injection, *J. Exp. Med.*, 1964, **119**, 225.
10. Svehag, S.-E., and Mandel, B., Progressive changes in primary properties of neutralizing antibody of rabbit origin to poliovirus, *Fed. Proc.*, 1962, **21**, 29.
11. Mandel, B., Reversibility of the reaction between poliovirus and neutralizing antibody of rabbit origin, *Virology*, 1961, **14**, 316.
12. Rappaport, I., Acid dissociation of aggregates of antibody and tobacco mosaic virus, with recovery of infectious virus, *Biochem. et Biophysica Acta*, 1961, **47**, 206.
13. Ostle, B., Statistics in Research, Ames, The Iowa State College Press, 1956, 16.
14. Svehag, S.-E., and Mandel, B., data to be published.
15. Boyd, W. C., Antibody-antigen reactions, in *Fundamentals of Immunology*, New York, Interscience Publishers, Inc., 3rd edition, 1956, 285.
16. Kabat, E. A., and Mayer, M. M., Precipitin reaction, in *Experimental Immunology*, Springfield, Illinois, Charles C Thomas, 2nd edition, 1961, 59-62.
17. Daniels, F., Chemical kinetics, in *Outlines of Physical Chemistry*, New York, John Wiley and Sons, Inc., 1954, 376.
18. Cowan, K. M., Interpretation of properdin levels determined by phage neutralization technique, *Science*, 1958, **128**, 778.
19. Toussaint, A. J., and Muschel, L. H., Neutralization of bacteriophage by normal serum, *Nature*, 1959, **183**, 1825.
20. Osawa, E., and Muschel, L. H., The bactericidal action of normal serum and the properdin system, *J. Immunol.*, 1960, **84**, 203.
21. Kallings, L. O., Studies on the inactivation of bacterial viruses by normal human serum. VI. Comparative studies of various phages and of individual sera of human and animal origin, *Acta Path. et Microbiol. Scand.*, 1961, **52**, 273.
22. Michael, G. J., and Rosen, F. S., Association of "natural" antibodies to Gram-negative bacteria with the  $\gamma_1$ -macroglobulins, *J. Exp. Med.*, 1963, **118**, 619.

23. Bawden, F. C., and Kleczkowski, A., The effects of heat on the serological reactions of antisera, *Brit. J. Exp. Path.*, 1942, **23**, 178.
24. Barlow, J. L., Van Vunakis, H., and Levine, L., Studies on the inactivation of phage by the properdin system. I. Evidence for complement, properdin and magnesium requirements, *J. Immunol.*, 1958, **80**, 339.
25. Cowan, K. M., Studies on the coliphage neutralizing activity of normal human serum, *J. Immunol.*, 1962, **88**, 476.
26. Muschel, L. H., The antibody-complement system and properdin, *Vox Sanguinis* 1961, **6**, 385.
27. Muschel, L. H., and Toussaint, A. J., Studies on the bacteriophage neutralizing activity of serums. II. Comparison of normal and immune phage neutralizing antibodies, *J. Immunol.*, 1962, **89**, 35.
28. Epstein, S. I., Doty, P., and Boyd, W. C., A thermodynamic study of haptent-antibody association, *J. Am. Chem. Soc.*, 1956, **78**, 3306.
29. Nelson, R. A., An alternative mechanism for the properdin system, *J. Exp. Med.*, 1958, **108**, 515.
30. Lepow, I. H., The properdin system: a review of current concepts, in *Immunochemical Approaches to Problems in Microbiology* (M. Heidelberger and O. J. Plescia, editors), New Brunswick, New Jersey, Rutgers University Press, 1961, 280.
31. Šterzl, J., Kostka, J., and Lanc, A., Development of bactericidal properties against Gram-negative organisms in the serum of young animals, *Folia Microbiol.*, 1962, **7**, 162.
32. Fishman, M., and Adler, F. L., Antibody formation initiated *in vitro*. II. Antibody synthesis in x-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen, *J. Exp. Med.*, 1963, **117**, 595.
33. Gustafsson, B. E., and Laurell, A.-B., Properdin titers in sera from germ-free rats, *Proc. Soc. Exp. Biol. and Med.*, 1960, **105**, 598.
34. Stelos, P., Comparative study of rabbit hemolysins to various antigens. I. Hemolysins to beef red cells, *J. Infect. Dis.*, 1958, **102**, 103.
35. Bauer, D. C., Mathies, M. J., and Stavitsky, A. B., Sequences of synthesis of  $\gamma$ -1 macroglobulin and  $\gamma$ -2 globulin antibodies during primary and secondary responses to proteins, *Salmonella* antigens, and phage, *J. Exp. Med.*, 1963, **117**, 889.
36. LoSpalluto, J., Miller, W., Jr., Dorward, B., and Fink, C. W., The formation of macroglobulin antibodies. I. Studies on adult humans, *J. Clin. Inv.*, 1962, **41**, 1415.
37. Uhr, J. W., and Finkelstein, M. S., Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage  $\phi$ X 174, *J. Exp. Med.*, 1963, **117**, 457.
38. Bellanti, J. A., Eitzman, D. V., Robbins, J. B., and Smith, R. T., The development of the immune response. Studies on the agglutinin response to *Salmonella* flagellar antigens in the newborn rabbit, *J. Exp. Med.*, 1963, **117**, 479.