SEQUENCES OF SYNTHESIS OF γ-1 MACROGLOBULIN AND γ-2 GLOBULIN ANTIBODIES DURING PRIMARY AND SECONDARY RESPONSES TO PROTEINS, SALMONELLA ANTIGENS, AND PHAGE*

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In a previous study (1) we observed that a single injection of various proteins, erythrocytes, and bacteria into rabbits caused the synthesis first of γ- macroglobulin antibodies and later γ-2 7S globulin antibodies. In extension of that study we sought to obtain more rigorous physicochemical evidence for the nature of the antibodies produced during the primary response and to gain more information about the primary and secondary antibody responses. Accordingly a very sensitive antibody, assay phage neutralization, was employed in conjunction with ion exchange chromatography and other methods for characterizing antibody. The two antibody responses were studied from the standpoint of the sequence of synthesis of the two molecular species of antibody. In an attempt to make a clear-cut distinction between these responses, both were elicited by a single injection of antigen.

Studies of the antibody response to T_2 bacteriophage supported the conclusions derived from experiments with other antigens (1). Comparison of the primary and secondary antibody responses revealed that each was associated with a different characteristic sequence of synthesis of the macroglobulin and 7S globulin antibodies.[1, 2]

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[1] Preliminary reports of these studies have appeared (2, 3).

[2] Recently, Benedict (4) reported that early in the course of immunization of rabbits with bovine albumin the antibodies were predominately macroglobulin but on continued immunization were mainly of a lower molecular weight.

Materials and Methods

The materials and methods previously used (1) are described only briefly; those not utilized previously are described completely.

Antigens.—The following antigens were employed: Purogenated® diphtheria toxoid, Lederle Laboratories, Pearl River, New York, alum-precipitated, containing 160 Lf units/ml; human serum albumin, prepared by alcohol fractionation, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania, containing a small amount of alpha globulin of immunoelectrophoretic analysis (5); bovine gamma globulin, crystalline, Armour Pharmaceutical Company, Kankakee, Illinois, containing a small amount of a contaminating second component by gel diffusion (6) and immunoelectrophoretic (5) analysis; keyhole limpet hemocyanin (KLH), Pacific Biomarine Supply Company, Los Angeles, prepared by centrifugation of the crude serum twice at 11,000 *G* for 10 minutes and once at 59,000 *G* for 3 hours. The pellet was collected and overlaid with saturated NaCl until used. It was dialyzed against buffered saline pH 7.2 (7) before use.

Immunization.—5 to 8 pound albino rabbits of either sex were injected with 5×10^{11} T_2 phage intravenously or into each of the hind foot-pads, with the following amounts of antigens: KLH, 4 mg of protein; diphtheria toxoid, 80 Lf units; HSA, 2 mg; BGG, 2 mg; *Salmonella typhosa*, Eli Lilly & Company, Indianapolis, 0.5 ml. The same amounts were employed for the second injection. All of the soluble antigens were alum-precipitated by addition of an equal volume of 10 per cent alum for use in immunization.

Antisera.—Bleedings were made from the marginal ear vein after application of xylene to a shaved portion of the tip of the ear. Serum was removed from the clotted blood and stored at 4–5°C or at −20°C. All sera were heated at 56°C for 30 minutes before assay.

Growth and Assay of Bacteriophage.—Escherichia coli strain R_2 and bacteriophage T_2, a wild type stock, which had been obtained originally from A. D. Hershey, were employed. Stock bacterial cultures were maintained on tryptose blood agar base (TBAB) (Difco Laboratories, Inc.) slants or plates. Bacterial suspensions for plating bacteriophage were grown in Penassay broth (Difco Laboratories, Inc., antibiotic medium III) at 37°C for 3 to 4 hours in a reciprocal shaker. The stock bacteriophage was produced by infection of the host organisms grown in a modified Fraser-Jerrel medium[3] (8). Phage was added to 1-hour bacterial cultures (approximately 4×10^8 cells/ml) at a multiplicity of 1:2, incubated with shaking at 37°C for 3 to 4 hours, and placed at 4°C overnight to permit completion of lysis. Following the removal of unlysed bacteria and debris by centrifugation at 5000 *G* for 30 minutes, the supernatants contained approximately 10^{11} viable phage particles per ml. These lysates were concentrated by further centrifugation at 18,000 *G* for 20 minutes, and the pellets were resuspended in modified Fraser-Jerrel medium to give stock concentrates containing between 1×10^{12} and 1×10^{13} viable phage per ml. These preparations were stable for several weeks when stored at 4°C in sterile tubes containing a few drops of chloroform.

The numbers of viable phage particles were assayed by a modification of the agar layer method described by Adams (9). Ten- or hundredfold dilutions of the phage suspension were made in Penassay broth in order to give final plaque count between 30 and 300 per plate. 0.1 ml of the appropriate phage dilution, 2 to 3 drops of a 3 hour broth culture of *E. coli*, and 2.5 ml of semisolid agar kept fluid at 50°C were added in rapid succession to a TBAB plate, and the plates were immediately swirled to insure even distribution of phage bacteria. Following

[3] Modified Fraser-Jerrel medium: To 1 liter of salt solution (1.05 per cent Na_2HPO_4, 0.45 per cent KH_2PO_4, 0.2 per cent NH_4Cl, and 0.03 per cent $MgSO_4 \cdot 7H_2O$) are added: 0.3 ml 1 M $CaCl_2$, 1.0 ml 1 per cent gelatin (in 0.5 per cent NaCl), 2.5 gm casamino acids (Difco Laboratories, Inc., Detroit), and 10 ml 50 per cent glucose (autoclaved separately and added immediately prior to inoculation).

overnight incubation at 37°C the plaques were counted and the number of viable phage particles were determined, assuming a plating efficiency of 100 per cent.

Antibody Assays.—The hemagglutination reaction was carried out according to previous descriptions (7, 10) with the exception that the supernatants remaining after sensitization of tannic acid-treated cells with a mixture of protein and pH 6.4 buffer were saved, frozen at −20°C, and employed to sensitize another batch of tannic acid–treated cells. Supernatants could be reutilized at least 4 times with no sacrifice of the specificity and sensitivity of the reaction (2). *Salmonella* O agglutinins were assayed with an ethanol-treated cell suspension of *Salmonella typhosa* which was adjusted to an optical density of 75 with the 40 filter in the Klett colorimeter (11). *Salmonella* H agglutinins were assayed with a formalin-treated cell suspension adjusted to the same optical density as the ethanol-treated suspension (11). Before assaying H agglutinins, the O agglutinins were absorbed from sera by incubation with a heavy suspension of ethanol-treated organisms for 2 hours at 37°C and removal of the bacteria by centrifugation (11). The mixtures of bacteria and serum were incubated at 56°C for 2 hours and overnight at about 5°C, and the end-points read by observation of the patterns of agglutintinated cells on the bottoms of the tubes. Phage-neutralizing antibodies were assayed as follows: 0.5 ml of each dilution of antiserum was mixed with 0.5 ml of a phage suspension containing approximately 3×10^6 particles per ml. Distilled water was routinely used as the diluent for the antiserum and nutrient broth as the diluent for phage because of the inhibiting effect of salts on the neutralization reaction (12, 13). The mixtures were incubated at 37°C for 30 minutes, placed in an ice bath, and assayed for viable phage as described. The percentage of phage neutralized by a given dilution of antiserum was calculated by comparison with the results of platings from control tubes which did not contain antibody.

The limitations of the expression of the antibody content of early antiphage sera in terms of their K values (9) stimulated the development of another assay. It was found convenient to use 90 per cent inactivation of phage as a reference point, and to define the number of neutralizing units in a given serum as the reciprocal of the dilution which inactivated 90 per cent of the phage in ½ hour at 37°C. Since the relationship between dilution and per cent inactivation is linear in this region, only three or four dilutions of antiserum need be assayed in order to determine the dilution causing inactivation of 90 per cent of the phage. A number of experiments indicated that the quantitation of neutralizing antibody by determining the dilution giving 90 per cent inactivation is a reliable method for measuring the 19S γ-1 globulin as well as the 7S γ-2 globulin phage antibodies.

Chromatography.—Separation of proteins by chromatography on substituted celluloses has recently been developed by Sober, Peterson, and collaborators (14, 15) and was employed in these studies to separate the different types of antibody. DEAE cellulose, type 20, was obtained from Brown Company, Hamlin, New Hampshire, and in all of these studies a single lot, having a capacity of 0.87 meq/gm was used. After a series of washes in distilled water, regenerating solution (0.125 M NaOH, 0.125 M NaCl), and 1 N HCl, it was equilibrated with the starting buffer and packed into the columns under 5 pound air pressure. Two concentrations of tris-phosphate buffer (made with phosphoric acid) were used in this work; 0.05 M tris, 0.004 M phosphate (pH 8.6) served as the starting buffer, and 0.35 M tris, 0.35 M phosphate (pH 3.9) as the limit buffer. A continuous gradient of increasing molarity and decreasing pH was obtained by the cone-sphere method of Peterson *et al.* (15). In our studies, 500 ml of limit buffer were used in the Erlenmeyer flask, and 1000 ml of starting buffer in the Florence flask mixing chamber. The gradient was always begun immediately after application of the serum to the column. For analytical experiments, the usual volume of serum used was 5 to 6 ml and the column size was approximately 1.9 × 24 cm containing about 60 ml of absorbent. A larger column measuring about 2.9 × 40 cm and containing about 250 ml was used for the preparative recovery of proteins from 15 to 20 ml of serum. In almost all experiments the maximum

flow rate (100 to 200 ml/hour) was allowed, and the eluate was collected in 25 ml amounts with the use of a radirac fraction collector (LKB Produkter Ab, Stockholm, Sweden). All chromatographic procedures were carried ot at room temperature with the exception of the preparative fractionation which was run at 5°C. Determinations of pH and optical density at 280 mμ were made on all fractions, which were then either assayed directly for neutralizing antibody or dialyzed and concentrated prior to assay. The total protein recovery was 80 to 98 per cent and the recovery of antibody activity was 50 to 100 per cent.

Zone electrophoresis.—3 to 5 ml of serum were placed in a block of polyvinyl chloride (pevikon) (16) 20 × 33 cm. Electrophoresis was carried out in a 0.1 μ barbital buffer, pH 8.6, for 18 22 hours at 375 to 400 volts and at a temperature of 5–10°C. 1 cm strips were cut from the block and the protein eluted with 5 ml saline by displacement filtration through sintered glass filter tubes of porosity A (Ace Glass Company, Vineland, New Jersey). The protein concentration of the eluates was determined by the Lowry method (17). The antibody content of the eluates was assayed directly or after concentration by pervaporation. It was ascertained that the heating of sera at 56°C for 30 minutes had no effect upon the electrophretic patterns or antibody activity of the fractions obtained by zone electrophoresis.

Density Gradient Ultracentrifugation.—0.25 ml of whole serum diluted with an equal volume of isotonic saline was layered over a continuous sucrose gradient from 10 to 40 per cent prepared as described by Edelman *et al.* (18). For analysis, nine to ten 0.5 ml samples were removed sequentially from the top of the tube with a 1 ml syringe and blunt No. 20 needle. Sucrose was removed from the fractions by dialysis against saline for 24 hours in the cold.

Although the sedimentation constants of antibodies appearing in the various fractions have not been determined, for convenience the antibodies appearing in the middle fractions of the density gradient system will be termed 7S gamma globulins and those in the bottom fractions macroglobulin or 19S gamma globulins, although the sedimentation constant of the latter could be greater. Some justification for these tentative designations is derived from a correlation of protein concentrations in the various fractions and the fractions containing maximal antibody activity. Invariably tubes 3 and 4 contained maximal protein concentrations, presumably representing albumin with a sedimentation constant of 4S, whereas lower molecular weight antibody appeared in fractions 4 and 5, presumably corresponding to a sedimentation constant of about 7S. Moreover, the higher molecular weight antibody in or studies has appeared in the same fractions of the sucrose density gradient system as rabbit and human antibodies which have a sedimentation coefficient of 19S (19).

Treatment with Mercaptoethanol.—Treatment with 2-mercaptoethanol was carried out by a modification of the method described by Deutsch and Morton (20). Equal volumes of sera diluted 1:10 and 1 M mercaptoethanol were mixed and incubated at room temperature for 24 hours. The diluent for both sera and mercaptoethanol was buffered saline, pH 7.2, made by mixing 100 ml 0.15 M NaCl and 100 ml of 0.15 M Na_2HPO_4. After incubation the mercaptoethanol was removed by dialysis for 72 hours against several changes of 0.015 M NaCl in the cold.

EXPERIMENTAL

Characterization of Antibody Response to a Single Injection of Phage

In these experiments, rabbits were injected intravenously with 5×10^{11} phage particles. The sera were collected at various intervals and were fractionated by zone electrophoresis, ultracentrifugal techniques, and anion exchange chromatography, and the phage-neutralizing activity of each fraction was determined. The effect of mercaptoethanol on the antibody activity in certain fractions was also investigated.

1. Zone Electrophoretic Fractionation.—The results of these studies were reminiscent of those of the earlier study with other antigens (1). For instance, a serum obtained 6 days after a single injection of phage showed a sharp peak of neutralizing activity in the main γ-globulin peak or the slower moving fractions. A serum taken 14 days after primary inoculation of phage revealed two distinct peaks of neutralizing activity which appeared to represent approximately equal neutralizing capacity. The faster moving γ-globulin corresponded to the fraction which contained neutralizing activity in the 6 day sample, but this serum also now contained antibody activity in the γ-2 globulin rich fraction.

Nine animals have been studied at various times during the primary response by these methods. The general trend of initial synthesis of antibody with mobility of a γ-1 globulin followed by production of antibody with the mobility of a γ-2 globulin was evident in all animals.

2. Ultracentrifugal Studies.—One experiment was performed in the Spinco model E analytical ultracentrifuge, according to the methods of Heimlich *et al.* (21). A partition cell (22) was employed so that the top and bottom portions could be recovered separately after centrifugation and assayed for antibody activity. Centrifugation was carried out at 52,640 RPM. A serum obtained 7 days after a single primary injection of phage with a neutralization titer of 100 units per ml was studied. The calculated sedimentation coefficient, which was not extrapolated to infinite dilution, was 14.9S and was similar to uncorrected values previously reported for macroglobulin antibody (23). Most of the neutralizing activity was found in the bottom of the cell and thus appeared to be associated with material of relatively high molecular weight.

Since these results suggested that the earliest antibody produced against phage was of relatively high molecular weight, the sucrose density gradient method of ultracentrifugation, which results in more clear-cut separation of fractions of different average molecular weight, was employed in the rest of these experiments.

The general pattern of sequence of synthesis of high and low molecular weight antibodies is summarized in Table I. The results illustrate the general trend of early synthesis of macroglobulin antibody, followed within a few days by the appearance of 7S antibody. Both types of antibody increase in titer for several days, after which the 7S variety predominates

A few experiments were done with sera obtained from rabbits months after a single injection of phage. These sera contained predominantly 7S antibody, although the presence of exceedingly small amounts of 19S antibody cannot be excluded.

3. Fractionation by Anion Exchange Chromatography.—Sera obtained 6 days after the injection of phage had been shown by zone electrophoretic and ultracentrifugal techniques to contain antibody activity only in the γ-1 19S-rich fractions. However, when such samples were applied to a DEAE cellulose

TABLE I

Phage-Neutralizing Activity of Ultracentrifugal Fractions of Sera Obtained during Primary Response

Days after primary injection		6	8	10	16
Titer of serum, *units/ml*		45	675	1500	3000
Dilution tested		1:2	1:10	1:25	1:100
Per cent of phage inactivated by fraction					
(Top)	1	—*	—	—	—
	2	—	—	—	—
	3	82.6	90.9	90.9	99.0
	4	85.4	90.9	91.2	99.8
	5	61.0	67.2	63.6	76.4
	6	83.8	89.8	90.6	94.8
	7	95.0	95.4	91.2	98.0
	8	97.2	94.4	95.6	93.2
	9	96.4	92.2	93.2	75.6
(Bottom)	10	91.0	92.0	84.2	57.0

* Less than 50 per cent inactivation.

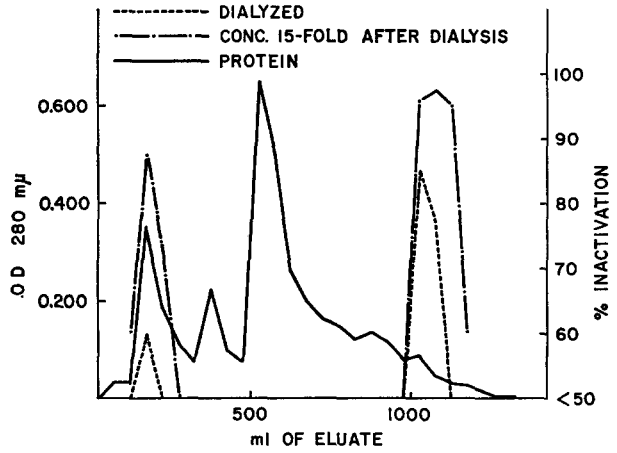

FIG. 1. Distribution of phage-neutralizing activity among fractions prepared by chromatography on DEAE cellulose of a rabbit serum obtained 6 days after a single intravenous injection of 1×10^{11} phage particles. 5 ml of serum was placed on a column 1.9 × 24 cm containing about 60 ml of adsorbent. The starting buffer was tris-phosphate (0.05 M tris, 0.004 M phosphate, pH 8.6) and the limit buffer also tris-phosphate (0.35 M tris, 0.34 M phosphate, pH 3.9). A continuous gradient of increasing molarity and decreasing pH was obtained by the cone-sphere method (15). 500 ml of limit buffer was used in the Erlenmeyer flask, and 1000 ml of starting buffer in the Florence flask mixing chamber. The eluate was collected in 25 ml volumes. The fractions were dialyzed against distilled water and concentrated by pervaporation. The fractions from the column are plotted on the abscissa as milliliters of eluate. The solid line is the protein profile and the dashed and dashed-dotted line the per cent phage neutralized by the dialyzed and dialyzed-concentrated fractions, respectively.

column and fractionated by means of graduent elution, no phage-neutralizing activity was detected in any of the undiluted fractions. These fractions were therefore first dialyzed against distilled water to eliminate the inhibitory effects of salt on the neutralization of phage (12, 13). The dialyzed fractions were concentrated by pervaporation. These treatments revealed a sharp peak of neutralizing activity in the macroglobulin region, and a smaller but still distinct level of activity associated with the main γ-2 globulins (Fig. 1).

When a serum obtained 10 days after a single injection of phage was fractionated on a DEAE cellulose column, two distinct peaks of neutralizing activity were evident, and their locations in the chromatogram were consistent

TABLE II

*Summary of the Chromatographic Studies of the Early Primary Response**

Days after primary injection	Units/ml	Approximate per cent of total actiivity	
		7S region	19S region
6	12	<10‡	>90‡
6	60	20	80
10	700	15	85
10	630	30	70
16	3000	40	60
63	6000	>99	<1

* Sera from different rabbits were used on each day.

‡ Fractions showed neutralizing activity only after dialysis and/or concentration.

with the findings of Peterson *et al.* (15) for the 7S γ-2 and 19S γ-1 globulins. Neutralizing activity in this serum was also associated with two distinct γ-1 and γ-2 globulin peaks on zone electrophoresis and with low and high molecular weight materials after centrifugation in a sucrose gradient.

The results of studies of the two species of antibody by different methods were correlated as follows. The fractions derived by chromatography on DEAE cellulose were concentrated about 50-fold in carbowax. An aliquot of each fraction was mixed with normal rabbit serum and subjected either to zone electrophoresis or to gradient centrifugation. All of the neutralizing activity from peak I was found to be associated with γ-2 7S globulins, whereas that from peak II was associated with γ-1 19S globulins.

Table II summarizes the chromatographic studies of primary response sera. Although the estimation of relative activity in each of the regions of the chromatogram is difficult because of the inhibition by high concentrations of salt and the greater lability of 19S antibody, a sequential appearance of two clearly distinguishable varieties of antibody is evident.

4. The Effect of 2-Mercaptoethanol on 7S and 19S Antibody.—Deutsch and

Morton (20) reported that macroglobulins could be dissociated by sulfhydryl reagents into smaller fragments having a molecular weight of about 160,000. Fudenberg and Kunkel (24) also found that the reductive dissociation of the macroglobulin red cell agglutinins in acquired hemolytic anemia resulted in the loss of hemagglutinating activity. In our earlier study (1) it was found that treatment with mercaptoethanol of rabbit antibodies to proteins and erythrocytes resulted in loss of specific hemagglutinating activity. It was, therefore, of interest to determine the effect of this reducing agent on the phage-neutralizing antibody whose reaction with antigen does not require aggregation and, therefore, conceivably could be achieved by an antibody fragment containing only one intact combining site.

TABLE III

The Effect of 2-Mercaptoethanol on 7S and 19S Antibody

Sample	Treatment	Neutralizing activity	
		Before treatment	After treatment
7S + NRS*	2-Mercaptoethanol	180‡	150
7S + NRS*	Buffered saline	180	150
19S + NRS*	2-Mercaptoethanol	150	2
19S + NRS*	Buffered saline	150	150

* Prepared by ion exchange chromatography. NRS = normal rabbit serum.

‡ Activity expressed as neutralizing units per milliliter.

A 16 day primary serum was separated by chromatography on DEAE cellulose into 7S antibody and 19S antibody fractions, and each of these fractions was mixed with normal rabbit serum. After incubation with mercaptoethanol or buffered saline, a 0.5 ml aliquot from each reaction mixture was immediately spun in a sucrose gradient. Each of the fractions obtained by this procedure was assayed for phage-neutralizing activity. None of the fractions derived from the mixture of 19S antibody with mercaptoethanol showed activity, whereas the appropriate fractions from the mixture of 19S antibody with buffered saline, or of 7S antibody with either mercaptoethanol or buffered saline showed the same activity as the untreated samples. Table III summarizes these results.

Characterization of Antibody Response to a Second Injection of Antigens

1. Response to Proteins.—Figs. 2 and 3 show the results of studies of sera from rabbits immunized with hemocyanin and diphtheria toxoid. Similar data were obtained in experiments with bovine γ-globulin and human serum albumin. Only the peak hemagglutination titers obtained upon assay of appropriate fractions after gradient ultracentrifugation are plotted; therefore, the combined titers of the two types of antibody are not equivalent to the titers

of the whole serum on a particular day. This discrepancy is greater with antisera which have high titers since hemagglutinating activity then tends to spread into fractions adjacent to those expected to contain maximal activity.

From the curves it is evident that the sequence of production of antibodies

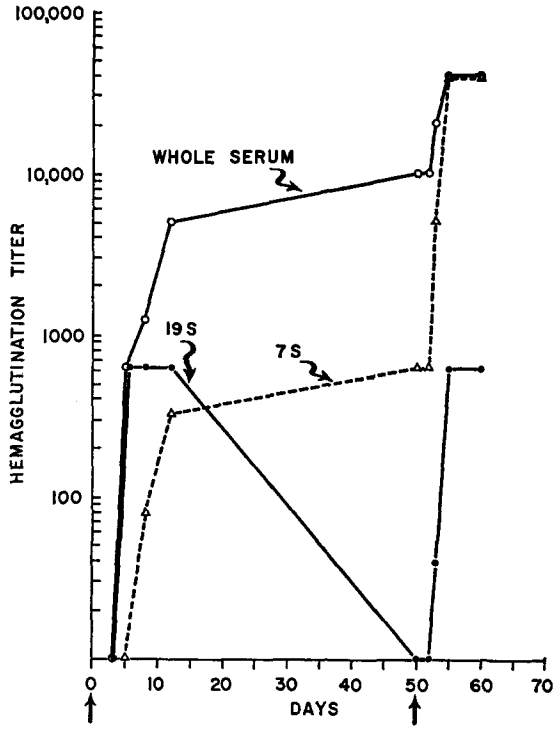

FIG. 2. Distribution of hemagglutinating activity for hemocyanin-conjugated erythrocytes among fractions prepared by sucrose density gradient ultracentrifugation of rabbit sera obtained at various times after a primary and secondary injection of 4 mg of alum-preclpitated hemocyanin into hind foot-pads. Only peak hemagglutinating titer of bottom (19S) or upper (7S) fractions are shown so that 19S and 7S titers for a particular day need not add up to the serum titer on that day. The actual titer is plotted on a logarithmic scale so as to accommodate the wide spread in titers.

associated with 7S and 19S material was strikingly different during the primary and secondary antibody responses. The primary response was characterized by the initial synthesis of macroglobulin antibody followed in a few days by the appearance of 7S γ-2 globulin antibody. While the 7S titers were continually rising the 19S titers were decreasing quickly. The second injection of antigen was followed by a prompt synthesis of high levels of 7S antibody and almost concomitant synthesis of low titers of 19S antibody. The titers of 7S

antibodies during the secondary response were many times those of the primary response. However, the titers of macroglobulin antibodies were usually the same in secondary as in primary response sera.

The serum of one rabbit immunized 6 months earlier with bovine γ-globulin

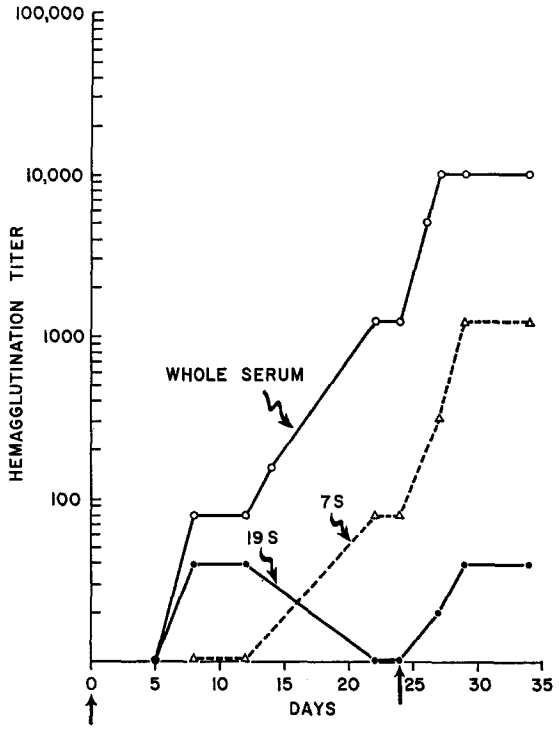

FIG. 3. Distribution of hemagglutinating activity for diphtheria toxoid–conjugated erythrocytes among fractions prepared by sucrose density gradient ultracentrifugation of rabbit sera obtained at various times after primary and secondary injections of 80 Lf units of alum-precipitated diphtheria toxoid into each hind foot-pad. See legend for Fig. 2 for other features of this experiment.

was studied. Hemagglutination titrations of the various fractions derived by gradient ultracentrifugation revealed that antibody activity was associated with 7S material.

Checks on the efficacy of separation of 7S and 19S fractions by gradient ultracentrifugation and the correlation of these results with the results of other methods were made. The possibility that the activity in the bottom fractions of secondary response antisera might be due to contamination of these fractions with small amounts of 7S antibody was eliminated by recentrifuging these fractions in another gradient. Again antibody activity was found only in the

bottom fractions. Further identification of the two types of antibody globulin produced during the secondary response was made by zone electrophoresis. The major antibody activity was associated with material with an electrophoretic mobility of γ-2 globulin while a lesser amount migrated as a γ-1 globulin.

2. Sequence of Synthesis of Anti-Salmonella O and H Antibodies during Primary and Secondary Antibody Responses.—We observed previously that the O antigen of *Salmonella typhosa* stimulated the production of macroglobulin agglutinins in the rabbit (1). Since the O antigen is a lipopolysaccharide and the H antigen a protein, it was of interest to determine the types and sequence of synthesis of antibodies to these components during the primary and secondary responses. Primary injection of these organisms caused the synthesis in a few days of macroglobulin anti-O agglutinins which declined rapidly in titer. Secondary and tertiary injections of this antigen caused the reappearance of macroglobulin anti-O agglutinins usually no higher, and usually somewhat lower than provoked initially. The H agglutinin response after primary injection of antigen comprised the usual sequence of macroglobulin antibody during the first 7 days followed by 7S agglutinins. The second injection of the microorganisms induced a sharp rise in 7S H agglutinins. Macroglobulin H agglutinins have been observed in two of four rabbits during the secondary response, about 9 days after the booster injection.

3. Characterization of the Antibody Response to a Second Injection of Phage.—*Zone electrophoretic fractionation:* The sera obtained from a rabbit 5 and 6 days after a single second injection of phage were pooled and fractionated by zone electrophoresis. The bulk of the neutralizing activity was associated with the γ-2 globulin peak, but there was the suggestion of a small peak of activity in the γ-1 globulin region. However, there was so much γ-2 globulin antibody in this serum that the small peak may have been contaminated with γ-2 globulin. When a pool of 12- and 14-day sera from the same animal was also studied, the antibody activity again was predominantly associated with the γ-2 globulin region. The activity peak was very broad, extended well over into the β-globulin region and suggested, consequently, that some of the antibody was γ-1 globulin.

Sucrose gradient ultracentrifugal studies: In view of the indecisive experiments with the zone electrophoretic technique, sera were next examined after centrifugation in a sucrose gradient. Table IV summarizes the findings of some of these experiments. It is evident that at the higher dilutions (the first column of figures for each animal) neutralizing activity is associated with low, probably 7S, molecular weight material, whereas at the lower dilutions activity may be associated with material of higher molecular weight, either 19S or aggregates of lower molecular weight substances.

Sera obtained 2, 4, and 11 months after the second injection of antigen were

spun in a sucrose gradient and the fractions tested at two dilutions. At 1:1000 a peak of activity was found associated with the fractions which contain 7S globulins but at a 1:100 dilution activity was also evident in the bottom fractions of two of the three samples. Further study by other methods obviously is needed to resolve the question of the nature of antibodies present in serum months after a second injection of antigen.

Anion exchange chromatography: When sera obtained 7 days after a second injection of phage were fractionated on a column of DEAE cellulose and the

TABLE IV

Phage-Neutralizing Activity of Ultracentrifugal Fractions of Sera Obtained during Secondary Response

Days after second injection		4		6		8		10	
Animal No.		41-33		42-51		42-51		42-49	
Titer of serum, *units/ml*		10,000-15,000		30,000		40,000		80,000	
Dilution tested		1:250	1:150	1:500	1:100	1:1000	1:100	10,000	1:1000
Per cent of phage inactivated by fraction									
(Top)	1	—	—	—		—		—	
	2	—		—		—		—	
	3	75.0		99.7		98.8		81.5	
	4	99.7		99.7		99.9		98.8	
	5	99.9		99.9		99.2		93.5	
	6	99.8		81.5	99.9	60.0	99.7	—	
	7	63.0	99.6	—	90.4	—	97.0	—	96.5
	8	—	91.9	—	53.0	—	83.0	—	69.2
(Bottom)	9	65.0	98.2	—	89.3	—	95.6	—	91.9

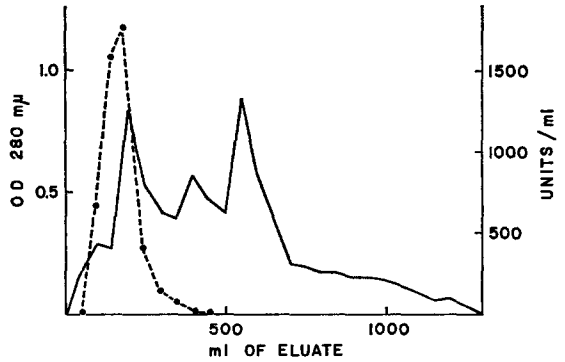

FIG. 4. Distribution of phage-neutralizing activity among fractions prepared by chromatography on DEAE cellulose of a rabbit serum obtained 10 days after a second intravenous injection of 1×10^{11} T_2 phage particles. Conditions are as described for Fig. 1, with the exception that fractions were dialyzed but not concentrated. Results are designated as in Fig. 1.

fractions assayed, neutralizing activity was found only in those fractions associated with 7S γ-2 globulins. Fig. 4 shows the fractionation of a serum taken 10 days after the secondary antigenic stimulus. It indicates a high peak of activity in the first protein peak off the column (containing γ-2 globulins). When the fractions which usually contain macroglobulins were tested without dilution they still showed less than 50 per cent inactivation of phage. Dialysis and concentration of the macroglobulin fractions from 7- to 10-day secondary sera prior to assay revealed low levels of antibody activity; however, this activity was not clearly separable from the tailing of the peak containing the 7S fractions and its significance must be dubious. The results of these experiments can be contrasted with the study of a 10 day primary serum which showed neutralizing activity in the macroglobulin fractions. A serum taken 4 months after a third injection of phage was also chromatographed on DEAE cellulose and the fractions assayed. Phage-neutralizing activity was essentially all associated with the 7S fractions, but some activity was detected in the 19S fractions after extensive dialysis and concentration. The latter activity appeared to be associated with the tail of the highly active 7S material, did not emerge as a distinct peak and was, therefore, not considered significant.

DISCUSSION

Additional physicochemical evidence is presented for the synthesis of two distinct species of antibody during the primary response of the rabbit to the foot-pad or intravenous injection of a number of different protein, phage, or bacterial antigens. Two types of antibody have been observed after the injection of bovine γ-globulin, human serum albumin, diphtheria toxoid, keyhole limpet hemocyanin, performate-oxidized ribonuclease,[4] *Salmonella typhosa* H antigen, α-amylase of *Bacillus subtilis*,[4] human type O erythrocytes, sheep erythrocytes, and T_2 bacteriophage. Although frequent cross-checks of the results of one method of characterizing these antibodies by the other methods have not disclosed any inconsistencies, homogeneous preparations of 19S and 7S antibodies are required for more rigorous characterization. Efforts are now being made to make such preparations.

The possibility that each of the different molecular types of antibody is directed against a different antigenic determinant on a single homogeneous antigen must be considered. The occurrence of macroglobulin and 7S globulin neutralizing antibodies to diphtheria toxin (1) suggests that both species of antibody combine with similar or vicinal antigenic determinants of the toxin. The macroglobulin and 7S globulin neutralizing antibodies for T_2 phage pre-

[4] The antibodies directed against the alpha amylase of *B. subtilis* thus far have been characterized only by zone electrophoresis as γ-1 and γ-2 globulins. The antibodies against performate-oxidized ribonuclease have been characterized only by sucrose gradient ultracentrigugation.

sumably both combine with tail protein(s) of the virus. The strongest evidence is provided by the finding that both high and low molecular weight antibodies can be produced against the same sulfanilazo haptenic determinant group in the same rabbit (25).

The loss of phage-neutralizing activity of macroglobulin fractions after treatment with mercaptoethanol probably is associated with reductive cleavage of the macroglobulin. This observation suggests that fragments of the 19S molecule are not simply 7S antibodies or even "incomplete" antibodies. Mercaptoethanol probably affects the combining site of macroglobulin antibody secondarily as a consequence of an alteration in configuration of the whole molecule by reduction of disulfide bonds. The loss of activity of 19S antibodies upon treatment with mercaptoethanol is in direct contrast with failure to affect the serological activity of 7S antibodies with sulfhydryl reagents unless the molecule is treated with a proteolytic enzyme (26). These observations suggest that the disulfide bond(s) are accessible in the native 19S antibody and inaccessible in the undegraded 7S molecule.

There is a distinct difference in the sequence of molecular forms of antibody synthesized by the rabbit in response to primary and secondary injections of T_2 phage, diphtheria toxoid, human serum albumin, bovine γ-globulin, keyhole limpet hemocyanin, and H antigen of *Salmonella typhosa*. The data with T_2 phage, depending upon a very sensitive assay, particularly support the conclusion that the primary injection of antigen elicited the synthesis of macroglobulin antibody[5] followed within a few days by the appearance of a lower molecular weight antibody. The second injection of a variety of antigens was followed by the rapid appearance of 7S γ-globulin antibody with peak titers many times the primary peak titers. Several days later macroglobulin antibody appeared against all of these antigens and attained peak titers the same or lower than the primary peak titers. The 7S antibody titers of the secondary response were 10 to 80 times higher than those of macroglobulin antibody. The antibodies which persisted for up to 6 months after a single injection of bovine γ-globulin, for 5 months after a single injection, or 11 months after a second injection of T_2 phage were predominantly 7S γ-globulin. This general scheme accommodates recent observations of the heterogeneous response of rabbits and other species to a variety of antigens (1, 4, 27–31). More study is required to determine whether this scheme applies to other antigens in the rabbit as well as in other species.

The only exceptions to this scheme involve the response to sheep and human erythrocytes, to the O antigen of *Salmonella typhosa*, and to the sulfanilazo hapten (25). Primary, secondary, and tertiary injections of the O antigen

[5] The studies with dialyzed and 15-fold concentrated fractions obtained by chromatography on DEAE cellulose revealed a small amount of 7S antibody in early primary antiphage sera. It is likely, however, that study of even earlier sera would have revealed 19S antibody exclusively.

resulted in the synthesis of macroglobulin antibody only. Following single injections of the human or sheep erythrocytes only macroglobulin agglutinins were produced. A second or third injection of these cells resulted in the synthesis of 7S γ-2 globulin antibodies.[6] Repeated courses of immunization of the hapten are required before 19S antibody appears (25). These observations suggest that the type of antibody produced may depend upon the chemical nature of the antigen since the proteins, including the tail antigen(s) of T_2 phage and flagellar antigen of *Salmonella*, induced the synthesis of two forms of antibody whereas the lipopolysaccharide O antigen and the polysaccharide antigens of erythrocytes led to the synthesis of macroglobulin antibodies only. The observations with the O antigen, if substantiated with other lipopolysaccharide and polysaccharide antigens, may help to explain the lack of an anamnestic response with this type of antigen, since anamnesis seems to be associated with the synthesis of 7S γ-2 globulin antibody. These observations may also explain the higher titers which may be observed with H compared to O antigens since the booster-type response is observed only with the flagellar antigen.

Recently LoSpalluto *et al.* (32) reported that neonatal infants, older children, and adult humans produced 19S and little or no 7S antibody after initial immunization with typhoid-paratyphoid antigens. In premature infants the change from 19S to 7S antibody to paratyphoids A and B was virtually complete at 13 weeks of age, whereas the change in typhoid H agglutinins from 19S to 7S antibodies was slower. In adults the change in typhoid H and paratyphoid A and B agglutinins from 19S to 7S required more than 6 months and few individuals showed a complete change. Booster injections of these organisms resulted in the production of 7S antibodies exclusively. Typhoid O agglutinins were found only in the 19S fraction in all of these groups and no change occurred with time or secondary immunization. Uhr *et al.* (33) recently found the antibody response to bacteriophage ϕX 174 in newborn premature infants to consist in the formation of 19S antibody which changed to 7S antibody 2 to 6 weeks after immunization. When compared to the data reported for the rabbit, it appears that age and species as well as type of antigen influence the nature, sequence and tempo of the synthesis of different molecular forms of antibody.

On the basis of the available data, the following working hypothesis has been developed. The primary injection of proteins induces 19S antibody globulin synthesis in certain cells. Since such synthesis can occur in neonatal animals in the apparent absence of plasma cells (34), it may be surmised that this synthesis does not require this type of cell. The small amount of 19S antibody synthesized and the rapidity with which this response ceases relative to the 7S globulin antibody response suggest that macroglobulin synthesis is performed by only a few cells, by short-lived cells, by cells of limited synthetic

[6] The studies of Stelos and Talmage (27) previously revealed that several injections of foreign erythrocytes were required before 7S agglutinins were synthesized by the rabbit.

capacity, or a combination of these effects. The short-lived nature of the primary macroglobulin response and failure to obtain a greater macroglobulin response to a second than to the first injection suggest that little or no replication of 19S-producing cells or templates occurs after the primary antigenic stimulus. The 19S response which follows the second injection would, therefore, be most readily explained as the result of the induction of antibody synthesis in a new group of unprimed cells.

It is also postulated that the primary injection of antigen causes the induction of 7S antibody globulin synthesis in cells other than those which synthesize macroglobulin antibody. Induction of this synthesis leads to a more prolonged response to the first as well as to the second antigenic stimulus. It is likely that the plasma cell is associated with 7S antibody synthesis. It is also likely that proliferation of some cell type of this series is involved both in the prolonged response as well as in the more prompt 7S antibody globulin response which follows after the second injection of antigen (35). The second injection of antigen apparently triggers 7S γ-globulin synthesis in cells somehow activated by the primary antigenic stimulus. Although the titer of 19S antibody during the secondary response is the same or lower than during the primary response, further study is required to determine whether this antibody appears sooner after the second injection; *i.e.*, whether the 19S response differs in any respect after the second antigenic stimulus.

Although much more evidence is needed, the available data suggest that different cells are involved in the synthesis of the 7S and 19S γ-globulin antibodies. The two antibodies are synthesized concurrently or at different times, depending upon the chemical composition of the antigen, whether synthesis follows a primary or secondary stimulus, and the age of the animal. Thus the 19S globulin antibody response is short-lived compared to the 7S and declines while the 7S is rising. Furthermore, lymph node fragments from primarily immunized animals synthesize macroglobulin antibody *in vitro* and do not shift to the synthesis of 7S globulin antibody during prolonged culture, whereas the contralateral node left *in situ* does make this shift (1). Further study is required to determine whether morphologically distinct cells synthesize the two species or antibody or whether morphologically similar cells synthesize both species of γ-globulin.

The present observations indicate a *qualitative* distinction between the primary and secondary antibody responses which has not hitherto been available. If our interpretation of the available data is correct, the determination of the type(s) of antibody synthesized following the injection of an antigen may serve to identify the response as primary or secondary in type.

The significance and implications of the sequential synthesis of two molecular types of antibody are unknown. Further studies of the structure and the cellular origins of these two types of antibody are needed. The present studies

as well as experiments reported previously (1) suggested that the two types of antibody may be synthesized either by spleen or lymph nodes. Although two different cell types are postulated as being responsible for the synthesis of the two forms of antibody, it is conceivable that these cells have a common precursor or that the 7S-forming cells are derived from the 19S-forming cells or that the two cells have independent stem cells. These problems are related to many others such as the relation of the type of antibody response to the nature of the antigen. The proteins may affect both the postulated 7S and 19S cells, whereas the lipopolysaccharides or polysaccharides may invoke a predominantly 19S cell response. Finally, it may be postulated that the macroglobulin-synthesizing system simply is an auxiliary system for antibody synthesis, perhaps the first to develop phylogenetically (31) and ontogenetically (34), rather than a prerequisite system for the quantitatively more significant 7S globulin antibody response.

SUMMARY

The nature of the antibodies produced by the rabbit during the primary and secondary responses to T_2 phage, proteins, and the O and H antigens of *Salmonella typhosa* has been determined. Immune sera have been fractionated by zone electrophoresis, sucrose density ultracentrifugation, and anion exchange chromatography. The resulting fractions have been assayed by phage neutralization or hemagglutination (antisera to proteins) or bacterial agglutination. In confirmation and extension of earlier work from this laboratory, the primary response to these antigens, with the exception of the O antigen of the *Salmonella*, included the early synthesis of 19S, γ-1 globulin antibody, and the later synthesis of 7S, γ-2 globulin antibody. The primary response to the O antigen consisted of the synthesis of only a macroglobulin agglutinin. The secondary response to the proteins, including the H antigen of the *Salmonella*, comprised the early synthesis of large amounts of the 7S γ-2 globulin antibody to the same level attained during the primary response. The secondary response to the phage consisted in the synthesis of 7S, γ-2 globulin antibody alone. Treatment of the macroglobulin phage-neutralizing antibody with mercaptoethanol resulted in complete loss of its neutralizing activity.

A working hypothesis to explain these observations was presented. A salient feature of this hypothesis was the suggestion that different cells synthesized the two distinct molecular forms of antibody. The significance of the sequential synthesis of the two forms of antibody is not known. It was proposed that the system for synthesis of macroglobulin antibody is an auxiliary system for antibody synthesis, perhaps the first to develop phylogenetically and ontogenetically. It is felt that the present observations indicate a clear-cut qualitative distinction between the primary and secondary responses to immunization whereby these responses might be identified in various experimental situations.

It is also felt that these findings with the primary and secondary responses to various antigens in the rabbit may be of widespread occurrence in nature among a variety of species.

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