[4] This is essentially the formula proposed (but proved only for $r = 2$) in the note cited in reference 1. A more explicit (but as yet unpublished) formula has recently been obtained by Glen Bredon by entirely different methods.

---

## ON THE DIFFERENT MOLECULAR FORMS OF ANTIBODY SYNTHESIZED BY RABBITS DURING THE EARLY RESPONSE TO A SINGLE INJECTION OF PROTEIN AND CELLULAR ANTIGENS*

BY D. C. BAUER† AND A. B. STAVITSKY

DEPARTMENT OF MICROBIOLOGY, WESTERN RESERVE UNIVERSITY SCHOOL OF MEDICINE

*Communicated by W. H. Taliaferro, August 21,1961*

In the course of attempts to enhance the primary antibody response to diphtheria toxoid, rabbits were injected with *Salmonella typhosa* vaccine followed by the toxoid. Antitoxoid appeared earlier and in higher titer in these rabbits than in controls, which received only toxoid. When the titers of the sera from *Salmonella*-primed animals were plotted against time after injection of toxoid, a biphasic curve with peak titers at about 9 and 15 days was obtained. This curve suggested that two distinct species of antidiphtheria toxoid were formed during the early response to a single injection of this antigen. Subsequent studies confirmed this suggestion. Since we were aware of previous reports of the synthesis of different molecular forms of antibody in man,[1-3] rabbit,[4] and horse[5, 6] following multiple injections of antigens, we wondered whether the rabbit generally formed more than one species of antibody to a single injection of various antigens. In the study to be described, it was found that the single injection of a variety of protein and cellular antigens into the rabbit regularly resulted in the synthesis first of a $\gamma$-1 macroglobulin antibody and several days later the synthesis of a $\gamma$-2 globulin antibody of lower molecular weight. This paper presents physico-chemical and serological evidence for the existence and the sequence of appearance of these two molecular species of antibody.

*Materials and Methods—Antigens:* Antigens were obtained from the following sources: Purogenated® diphtheria toxoid—Lederle Laboratories; bovine $\gamma$ globulin (BGG)—Armour Laboratories; keyhole limpet hemocyanin (KLH)—Pacific Biomarine Supply Co., Los Angeles, California; typhoid-paratyphoid A and B or typhoid vaccine—Eli Lilly Co.; sheep erythrocytes—Cappel Laboratories; human serum albumin (HSA)—prepared by alcohol fractionation, Merck, Sharpe and Dohme Co.; Diphtheria toxin (76 K-A) containing 32 Lf units per ml was obtained from the Commonwealth of Massachusetts, Department of Public Health, Division of Biologic Laboratories, Boston, Massachusetts. This toxin was utilized for the assay of diphtheria antitoxin by the neutralization assay. A 5-times recrystallized preparation of diphtheria toxin[7] was obtained from Dr. C. G. Pope, Wellcome Research Laboratories, Beckenham, Kent, England. It contained 3,000–3,200 Lf units per mg of protein nitrogen. The diphtheria toxoid, recrystallized diphtheria toxin, and hemocyanin were shown to be heterogeneous by gel diffusion[8] and immunoelectrophoretic[9] analysis, but by these same criteria the bovine $\gamma$ globulin and human serum albumin consisted essentially of one component with a small amount of a contaminating second component.

*Radioactive antigen:* $I^{131}$-labelled HSA was obtained from Abbott Laboratories. This preparation contained some $\alpha$-globulin as judged by immunoelectrophoretic analysis.

*Immunization:* Albino rabbits of either sex and weighing 3–4 kilos were injected in each of the

hind foot pads with the following amounts of antigens: diphtheria toxoid—80 Lf units; BGG, KLH, and HSA—2 mg of protein; typhoid-paratyphoid vaccine and 50% suspension of washed sheep erythrocytes—0.5 ml. The same amounts of antigens were used to restimulate the antibody response. All of the soluble antigens were alum-precipitated for use in immunization.

*Antisera:* Bleedings were made from the marginal ear vein. Serum was removed from the clotted blood and stored at 4–5°C or −20°C. All sera were inactivated at 56°C for 30 min before analysis.

*Antibody assays: The hemagglutination and hemagglutination-inhibition* procedures were performed as described previously.[13] The specificity of hemagglutination was frequently tested by the inhibition procedure. For the assay of hemagglutinins to sheep erythrocytes, the cells were standardized as in the tanned-cell procedure.[13] *Salmonella* O agglutinins were assayed with an ethanol-treated suspension of *Salmonella typhosa* which was adjusted to an optical density of 75 with the 40 filter in the Klett colorimeter. *Salmonella* H agglutinins were assayed with a formalin-treated cell suspension adjusted to the same optical density as the ethanol-treated suspension. The O agglutinins were absorbed from sera before assaying for the H agglutinins. Antibody was also detected by the *binding of radioactive antigen,* based on the method of Feinberg.[10] Labeled antigen was mixed either with diluted antiserum or with a fraction of serum isolated by zone electrophoresis[11] or by centrifugation in a sucrose gradient.[12] The mixture was incubated for two hours at +5°C. Sufficient sheep anti-rabbit $\gamma$-globulin was then added to precipitate all of the rabbit $\gamma$-globulin in the mixture and incubation carried out for 1–2 hr at 37°C. Precipitation was completed by further storage for 1–2 days at +5°C. The resulting precipitate was washed twice with 5 ml of pH 7.2 buffered saline[13] and finally dissolved in 0.1 ml 0.1 *N* NaOH and 0.4 ml distilled water. 0.2 to 0.3 ml of dissolved precipitate was plated on aluminum planchets, dried, and counted in a Nuclear-Chicago automatic gas flow counter. As controls, the labeled antigens were mixed with normal rabbit serum or with a heterologous antiserum, before the addition of the sheep anti-rabbit $\gamma$-globulin. The resulting complex was treated as above. The resulting radioactivity was deducted as a blank from the values obtained with the specific antisera or fractions thereof. *Interfacial precipitation tests* were performed in 6 × 50 mm tubes by layering the antigen over the antiserum and observing the formation of a precipitate at the interface.

*Neutralization assay* for diphtheria antitoxin was performed according to the method of Fraser[14] by incubation of toxin-antiserum or toxin-zone electrophoretically-separated fraction mixtures for varying periods at room temperature or +5°C before intradermal inoculation into the shaved skin of rabbits.

*Zone electrophoresis:* Three to five ml of serum were placed in a trough cut in a block of polyvinyl chloride (Pevikon)[11] 20 cm by 33 cm. Electrophoresis was carried out in a 0.1 $\mu$ barbital buffer, pH 8.6, for 18 to 22 hr at 375 to 400 volts and at a temperature of 5° to 10°C. One-cm strips were cut from the block and the protein eluted with 5 ml saline by displacement filtration or centrifugation. The protein concentration of the eluates was determined by the Lowry method.[15] The antibody content of the eluates was assayed directly or after concentration by pervaporation. It was determined that the heating of sera at 56°C for 30 min to inactivate complement had no effect upon the electrophoretic patterns or antibody activity of the fractions obtained by zone electrophoresis.

*Density gradient ultracentrifugation:* Whole serum, diluted 1:1 with isotonic saline, was ultracentrifuged for 16–17 hr at 35,000 r.p.m. in a continuous sucrose gradient prepared as described by Edelman *et al.*[12] For analysis, 0.5-ml samples were removed sequentially from the top of the tube with a 1-ml syringe and #20 needle. Sucrose was removed from the fractions by dialysis against saline for 24 hr.

*Results—Enhancement of primary antibody response to diphtheria toxoid:* As a basis for the study of the enhancement of the synthesis of antitoxoid by *Salmonella,* 80 Lf units of alum-precipitated diphtheria toxoid was injected into the hind foot pads of rabbits. Hemagglutinating antibody appeared in low titer within 8–15 days with an average of 10 to 11 days. The antibodies produced during the primary response in these rabbits were stable upon storage at +4°C or −20°C. The results of a typical experiment are illustrated in Figure 1, in which diphtheria toxoid was

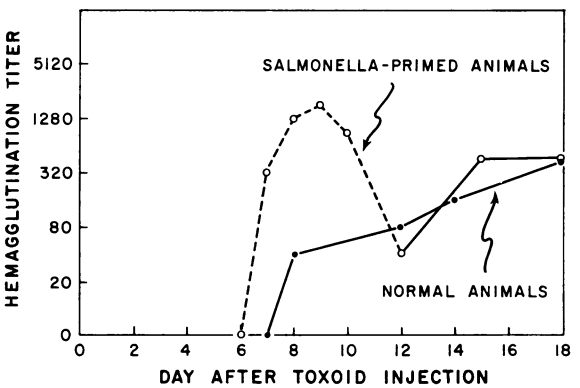


FIG. 1.—The effect of prior injection of *Salmonella* vaccine on the primary antibody response to diphtheria toxoid. The data represent the average response of 12 rabbits treated with *Salmonella* vaccine and 6 rabbits which received only the toxoid. The dashed line represents the antibody formed which lost its hemagglutinating activity during *in vitro* storage in the cold and the solid line that antibody which did not lose this activity under these circumstances.

employed as the antigen. Similar results were obtained when the recrystallized diphtheria toxin was utilized as the antigen.

The injection of 0.5 ml of the *Salmonella* vaccine (typhoid or typhoid-paratyphoid) into the foot pads of rabbits 2–5 days before the inoculation of diphtheria toxoid regularly resulted in the appearance of antibody detectable by hemagglutination 7–8 days after the injection of toxoid. The peak titers at this time were over 10 times those obtained in unprimed animals. Between the ninth and twelfth day after toxoid was injected, the titers of the primed animals fell to about 2 per cent of the peak titers. Several days later the curve of the antibody ascended again and attained a peak titer similar to that found in the unprimed rabbits. Figure 1 also depicts the response in primed rabbits.

The antibody which appeared first in the *Salmonella*-primed rabbits consistently lost over 75 per cent of its hemagglutinating activity upon storage at $+5°$ or $-20°C$ in contradistinction to the antibody in the unprimed animals, which did not lose its serological activity under these conditions. The antibody which appeared later in the primed animals did not, however, lose hemagglutinating activity in the cold.

Fractionation of the early response sera by zone electrophoresis followed by hemagglutination titrations of the resulting fractions revealed that the antibody which appeared initially in *both* primed and unprimed rabbits had the mobility of a $\gamma$-1 globulin, whereas the antibody which appeared later had the mobility of a $\gamma$-2 globulin. The specificity of these antibodies in primed and unprimed animals was established by the hemagglutination-inhibition reaction.[13]

Attempts to enhance antibody synthesis to bovine $\gamma$-globulin, hemocyanin, and human serum albumin by the preinjection of rabbits with *Salmonella* vaccine thus far have been inconclusive. While such animals formed both $\gamma$-1 and $\gamma$-2 globulin antibodies, as will be discussed in the next section, the $\gamma$-1 globulin antibodies to these antigens did not lose their serological activity upon storage in the cold as did the $\gamma$-1 globulin antibodies to diphtheria toxoid synthesized in primed rabbits.

*Physico-chemical properties of antibodies produced during early antibody response to proteins:* The findings of $\gamma$-1 and $\gamma$-2 globulin antibodies to diphtheria toxoid recalled the observation by Stelos and Talmage[4] first of $\gamma$-1 and then $\gamma$-2 globulin antibodies to sheep red cell antigens in rabbits which had been injected repeatedly with these cells. In addition, the observation of two forms of antibody globulin in response to a single inoculation of diphtheria toxoid prompted us to explore the possibility that these two species of antibody were formed consistently in response to a *single* injection in the rabbit of a variety of antigens. Eventually, it was found that the injection of proteins other than diphtheria toxoid into *unprimed* rabbits also resulted first in the synthesis of antibodies with the mobility of $\gamma$-1 globulins. Figure 2 shows the results of an electrophoretic separation of a 5-day antiserum to KLH. Anti-KLH activity is present in the faster-migrating $\gamma$-1 globulin fractions. Three days later, some $\gamma$-2 globulin antibody was demonstrable (Fig. 2). Similar results were obtained with antisera to BGG and HSA collected at various times after immunization.

These early antisera have been characterized further by ultracentrifugation in a sucrose gradient.[12] By this procedure, antibody activity was detectable only in the fastest sedimenting fractions, indicating that the antibody was a macroglobulin. Fractionation of sera obtained from later bleedings showed the gradual appearance of an antibody with a lower sedimentation coefficient. The results of a typical experiment with antisera to BGG are shown in Table 1. Protein determinations[15]

TABLE 1

DENSITY GRADIENT ULTRACENTRIFUGATION OF PRIMARY RESPONSE ANTISERA TO BGG IN UNPRIMED RABBITS

| | 7 day | | 14 day | | 30 day | |
|---|---|---|---|---|---|---|
| Fraction | Mg protein/ml | HA titer | Mg protein/ml | HA titer | Mg protein/ml | HA titer |
| 1 (top) | 0.16 | 0* | 0.10 | 0 | 0.04 | 0 |
| 2 | 2.30 | 0 | 3.76 | 0 | 1.70 | 0 |
| 3 | 4.80 | 0 | 5.00 | 0 | 4.80 | 160 |
| 4 | 4.16 | 0 | 3.90 | 160 | 4.60 | 320 |
| 5 | 1.40 | 0 | 2.24 | 80 | 1.70 | 160 |
| 6 | 1.00 | 0 | 0.92 | 20 | 0.92 | 40 |
| 7 | 0.74 | 40 | 0.36 | 0 | 1.42 | 0 |
| 8 | 0.20 | 80 | 0.18 | 20 | 0.93 | 0 |
| 9 (bottom) | 0.70 | 160 | 0.22 | 40 | 1.20 | 20 |

* O titer = less than 10. HA = reciprocal of hemagglutination titer.

on each of the fractions showed a maximum concentration in tube 3. Assuming this to be primarily albumin with a sedimentation coefficient of 4*S*, the lighter antibody is probably of the usual 7*S* type. Whether the heavier antibody has a sedimentation coefficient of 19*S* has not been determined precisely, but this antibody does appear in the same fractions as rabbit and human antibodies which have a sedimentation coefficient of 19*S*.[6] Following zone electrophoresis of the 10-day serum from the same rabbit, antibodies were associated with both $\gamma$-1 and $\gamma$-2 globulin fractions. Only traces of antibody activity were apparent in the $\gamma$-1 globulin fraction from a 30-day sample of serum (Table 1).

In Table 2, some physical properties of primary response antibodies to four proteins are presented. Diphtheria toxoid, KLH, BGG, and HSA stimulated first the production of $\gamma$-1 macroglobulin antibodies. Three to five days later, antibody activity was also associated with $\gamma$-2, 7*S* globulins. It is apparent from the results

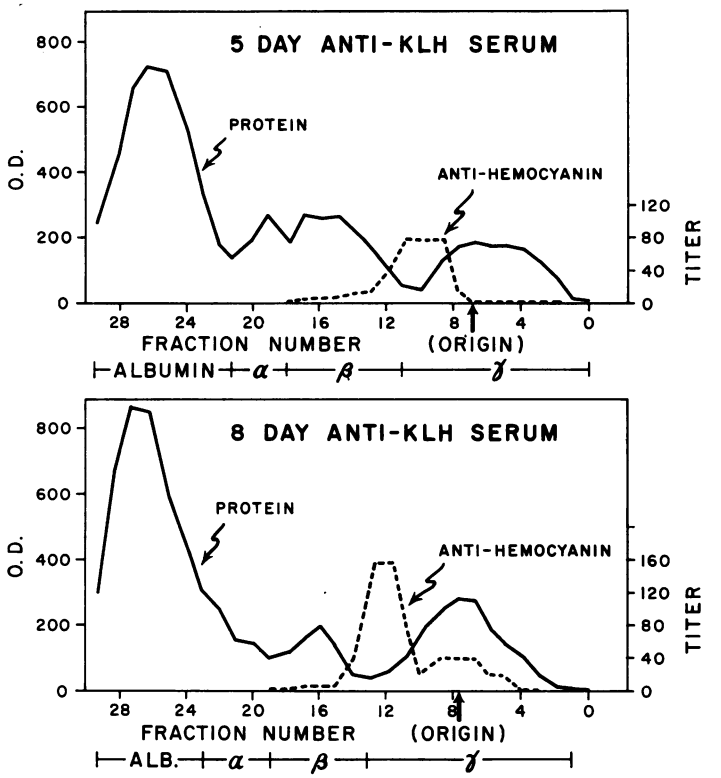


FIG. 2.—Zone electrophoresis of primary response antirsera to hemocyanin five and eight days after a single injection of the antigen. Five ml serum eletrophoresced for 21 hr at 400 volts. One-cm strips eluted with 5 ml 0.15 *M* phosphate buffered saline, pH 7.2.

with the 24-day anti-diphtheria toxoid serum that the γ-1 globulin antibody is gradually lost.

Table 3 presents data on the binding of radioactive HSA by γ-1 and γ-2 globulin fractions according to the method of Feinberg.[10] It is clear that these antibodies, presumably macroglobulins, bind specific antigen specifically because uptake was blocked by preincubation of the whole serum with HSA but not by preincubation with egg albumin.

Deutsch and Morton[16] reported that human 19*S* macroglobulins were dissociated into 7*S* components when treated with sulfhydryl compounds. As shown in Table 4, incubation with 2-mercaptoethanol of diluted early-response sera, γ-globulin or 19*S* and γ globulin fractions of these antisera abolished their hemagglutinating and precipitating activities. These activities were not lost upon similar treatment of sera collected later during immunization or of γ-2 or 7*S* γ globulin antibody fractions.

*Specificity and homogeneity of macroglobulin antibodies:* The specificity of the macroglobulin antibodies was examined by the hemagglutination–inhibition technique.[13] In all cases the hemagglutination activity of whole serum or serum fractions was inhibited only by specific antigen. It is evident, therefore, that the macroglobulin antibodies are highly specific.

TABLE 2

PHYSICAL PROPERTIES OF PRIMARY RESPONSE ANTIBODIES TO PROTEINS

| Antigen | Day after primary injection | Gamma-1 | Gamma-2 | Heavy* | Light* |
|---|---|---|---|---|---|
| BGG | 7 | −† | − | +‡ | 0§ |
| " | 10 | + | + | + | + |
| " | 30 | + | + | + | + |
| " | 180 | − | − | 0 | + |
| Diph. Tox. | 8 | − | − | + | 0 |
| " | 12 | + | + | + | + |
| " | 24 | 0 | + | 0 | + |
| KLH | 5 | + | 0 | + | 0 |
| " | 8 | + | + | + | + |
| " | 50 | − | − | 0 | + |
| HSA | 7 | − | − | + | 0 |
| " | 11 | − | − | + | + |
| " | 17 | + | + | − | − |

* On the basis of ultracentrifugation in a sucrose gradient.
† (−) not done.
‡ (+) antibody present in fraction by hemagglutination.
§ (0) antibody not present in fraction by hemagglutination.

TABLE 3

SPECIFIC BINDING OF $I^{131}$- HSA BY RABBIT ANTI-HSA SERUM AND BY FRACTIONS ISOLATED FROM SUCH A SERUM BY ZONE ELECTROPHORESIS

| Serum # | Reaction Mixture | CPM/precipitate |
|---|---|---|
| 4219 | γ-1 globulin + HSA* | 456 |
| 4219 | γ-2 globulin + HSA* | 1730 |
| 4295 | whole serum + HSA* | 4300 |
| 4295 | " " + HSA | 378 |
| 4295 | " " + EA | 4280 |

HSA* = $I^{131}$ HSA; Reaction mixture: globulin fractions from serum # 4219 plus HSA* before addition of sheep anti-rabbit γ-globulin serum. Whole serum + HSA* = serum plus pH 7.2 buffered saline buffer for 2 hr at + 5°C before addition of 1 mg of HSA*. Whole serum + HSA = serum + 1 mg non-radioactive HSA for 2 hr at + 5°C before addition of HSA*. Whole serum + EA = serum + 1 mg non-radioactive EA for 2 hr at + 5°C before addition of HSA*.

TABLE 4

EFFECT OF 2-MERCAPTOETHANOL ON VARIOUS ANTISERA AND ANTIBODY FRACTIONS

| Antiserum to | Type of sample* | Titer† Buffer | Titer† −SH |
|---|---|---|---|
| KLH | 7-day serum | 2,560(+)‡ | 0(−) |
| " | γ-1 globulin | 80 | 0 |
| " | 50-day serum | 320(+) | 320(+) |
| " | 7S fraction | 320 | 320 |
| BGG | 7-day serum | 80 | 0 |
| " | 19S fraction | 40 | 0 |
| " | 35-day serum | 640 | 640 |
| HSA | 7-day serum | 640(+) | 0(−) |
| " | 19S fraction | 320 | 0 |
| " | hyperimmune serum | 5,120(+) | 5,120(+) |

* 7-day serum collected 7 days after injection of 2 mg alum-precipitated KLH into each hind foot pad; γ-1 globulin prepared by zone electrophoresis; 7S and 19S fractions prepared by sucrose gradient ultracentrifugation.
† Titers after treatment of $^1/_{10}$ dilution of sera in pH 7.2 buffered saline[13] or undiluted globulin fractions with equal volume of pH 7.2 buffered saline (Buffer) or 0.1 *M* 2-mercaptoethanol (-SH) for 48 hr at room temperature followed by dialysis against pH 7.2 buffered saline for 24 hr.
‡ Results of interfacial precipitin tests are shown in parentheses.

The possibility remained that each of the species of antibody produced against KLH was stimulated by different antigenic component(s) of this antigenic preparation. This possibility first was examined by the Ouchterlony technique of double diffusion in agar.[8] An anti-KLH serum which contained over 95 per cent γ-1 globulin antibody and an anti-KLH serum in which essentially all of the activity was in the γ-2 globulin fraction were placed in separate peripheral wells and KLH

at a concentration of 5 mg./ml. in the center well. The γ-1 globulin antibody reacted with the KLH to give 4 discrete lines which were continuous with the 4 lines formed with the γ-2 globulin antibody. A similar relationship was demonstrated by micro-immunoelectrophoretic analysis.[9] KLH was separated into a number of antigenic components by agar electrophoresis. Each of the antisera which had been employed in the double diffusion experiment was then placed in a trough on each side of the separated KLH components. Seven discrete arcs of precipitate were formed between the KLH and the γ-1 globulin antibody. Seven arcs of precipitate were formed between the KLH and the γ-2 globulin antibody, corresponding in position to the lines on the other side of the slide. These results indicate that the γ-1 and γ-2 globulin antibodies were directed against the same seven components in the KLH preparation. Four other arcs of precipitate were formed with KLH and the γ-2 globulin antibody which were not formed with the γ-1 globulin antibody.

The BGG preparation contained a minor impurity which has not been identified. Nevertheless, by the Ouchterlony technique,[8] it was shown that γ-1 and γ-2 globulin antibodies were formed against both the major and minor components in the BGG preparation.

*Effect of temperature on hemagglutinating and neutralizing activity of various antisera and antibody fractions:* Recently Iseki *et al.*[17] produced cold incomplete agglutinins and precipitins to various Lewis blood group antigens in rabbits by immunization with secretor and nonsecretor human saliva. Since cold agglutinins in man have been found to be macroglobulins,[18] we wondered whether rabbit antiprotein macroglobulin antibodies had a lower temperature optimum than did lower-molecular-weight antibodies. The data presented in Table 5 indicate that

TABLE 5

EFFECT OF TEMPERATURE ON HEMAGGLUTINATING AND TOXIN-NEUTRALIZING ACTIVITY OF VARIOUS RABBIT ANTISERA AND ANTIBODY FRACTIONS

| Type of sample | Assay | Conditions | Titer | Neutraliz. |
|---|---|---|---|---|
| 9-day anti-BGG serum #1 | HA† | 37°C | 0 | |
| " " " " | " | 5°C | 320 | |
| 9-day anti-BGG serum #2 | " | 37°C→5°C | 640 (37°C)<br>2,560 (5°C) | |
| 9-day anti-KLH serum | " | 37°C | 640 | |
| " " " " | " | 5°C | 10,240 | |
| 7-day anti-HSA serum | " | 37°C | 160 | |
| " " " " | " | 5°C | 160 | |
| Hyperimmune anti-BGG serum | " | 37°C | 20,480 | |
| " " " " | " | 5°C | 20,480 | |
| Hyperimmune anti-KLH serum | " | 37°C | 2,560 | |
| " " " " | " | 5°C | 5,120 | |
| γ-1 globulin fraction (anti-Neut. diphtheria toxoid)* | | 23°C | | 0 |
| | | 5°C | | ‡ |

* γ-1 globulin prepared by zone electrophoresis from early antiserum from rabbit primed with *Salmonella*.
† HA = incubation overnight at indicated temperature; neutralization = incubation for 1 hr at 23°C or 5°C; 37°→5°C = incubation at 37°C overnight followed by reading of titer, resuspension of cells, incubation at 5°C for 6–12 hr and rereading of titer.
‡ Titers were specific, inhibited only by specific antigen.

some of these early-response sera and γ-1 globulin antibodies (anti-BGG, KLH, diphtheria toxin) indeed are more active at the lower temperature. Since the antibodies in these anti-BGG and anti-KLH sera were localized in the bottom fractions by sucrose-gradient ultracentrifugation, it is presumed that they are

macroglobulins. A similar determination, presumably owing to its lower titer, could not be made on the anti-HSA serum, which did not yield higher titers at 5° than at 37°C. The effect of temperature could be demonstrated in 2 steps by incubating first at 37°C, reading, resuspending the cells and incubation at 5°C, and reading again. The hyperimmune sera did not show enhanced hemagglutinating activity at the lower temperature.

The observation of neutralization of toxin by γ-1 globulin fractions from *Salmonella*-primed rabbits at 5°C but not at 23°C resolved an apparent discrepancy between the results of hemagglutination and neutralization assays on early anti-diphtheria toxoid sera or γ-1 globulin fractions from such sera. The earlier neutralization assays had been carried out at room temperature, which accounts for their negative results despite the positive hemagglutinating activity of these preparations. Thus far, the neutralizing properties at 5°C of anti-diphtheria toxoid sera or γ-1 globulin fractions from such sera of unprimed rabbits have not been determined.

*Physico-chemical properties of antibodies to cellular antigens:* Table 6 summarizes

TABLE 6

PHYSICAL PROPERTIES OF PRIMARY RESPONSE AND HYPERIMMUNE ANTISERA TO CELLULAR ANTIGENS

| Antigen | Day after primary injection | Gamma-1 | Gamma-2 | Heavy* | Light* |
|---|---|---|---|---|---|
| Sheep RBC | 11 | −† | − | +‡ | 0§ |
| " " | 15 | + | 0 | − | − |
| " " | Hyperimmune | + | + | + | + |
| *S. typhosa* O | 5 | − | − | + | 0 |
| " | 10 | + | 0 | + | 0 |
| " | 17 | − | − | + | 0 |
| " | Hyperimmune | + | 0 | + | 0 |
| *S. typhosa* H | 5 | − | − | + | 0 |
| " | 10 | + | + | + | + |
| " | Hyperimmune | + | + | + | + |

* On the basis of density gradient ultracentrifugation.
† (−) not done.
‡ (+) antibody present by agglutination.
§ (0) antibody not present by agglutination.

the results of experiments on the early antibody response to cellular antigens. They indicate that the first detectable antibodies to *Salmonella* O antigen, *Salmonella* H antigen, and sheep red cells are γ-1 macroglobulins. γ-2 globulin antibodies to *Salmonella* O antigen were not synthesized even following 3 injections of these organisms. γ-2 globulin antibodies to the H antigen were synthesized following the primary injection. γ-2 antibodies were not produced against sheep erythrocytes following a single injection but did appear after two injections of these cells.

*Patterns of hemagglutination with macroglobulin antibodies:* In the course of experiments with early response antisera, it was frequently noticed that these sera produced patterns of hemagglutination which were difficult to designate positive or negative on the basis of our usual criteria (see patterns in Stavitsky[13]). Nevertheless, it was possible to demonstrate by antigen-binding,[10] precipitin reactions in gels,[8, 9] interfacial precipitation reactions, and hemagglutination-inhibition reactions that these sera contained antibody. Eventually, we learned to distinguish the patterns produced by some of the early response antisera from those elicited by

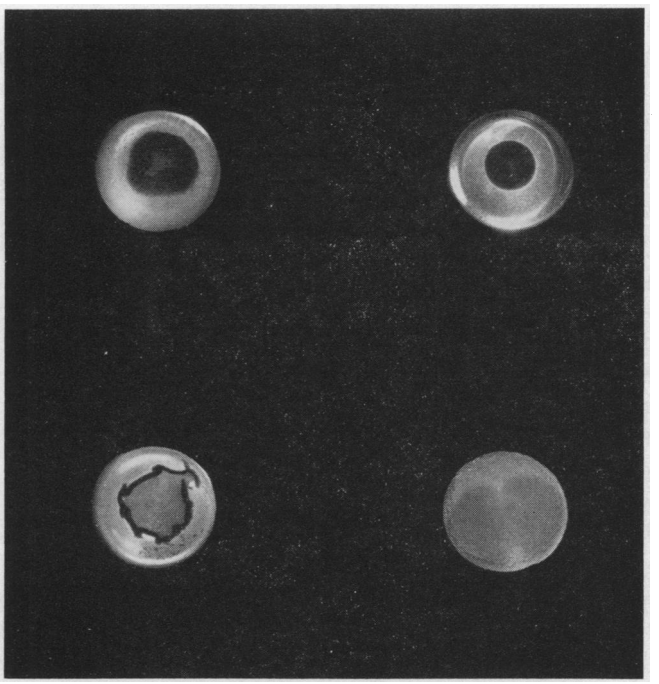

FIG. 3.—Patterns of hemagglutination produced during various phases of the antibody response in the rabbit. Top row, left to right ++++, −. Bottom row, left to right +++, ++.

hyperimmune sera.[19] Figure 3 illustrates some of these patterns. The top row left is what we originally designated ++++[13]. This is seen consistently with early anti-BGG and anti-BSA sera, occasionally with early anti-KLH, anti-HSA, and anti-diphtheria toxoid sera. This pattern is occasionally seen at the bottom of the first tube (lowest dilution) with hyperimmune sera against a variety of protein antigens and may reflect the presence of small amounts of macroglobulin antibody in these sera. The upper right is a negative reaction. The bottom left is a +++ reaction, less commonly seen with the $\gamma$-globulin-containing antisera, but frequently observed with hyperimmune sera. The bottom right pattern is ++, the most common type of reaction with hyperimmune anti-protein sera, less commonly seen with the early antisera. The zone electrophoretically-separated fractions of the early response sera do, however, yield the ++ or +++ pattern.

*Organ site of synthesis of macroglobulin antibody:* Rabbits were injected into the hind foot pads with *Salmonella* vaccine on day 1 and diphtheria toxoid on day 6. The left popliteal lymph nodes were removed when the rabbits were synthesizing only $\gamma$-1 globulin antibody. These nodes were cultured *in vitro*,[20] and continued to produce only $\gamma$-1 globulin antibody for eight days. The contralateral lymph nodes left *in situ* began to produce $\gamma$-2 in addition to $\gamma$-1 globulin antibody several days after removal of the left node.

*Discussion—Enhancement of primary antibody response to diphtheria toxoid:* The injection of *Salmonella* before diphtheria toxoid increased the antitoxoid titers compared to unprimed rabbits—over 10 fold for $\gamma$-1 globulin antibodies. The mechanism of this enhancement is not understood. The endotoxin of *Salmonella*

species may be responsible for enhancement by analogy with previous findings of enhanced antibody production in rabbits inoculated with the endotoxin of this and other organisms.[21] However, there are at least two apparent differences between the present results and previous ones. Previously, maximal enhancement of antibody synthesis was obtained when endotoxin was given simultaneously with or within 1 day *after* antigen.[21] Little or no effect on antibody formation was observed when endotoxin was given 1 day *before* antigen.[21] In the present study, enhancement was observed when the bacteria were given 2–5 days *before* antigen. It is conceivable that this discrepancy is only apparent and associated with the utilization of soluble endotoxin in the previous work and bacterial cells of this study. Secondly, endotoxin enhanced antibody formation to a variety of antigens,[22] whereas the *Salmonella* vaccine did not appear to promote antibody synthesis to antigens other than diphtheria toxoid.

The basis for the loss of serological activity of the macroglobulin diphtheria antitoxin produced in *Salmonella*-primed rabbits upon cold storage is unknown. Detailed study of the structure of the macroglobulin antibodies produced in primed and unprimed rabbits is, therefore, indicated. It is, for instance, possible that the loss of serological activity follows the spontaneous dissociation of the 19*S* into 7*S* units. The retention by these sera of neutralizing capacity for diphtheria toxin even after loss of hemagglutinating activity (Table 5) suggests that at least some of the antibody combining sites are not appreciably altered by storage in the cold.

The bisphasic curve (Fig. 1) is characteristic of *Salmonella*-primed rabbits given diphtheria toxoid. Since this is not observed in unprimed animals, the sharp descent of the curve for the macroglobulin antitoxoid may well be associated with a loss of hemagglutinating activity of the antibody *in vivo* by analogy with the loss of serological activity upon storage of these early antisera in the cold *in vitro*. In rabbits not primed with *Salmonella*, coexistence of the two molecular species of antibody was observed upon injection of BGG, KLH, diphtheria toxoid, HSA, $T_2$ bacteriophage, and *B. subtilis amylase*. The rapid decline of the macroglobulin antibody in these antisera may be due to the short half-life of the macroglobulin (see Taliaferro and Talmage[23]) and a decreased rate of macroglobulin synthesis.

*Heterogeneity of early antibody response:* Previous studies in man,[1–3] rabbit,[4] and horse[5, 6] injected repeatedly with a number of antigens indicated that more than one molecular form of antibody could be formed to multiple injections of antigen. Some of these studies showed that macroglobulin antibodies were formed early in immunization and lower-molecular-weight antibodies upon continued immunization.[24] In the present study, physical methods of separation of antisera into different fractions were combined with hemagglutination assay for antibody in these fractions to demonstrate that similar molecular heterogeneity of antibody was produced by a *single* injection of antigen. Antibody was also demonstrated in these sera by binding of radioactive antigens[10] (Table 3), specific co-precipitation on antigen-antibody precipitates,[25] gel diffusion,[8] immunoelectrophoresis,[9] and interfacial precipitin reactions. However, the electrophoretic mobility and sedimentation coefficients of the antibodies in these sera could not have been characterized readily without the hemagglutination method because thus far antibody activity has been detected in fractions from zone electrophoresis only by the hemagglutination and antigen-binding methods. Presumably, only the latter two methods

have adequate sensitivity to detect the small concentrations of macroglobulin antibody formed during the primary antibody response.

The evidence indicates that the rabbit responds to a single injection of diphtheria toxoid, KLH, BGG, HSA, and *Salmonella typhosa* H antigen by the synthesis first of $\gamma$-1 macroglobulin antibody followed within several days by the additional synthesis of a $\gamma$-2, 7*S* antibody. The localization following zone electrophoresis of antibody activity (hemagglutination, binding of radioactive antigens) exclusively in the $\gamma$-1 globulin fraction provides evidence for presence of $\gamma$-1 globulin antibody in the earliest sera. These sera contained macroglobulin antibodies on the basis of their behavior upon ultracentrifugation in a sucrose gradient and their loss of serological activity upon treatment with 2-mercaptoethanol, which has been shown to cause dissociation of 19*S* macroglobulins into 7*S* units.[16] However, more complete characterization of the macroglobulin antibodies by ultracentrifugation of antisera and by isolation of a purified macroglobulin antibody and determination of its molecular weight is needed for rigorous proof.

In addition to the antigens noted, it has been found that the intravenous injection of $T_2$ bacteriophage into rabbits results in the formation of a $\gamma$-1, macroglobulin neutralizing antibody and later a $\gamma$-2 globulin neutralizing antibody.[26] The earliest rabbit antisera obtained following the injection of an amylase of *B. subtilis* showed enzyme-neutralizing activity in both $\gamma$-1 and $\gamma$-2 globulin fractions of the serum.[25] Since these two forms of antibody were produced against a number of components in the KLH preparation, it is clear that the early antibody response in the rabbit upon single injection of a variety of antigens is quite generally heterogeneous. It is not known whether the $\gamma$-1 or $\gamma$-2 globulin antibodies react with the same antigenic determinant groups in each antigen, although the occurrence of neutralizing antibodies for diphtheria toxin in both fractions suggests that they both combine with the same grouping on the toxin. Other antigens are being studied to determine just how general the response is in the rabbit and other species.

The macroglobulin antibodies in the rabbit seem to be more active in the cold both as regards hemagglutinating and toxin-neutralizing activity. Presumably, the cold anti-Lewis group agglutinins and precipitins observed in the rabbit by Iseki *et al.*[17] were also macroglobulins. It is, therefore, quite possible that a lower temperature optimum is one of the characteristic properties of macroglobulin compared to lower-molecular-weight antibodies. This lower temperature optimum may be a reflection of a tendency of these antibodies to dissociate from the antigen-antibody complex at the higher temperatures, but this remains to be determined. It may be pertinent that Jerne[27] found that first-stimulus anti-diphtheria toxins from guinea pigs, horses, ferrets, rabbits, and humans were of low avidity. He did not, however, determine whether these antibodies were $\gamma$-1 macroglobulins.

The present data provides the first examples of the synthesis of $\gamma$-1 macroglobulin antibodies to protein antigens in a species other than man. The synthesis by humans of macroglobulin antibodies to insulin,[2] *Salmonella* H antigens,[28] and thyroglobulin[29] has been reported. The so-called rheumatoid factor, which may be an antibody to human gamma globulin, also appears to be a macroglobulin.[30] However, the shift in synthesis from macroglobulin to lower-molecular-weight

antibody in these circumstances in man has not been reported. Further studies are, therefore, needed to ascertain whether the sequence of antibodies synthesized in the rabbit also occurs in other species.

Preliminary studies of hyperimmune rabbit anti-diphtheria toxoid and anti-BGG revealed the presence of small amounts of $\gamma$-1 macroglobulin antibody. It is, therefore, likely that all hyperimmune rabbit antisera contain small or varying amounts of macroglobulin antibody. This finding is not surprising in view of the appearance of small amounts of specific $\gamma$-1 macroglobulin antibody each time a protein was introduced into the rabbit.[25] The presence of varying amounts of macroglobulin and 7*S* antibodies with different avidities and serological reactivities in a single antiserum may account for some of the discrepancies between the results of varying assays, such as hemagglutination and quantitative precipitin reactions, applied to a single antiserum.[31]

The only exception to this general picture noted thus far is in response to the *Salmonella typhosa* O antigen. Only the $\gamma$-1 macroglobulin agglutinin for this antigen has been detected despite the injection of typhoid antigen several times.

*Organ and cellular sites of synthesis of $\gamma$-1 and $\gamma$-2 globulin antibodies:* Under the conditions of the present experiments, only the popliteal lymph nodes produced significant amounts of antibody. $\gamma$-1 and $\gamma$-2 globulin antibodies can, therefore, be produced in a single organ. In the experiments with $T_2$ bacteriophage,[26] antigen was injected intravenously, and there is some evidence that the spleen produced both molecular species of antibody. The heterogeneity of the early antibody response cannot, therefore, be attributed to the synthesis of antibody in more than one organ, although the participation of more than one organ in these syntheses in the intact animal has not been excluded.

The role played by the macroglobulin-producing cells in the overall antibody response is obscure, partly because of lack of knowledge of the cellular origin of macroglobulins. The lag of 3–5 days between the synthesis of detectable $\gamma$-1 globulin antibody and the formation of $\gamma$-2 globulin antibody suggests that the same cells are not simultaneously producing both globulins. The inability of lymph-node fragments from primarily immunized animals to shift from the synthesis of $\gamma$-1 to the production of $\gamma$-2 globulin antibody *in vitro* also suggests that cellular differentiation may occur following the injection of antigen, probably during the interval between the appearance of macroglobulin and the appearance of $\gamma$-2 globulin antibodies. Several possible relationships between the cell types producing the two antibodies are apparent. (1) Cells producing $\gamma$-1 antibody differentiate into $\gamma$-2 globulin synthesizing cells. (2) The $\gamma$-1 and $\gamma$-2 globulin synthesizing cells differentiate independently from a common stem cell. (3) These two cell types represent completely independent and unrelated lines of cells.

Further study of the cellular origin, sequential synthesis, physicochemical, and immunochemical properties of the $\gamma$-1 macroglobulin and $\gamma$-2 globulin antibodies undoubtedly will contribute to better understanding of many facets of the antibody response. A subsequent paper concerning these two species of antibody will present various types of data which furnish some insight into the nature of the primary and secondary antibody responses.

*Summary.*—Priming of rabbits with *Salmonella* before diphtheria toxoid results in an accelerated and enhanced production of antitoxoid. The synthesis of $\gamma$-1

globulin antitoxoid is enhanced some 10 fold compared to unprimed animals. The $\gamma$-1 antitoxoid produced in primed animals loses hemagglutinating but not neutralizing activity upon storage in the cold for several days. The $\gamma$-2 antitoxoid synthesized in these animals several days later does not lose hemagglutinating activity under these conditions.

The early antibody response to a single injection of a number of protein and cellular antigens in unprimed rabbits is also heterogeneous and consists of the initial synthesis of $\gamma$-1 macroglobulin antibody followed by the synthesis of $\gamma$-2 globulin, 7*S* globulin antibody. Neither antibody loses serological activity upon storage in the cold. The $\gamma$-1 macroglobulin antibody loses specific hemagglutinating and precipitating activity upon treatment with 2 mercaptoethanol, but the 7*S* antibody does not lose these activities under these conditions. The $\gamma$-1 macroglobulin antibodies seem to have a lower temperature optimum than the $\gamma$-2 7*S* globulin antibodies. Lymph-node fragments from primarily immunized animals which synthesized the macroglobulin antibody *in vitro* did not shift to the synthesis of the $\gamma$-2 globulin upon prolonged culture. Some problems arising from the above observations and possible explanations are presented and discussed.

The authors appreciate the diligent and intelligent technical assistance of Mr. Weldon W. Harold in the course of this investigation. They also acknowledge gratefully the advice of Dr. C. F. Hinz, Jr., in connection with the immunoelectrophoretic analyses.

* This investigation was supported by a research grant (E-1865) from the Allergy and Infectious Diseases Institute of the U. S. Public Health Service.

† Present address: Department of Microbiology, Indiana University Medical Center, Indianapolis, Indiana.

[1] Kuhns, W. J., *J. Immunol.*, **75**, 105 (1955).

[2] Berson, S. A., and R. S. Yalow, *J. Clin. Invest.*, **36**, 642 (1957).

[3] Sehon, A. H., M. Kaye, E. McGarry, and B. Rose, *J. Lab. Clin. Med.*, **45**, 765 (1955).

[4] Stelos, P., and D. W. Talmage, *J. Infectious Diseases*, **100**, 126 (1957).

[5] Kekwick, R. A., and B. R. Record, *Brit. J. Exptl. Pathol.*, **22**, 29 (1941).

[6] Kabat, E. A., *J. Immunol.*, **47**, 513 (1943).

[7] Pope, C. G., and M. F. Stevens, *Brit. J. Exptl. Pathol.*, **39**, 139 (1958).

[8] Ouchterlony, O., *Acta Pathol. Microbiol. Scand.*, **25**, 186 (1948).

[9] Scheidegger, J. J., *Internat. Arch. Allergy and Appl. Immunol.*, **7**, 103 (1955).

[10] Feinberg, R., *J. Immunol.*, **81**, 14 (1958).

[11] Müller-Eberhard, H. J., *Scand. J. Lab. Invest.*, **12**, 33 (1960).

[12] Edelman, G. M., H. G. Kunkel, and E. C. Franklin, *J. Exptl. Med.*, **108**, 105 (1958).

[13] Stavitsky, A. B., *J. Immunol.*, **72**, 360 (1954).

[14] Fraser, D. T., *Tr. Roy. Soc. of Canada, Section V*, **25**, 175 (1931).

[15] Lowry, O. H., N. J. Rosebrough, F. A. Lewis, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[16] Deutsch, H. F., and J. F. Morton, *Science*, **125**, 600 (1957).

[17] Iseki, S. S. Masaki, and K. Shibasaki, *Proc. Japan Acad.*, **33**, 492 (1957).

[18] Gordon, R. S., Jr., *J. Immunol.*, **71**, 220 (1953).

[19] Dr. Richard Porter (personal communication) has observed similar hemagglutination patterns in studies of the early antibody response to bovine serum albumin in rabbits.

[20] Stavitsky, A. B., and B. Wolf, *Biochem. et Biophys. Acta.*, **27**, 4 (1958).

[21] Kind, P., and A. G. Johnson, *J. Immunol.*, **82**, 415 (1959).

[22] Johnson, A. G., and S. Gaines, and M. Landy, *J. Exptl. Med.*, **103**, 225 (1956).

[23] Taliaferro, W. H., and D. W. Talmage, *J. Infectious Diseases*, **99**, 21 (1956).

[24] Kabat, E. A., *Experimental Immunochemistry* (Springfield, Illinois: Charles C Thomas, 1961), 2nd ed., p. 326.

[25] Bauer, D. C., and A. B. Stavitsky (unpublished observations).

[26] Mathies, M. M., D. C. Bauer, and A. B. Stavitsky (unpublished observations).
[27] Jerne, N. K., *Acta Pathol. Microbiol. Scand.*, Suppl. LXXXVII, 1 (1951).
[28] Smith, R. T., in *Cellular Aspects of Immunity*, Ciba Foundation Symposium, ed. G. E. W. Wolstenholme and Maeve O'Connor (London: J. and A. Churchill, Ltd., 1960), p. 348.
[29] Korngold, L., G. van Leeuwen, and J. L. Brewer, *J. Lab. Clin. Med.*, **53**, 517 (1959).
[30] Edelman, G. M., H. G. Kunkel, and E. C. Franklin, *J. Exptl. Med.*, **108**, 105 (1958).
[31] Stavitsky, A. B., *J. Immunol.*, **72**, 368 (1954).

---

## *TWO CHANGES IN THE USUAL EQUATIONS OF A PLASMA REQUIRED FOR MAGNETODYNAMIC WORK*

BY JOSEPH SLEPIAN

1115 LANCASTER STREET, PITTSBURGH 18, PENNSYLVANIA

*Read before the Academy, April 24, 1961*

1. *Spitzer's Equations of a Plasma.*—In the book by L. Spitzer, Jr.,[1] are given on page 21 the two usually used vector equations for a plasma, corresponding to a plasma being a mixture of two gases, the electron gas and the ion gas.

$$\rho \frac{\partial \boldsymbol{v}}{\partial t} = j \times \boldsymbol{B} - \nabla p - \rho \nabla \Phi. \qquad \text{(2-11) Spitzer}$$

$$\frac{m_e c^2}{n_e e^2} \frac{\partial j}{\partial t} = \boldsymbol{E} + \boldsymbol{v} \times \boldsymbol{B} + \frac{c}{e n_e} \nabla p_e - \frac{c}{e n_e} j \times \boldsymbol{B} - \eta j. \qquad \text{(2-12) Spitzer}$$

We shall make two major corrections to these equations. Applying these to a simple problem, we find that, contrary to the teaching of the book, the containment time of the plasma is independent of the magnitude of the impressed magnetic field.

2. *The Special Case Considered Here.*—Take a very long straight circular tube of such a nature that all ions and electrons which reach it are condensed or are removed from the gas space inside. Let the tube be of such length that the velocity of the plasma in the central part of the tube may be considered two-dimensional, $\boldsymbol{v} = \boldsymbol{v}(\boldsymbol{v}_r, \boldsymbol{v}_\theta, 0)$ for the time considered, $\boldsymbol{v}$ being very nearly the same as that of the positive ion $\boldsymbol{v}_i$. Let the velocity of the electrons there be similarly two dimensional, $\boldsymbol{w} = \boldsymbol{w}(\boldsymbol{w}_r, \boldsymbol{w}_\theta, 0)$. Let a uniform constant magnetic field $\boldsymbol{B}_0$ exist along the tube.

3. *The Modified Equations of Spitzer.*—The modified equations of the plasma are as follows.

$$\rho\left(\frac{\partial \boldsymbol{v}}{\partial t} + \boldsymbol{v} \cdot \nabla \boldsymbol{v}\right) = j \times \boldsymbol{B} - \nabla p_e - \nabla p_i. \qquad (1)$$

$$0 = \boldsymbol{E} + \boldsymbol{v} \times \boldsymbol{B} + \frac{c}{en} \nabla p_e - \frac{c}{en} j \times \boldsymbol{B} - \eta' j. \qquad (2)$$

In equation (1), we do not make the assumption 1 on page 20 of Spitzer's book[1] that $\boldsymbol{v} \cdot \nabla \boldsymbol{v}$ is negligible. This constitutes the first modification. We take $\nabla \Phi$ as zero. We write $p = p_e + p_i$.

We let the first term of (2-12) be negligibly small. For the second modification, we write $\eta'$ for $\eta$ to remind us that $\eta'$ is a function of $(r,\theta)$ as well as of the local characteristics of the plasma, due to the fact that the forces exerted by the relatively moving electrons upon the ions are due to microscopic high-frequency fields