Antibody Response to Immunization by Different Routes

Abstract. *Rabbits were injected with human serum albumin once intravenously or intradermally with this antigen plus complete Freund adjuvant. The hemagglutinating titers of the antiserum and of the heavy and light antibody fractions were followed for several months. Circulation of heavy antibody in the intravenously injected group disappeared in 1 month, but in the intradermally injected group, it rose to a maximum titer, then declined, and then showed a second rise in titer. Light antibody showed increasing and then sustained titers over most of the time period. Heavy antibody failed to precipitate with antigen, even when it showed high hemagglutinating titers. Light antibody, in contrast, gave precipitation even when tested at much lower hemagglutination titers.*

The sequential formation of the two major molecular forms of the antibody molecule, as stimulated by various antigens, has been described (*1–4*) for a number of different host animals, including rabbit (*1*), chicken (*2*), guinea pig (*3*), and human (*4*). In many examples of primary immunization there is a production of both 19*S* and 7*S* antibodies to the antigen, and it has been frequently stated that 19*S* production occurs earlier. The appearance of 7*S* antibody may be delayed for a few days after the other form, or it may await a second injection of antigen. Reported measurements of antibody titer have usually been made by passive hemagglutination procedures or by virus neutralization; a few have included precipitation analysis. A very recent study of antigen binding strength at several times during immune response (*5*) showed that the dissociation characteristics of the various serums were quite similar, regardless of the amount of injected antigen, its route, or the number of injections. There seems to be no report on any extensive comparison of 19*S* and 7*S* production by different routes of inoculation or on the observations attendant on a single injection observed over a long period of time. These two parameters of immunization have now been studied, and the essential features of the data are reported below.

The human serum albumin (HSA) was examined by analytical ultracentrifugation, immunoelectrophoresis, and optical electrophoresis; it contained at least 95 percent albumin plus very small quantities of two fast α-globulins. Primary antiserums to human serum albumin were prepared in six rabbits; the resultant antiserums were tested with the same preparation. All rabbits were initially bled before inoculation. They were injected only once, either intravenously in the marginal ear vein or intradermally in the inguinal region. Intravenous injections consisted of one injection of 40 mg of human serum albumin for each of three rabbits. Intradermal injections consisted of a total dose of 7 mg, incorporated into complete Freund adjuvant, spread in eight injection sites for each of three rabbits. In both groups of rabbits, trial bleedings were taken every few days for the 1st month and at monthly intervals after this.

Serum fractionation in an ultracentrifugal density gradient (*6*) was the basis for our separation procedure. The gradient ranged from 10 to 40 percent sucrose. Before the serum was added, the gradient was chilled at 4°C for at least 2 hours. Immediately before centrifugation 1.0 ml of a mixture of equal amounts of the serum and 0.15*M* NaCl was gently stirred into the top zone of sucrose. A swinging bucket rotor (SW-39) was employed, and centrifugation for 18 hours at 35,000 rev/min was carried out in a Spinco model L ultracentrifuge. After centrifugation, fractions were collected in drops through a hole punctured in the bottom of the cellulose tube with a 26-gage needle. In order to locate the heavy and light antibody fractions, the two major protein peaks obtained by this method, 4 drops were collected per fraction, and each fraction was suitably diluted with 0.15*M* NaCl. Optical density readings at 280 mμ were taken on a Beckman DU spectrophotometer and immunoelectrophoretic analyses were made on the dialyzed fractions. After a number of such analyses, it was found that the first 12 drops could be collected as fraction *a*, or the heavy antibody fraction. The next 8 drops could be collected as fraction *b*, or the safety fraction between the two protein peaks (*a* and *c*). The remainder was collected as fraction *c*, or the light antibody fraction. By osmotic concentration with dry sucrose, these fractions were each adjusted to approximately 0.5 ml, the original volume of the serum added to the gradient tube. Finally, all fractions were dialyzed exhaustively against the phosphate-buffered saline, *p*H 7.2, and then against 0.15*M* NaCl. The fractions were tested within several days after fractionation. The amount of protein in these fractions was considered to be a fair approximation of the concentration of these proteins in whole serum.

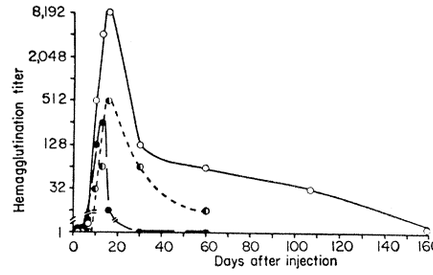

Fig. 1. Hemagglutination titer plotted against number of days after the injection for rabbit 1. Inoculation of 40 mg of human serum albumin was made by the intravenous route. Titers are plotted for the whole serum (—○—), the heavy antibody fraction (—●—), and the light antibody fraction (– – ◐ – –).

The tanned-cell hemagglutination test with a 6 percent suspension of human O, Rh+ red blood cells was a modification of that described by Boyden (*7*). The cells were coated with human serum albumin (0.50 percent) and tested with twofold serial dilutions of inactivated rabbit antiserum, density gradient fractions of antiserum, and normal rabbit serum (obtained prior to immunization), with 1:100 normal rabbit serum as diluent. The entire procedure was carried out in phosphate-buffered saline, *p*H 7.2. The readings of the cell patterns were made according to Stavitsky (*7*). A modification of the method of Ouchterlony (*8*) was utilized for gel-diffusion precipitation. The wells were 5 mm in diameter and 5 mm apart, edge-to-edge, in a 1 percent agar gel. The conditions were varied, however, in studying the density gradient fractions. Agar was prepared in concentrations from 0.7 to 1.0 percent. The well diameter was varied from 3 to 7 mm, as was the distance between wells. In each study, an antigen dilution series was made in order to insure the accuracy of the number and geometry of the precipitation lines. All of the normal serums were tested, and wells were often filled with 0.15*M* NaCl to test for nonspecific reactions. The plates were incubated at room temperature.

Some of the samples were tested for mercaptoethanol sensitivity by

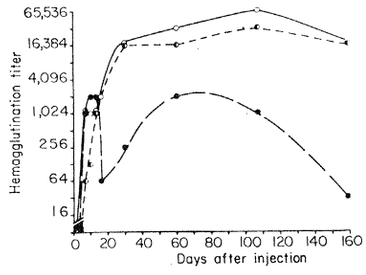

Fig. 2. Hemagglutination titer plotted against number of days after the injection for rabbit 4. Inoculation of 7 mg of human serum albumin was made by the intradermal route, incorporating complete Freund adjuvant. Titers are plotted for the whole serum (—○—), the heavy antibody fraction (—◐—), and the light antibody fraction (– – ● – –).

means of treatment, at a final protein concentration of 1 percent or less, with an equal volume of 0.2*M* 2-mercaptoethanol, as outlined by Deutsch and others (*9*). After incubation at room temperature for 5 hours, the mixtures were then dialyzed against 0.02*M* iodoacetamide. Controls were treated with phosphate buffer instead of the mercaptoethanol and iodoacetamide.

The hemagglutination titers of the primary antiserums and of their density-gradient fractions were followed for several months. All six rabbits produced demonstrable antibody within 7 to 10 days and all of them produced antibody of both molecular types. One example from each group of rabbits is illustrated in Figs. 1 and 2. The figures are graphs of the hemagglutinating titers of these antiserums obtained at various intervals after the original injection. Detection of heavy antibody started earlier than that of light antibody after intravenous injection (Fig. 1). The maximum titer of heavy antibody (1:256) was reached at 13 days, and the maximum titer of light antibody (1:512) was reached at 16 days after the antigen injection. The titer of the heavy fraction dropped to zero by 30 days, but the titer of the light fraction was significantly high at 60 days and at 108 days. By day 159, the titer of the antiserum itself was very low. The other two rabbits that were immunized intravenously showed quite similar results, although the actual titers varied somewhat.

In one of the rabbits injected intradermally, synthesis of heavy antibody occurred slightly before synthesis of light antibody (Fig. 2). After the original increase of the titer of light antibody, there occurred a plateau in titer, which continued for several days. After this, the titer of light antibody continued to increase and to remain high even at 159 days after antigen injection. The initial increase of the titer of heavy antibody, rising more quickly than that for the light one, was followed by a decline by day 16. This decline was then superseded by a rise again in the titer of this type of antibody, and the titer rose to its initial level, but no higher. This was observed for all three rabbits in the group. The recovery remained substantial for 2 months in every case; the subsequent decline varied in sharpness for each rabbit. Detectable amounts of heavy antibody, however, were still observed even at 159 days. In comparing all six rabbits, the maximum titer of heavy antibody was not appreciably dissimilar in the two groups. On the other hand, the peak titer of light antibody obtained from rabbits injected intradermally did exceed markedly that which was obtained from rabbits injected intravenously.

Experiments were carried out in gel diffusion to evaluate the precipitating capacity of the various antiserums and fractions. Two of the three rabbits injected intravenously produced antiserums which precipitated HSA in these tests, and all three rabbits injected intradermally produced precipitating antibody, observable in bleedings by 10 or 13 days after injection. In all cases of precipitating antibody, the hemagglutination titer of the antiserum was at least 1:512. A number of the density-gradient fractions were also tested. All attempts to find precipitating antibody by gel diffusion in the heavy antibody fraction resulted in failure. Even in those cases where the hemagglutinating titer of this antibody was at least 1:2000, or higher, there was no evidence of a precipitating antibody. On the other hand, light antibody fractions showed precipitation even when their hemagglutination titers were only 1:256, illustrating differences in quality of precipitation in the two molecular forms of antibody.

It is not yet understood how the intradermally induced heavy antibody differs in the late bleedings (after the drop in titer) from the heavy antibody in the early bleedings (before the drop). One criterion applied to this question was the sensitivity to mercaptoethanol treatment. From two of these rabbits, the antibodies were tested from the 7-day and 60- or 159-day bleedings, and the hemagglutination assays showed that the heavy antibody from both early and late antiserums lost their hemagglutinating capacity after treatment with mercaptoethanol, whereas the control solutions maintained their activity. The light antibody always maintained activity after mercaptoethanol treatment.

The duration of production of macroglobulin antibody has been shown to depend on the route and manner of injection. Although the amount of antigen injected intravenously was five times as much as that injected intradermally, the macroglobulin response was short-lived in the first group, but continued for many months in the second group, a distinction that cannot be attributed to the difference in dose. While a somewhat similar biphasic curve for the titer of 19*S* antibody was reported by Uhr *et al.* (*3*) for guinea pig antibody to bacteriophage, it should be noted that this only occurred after a second (intravenous) injection, given at 9 days. Apparently, none of the previous investigators permitted a single injection to manifest itself for a sufficiently long time. It may well be that the early and late antibodies in our study differ by being responsive to different antigenic groups in the albumin molecules, or the difference may indicate stages in proliferation of antibody-forming cells. It may be significant that the intradermal injections included complete Freund adjuvant, and this in itself might induce a different cellular response. It is not known whether the heavy and light antibodies were stimulated by different antigenic determinants. That this condition is not essential is indicated by the finding that both heavy and light antibody can be produced in the same rabbit in response to a small haptenic group, as shown recently by Bauer (*10*), and by Groff in this laboratory (*10*). Additional studies have shown by means of the antigen-binding capacity method of Farr (*11*) that heavy and light antibody do differ in hemagglutinating activity per unit of antigen-binding activity.

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Nicotinic Acid Biosynthesis: Control by an Enzyme that Competes with a Spontaneous Reaction

Abstract. *Extracts of livers from diabetic rats contain normal amounts of the enzymes needed to convert 3-hydroxyanthranilic acid to nicotinic acid nucleotide. The decreased capacity of diabetic animals to synthesize nicotinic acid is therefore attributed to increased amounts of picolinic carboxylase, which competes for a common intermediate with the spontaneous reaction in which quinolinic acid is formed as a precursor of nicotinic acid. These studies were facilitated by the synthesis of 3-hydroxyanthranilic acid labeled with carbon-14 in positions 3 and 6.*

Control of biochemical processes is known to occur by many devices, including repression and derepression of gene expression, enzyme inhibition in feedback control, and substrate limitation—as in control of utilization of inorganic phosphate. An unusual opportunity for control of a biological process exists in the synthesis of nicotinic acid, since one step in the sequence of reactions that starts with the oxidation of tryptophan is the nonenzymatic formation of quinolinic acid from the oxidation product of 3-hydroxyanthranilic acid (*1*). In normal animals there is very little enzymatic reaction to interfere with the formation of quinolinic acid, but in animals with certain endocrine disturbances, in particular with diabetes, there is a great increase in the concentration of picolinic carboxylase in the liver. Diabetic animals excrete only small amounts of nicotinic acid metabolites. Therefore, it was previously suggested that the relative activity of picolinic carboxylase, which utilizes the precursor of quinolinic acid as a substrate, could control the amount of quinolinic acid formed, which would in turn determine the amount of nicotinic acid synthesized (*2*). At that time the terminal steps in nicotinic acid biosynthesis were not known. The discovery by Nishizuka and Hayaishi (*3*) of the enzymatic synthesis of nicotinic mononucleotide from quinolinic acid and PRPP (*4*) has made it possible to demonstrate that the formation of quinolinic acid is indeed limiting in systems containing large amounts of picolinic carboxylase. Our experiments show that the enzyme of Nishizuka and Hayaishi is present in livers of diabetic rats, since these livers are able to convert quinolinic acid to nicotinic acid, although they do not carry out the corresponding conversion when 3-hydroxyanthranilic acid is the substrate.

In order to follow the reactions of 3-hydroxyanthranilic acid in these studies, we have devised a synthesis for labeling this compound with carbon-14 in positions 3 and 6. This pattern of labeling permits the formation of quinolinic acid with half the radioactive carbon in the ring (which persists in the ring of nicotinic acid) and half in the carboxyl group that is converted to carbon dioxide when the nucleotide is synthesized.

Anthranilic acid and 3-hydroxyanthranilic acid labeled with C^{14} at positions 1 and 2 have been synthesized from 1,3-butadiene and C^{14}-labeled maleic anhydride (*5*). The same scheme (Fig. 2) has been used in the present investigation starting with 1,3-butadiene labeled at carbons 1 and 4 that had been prepared by vapor-phase micropyrolysis of 1,4-C^{14}-labeled 1,4-butanediol diacetate according to Hooton (*6*).

The 1,4-butanediol diacetate-1,4-C^{14} was prepared as follows. 1,4-Butanediol-1,4-C^{14} (0.05 ml) (*7*), 0.5 g of 1,4-butanediol, and 1.6 g of acetic anhydride were kept on the steam bath for 1 to 2 hours, cooled, and treated with ice.

Table 1. Nicotinic acid mononucleotide synthesis by enzymes from livers of normal and diabetic rats. The experiment in duplicate was carried out as described.

Enzyme	Count/min	
	Diabetic	Control
3-HA	69	523
	52	539
"Quin"	650	520
	643	563

After all ice had melted, ether and excess dilute potassium carbonate were added. The ether layer was washed with potassium carbonate solution, dried over sodium sulfate, and evaporated to dryness, giving 0.9 g of liquid with an index of refraction n_D^{20} 1.4250 (*8*); an infrared spectrum taken on a smear showed a maximum absorption (λ_{max}) at 5.75 μ.

The diester was pyrolyzed in a Vycor tube (20 cm in total length and 0.8-cm inside diameter) packed with pyrex helices and heated externally by a 10-cm furnace maintained at 630°C. The tube was connected directly to a 2.1-m chromatographic column containing one part of silicone grease on six parts of Chromosorb P 30/60 by weight, maintained at room temperature. Eighteen 50-μl portions (total 0.9 g) of the diacetate were injected at 60-second intervals through a serum cap at the top of the tube by means of a microsyringe while helium was passed through the tube from a side arm, attached just below the serum cap, at the rate of 1 ml per second. As indicated by the peak that appeared in the chromatogram, each portion of the diacetate produced 75 to 90 percent of 1,3-butadiene-1,4-C^{14} which was collected at the exit of the column in two test tubes (20 by 150 mm) connected in series, each containing 1.0 g of maleic anhydride in 15 ml of benzene (cooled to 5° to 10°C). At the end of the pyrolysis period the test tubes were stoppered firmly and kept at room temperature for 24 to 48 hours. The solutions were then combined and treated with excess "cold" butadiene, and the mixture was allowed to stand for an additional 24 hours. The solution was concentrated to 8 to 10 ml, treated with an equal volume of ligroin (bp 30° to 60°C), and cooled to −15°C. Filtration gave 2.3 g of 1,2,3,6-*cis*-tetrahydrophthalic anhydride-3,6-C^{14}, mp 100°-102°C; infrared absorption showed $\lambda_{max}^{CHCl_3}$ 5.4 and 5.63 μ (*9*); this was converted to 1.0 g of anthranilic acid-3,6-C^{14} (I) mp