Proceedings of the National Academy of Sciences
Vol. 66, No. 2, pp. 411–418, June 1970

Electrophoretically Homogeneous Anti-DNP Antibodies with Restricted Isoelectric Points Elicited in Mice by Immunization with the Antigen Papain-S-DNPL*

Gary N. Trump† and S. J. Singer‡

DEPARTMENT OF BIOLOGY, UNIVERSITY OF CALIFORNIA, SAN DIEGO (LA JOLLA)

Communicated March 2, 1970

Abstract. The primary immunization of outbred mice with the antigen Pap-S-DNPL results in the generation of low titers of anti-DNP antibody which in about one-third of the responding animals is as homogeneous as a myeloma protein by the combined criteria of (*a*) isoelectric focusing in gels and (*b*) gel electrophoresis of the antibody light chains. The electrophoretically homogeneous antibodies show a marked restriction of isoelectric points near pH 5.0. Such marked selectivity of the antihapten antibodies appears to result from the chemical and structural homogeneity of the Pap-S-DNPL antigen.

An analysis of the origins of antibody (Ab) heterogeneity led us some years ago to suggest that homogeneous antihapten Ab might be elicited by the immunization of animals with a chemically and structurally homogeneous hapten-protein conjugate.[1] Such a conjugate (Pap-S-DNPL) was prepared by the highly specific reaction of one molecule of α-*N*-iodoacetyl, ϵ-*N*-2,4-dinitrophenyl-L-lysine (IADL) with the SH group of cysteine-25 in the active site of papain.[2, 3] Immunization of rabbits with Pap-S-DNPL resulted in the production of low titers of anti-2,4-dinitrophenyl (anti-DNP) Ab which in a large number of individual animals exhibited predominantly single bands of light chains in gel electrophoresis.[2] In this paper, these studies have been extended in two directions. First, mice have been immunized with Pap-S-DNPL, in order to take advantage of several important features of the mouse immune system that are more favorable than the rabbit's. Second, we have developed and applied methods whereby the anti-DNP Ab produced in individual mice can be conveniently examined for their degree of heterogeneity by two independent and powerful criteria: isoelectric focusing of the whole Ab, and electrophoresis of the light chains derived from the same Ab. By these means, it has been demonstrated that in about a third of the mice that show a significant primary anti-DNP Ab response to the antigen Pap-S-DNPL, the Ab is as homogeneous as a myeloma immunoglobulin, and the anti-DNP Ab from another third of the responding mice exhibit only a few electrophoretic components. Furthermore, these homogeneous Ab are characterized by a narrow range of isoelectric points near pH 5.0.

Materials and Methods. The preparation and characterization of the antigen, Pap-S-DNPL, has been described elsewhere.[2, 3] Retired-breeder female Swiss-Webster mice, about 6 months old (Simonson Laboratories, Gilroy, Calif.) were given three injec-

tions of Pap-S-DNPL without adjuvant in phosphate-buffered saline: 35 μg intravenous on day 0, 175 μg intraperitoneal on day 2, and 70 μg intramuscular on day 4. The course of anti-DNP Ab production was followed by Farr tests with the antigen ^{125}I-DNP-BSA[2] on bleedings from representative mice. In initial experiments, it was found that the maximum primary response was reached about 10 days after the first injection. In order to produce large volumes of Ab-containing fluids, the mice were injected intraperitoneally with a suspension of Sarcoma-180 cells[4] in mouse ascites fluid on day 3. By day 10, the maximum amount of ascites fluids were produced; these were tapped, heparinized, and the Ab-containing supernatants removed by centrifugation from the ascites cells. The anti-DNP Ab from the fluids of individual mice was adsorbed on small columns of DNP-Sephadex, the Ab was specifically eluted with 2,4-dinitrophenol, and was carefully iodinated[5] with ^{125}I as in our previous studies.[2] The ^{125}I-anti-DNP Ab was then divided into two aliquots: (1) for isoelectric focusing of the whole Ab, and (2) for gel electrophoresis of the light chains of the Ab. Aliquot (1) was dialyzed against de-ionized 5 *M* urea and uniformly mixed with an acrylamide-ampholine solution[6] to give a final acrylamide concentration of 5%. The sample was then photopolymerized in 0.5 × 12-cm gel columns. Aliquot (2) was dialyzed against 0.55 *M* Tris buffer, pH 8.2, was reduced with 0.22 *M* mercaptoethanol for 1 hr at room temperature and alkylated with iodoacetamide,[7] and was then dialyzed against 1 *M* propionic acid and lyophilized. The dried sample was then taken up in 8 *M* urea and applied to a 7% polyacrylamide gel equilibrated with a pH 7.5 buffer modified from that used by Williams and Reisfeld.[8] The isoelectric focusing experiment was performed for 18 hr at 4°C at 380 v; the light-chain electrophoresis was performed for 4 hr at room temperature at 160 v. The gels were then removed, sliced into 1-mm sections, and the sections counted in a Baird-Atomic automatic gamma counter. From these data, the distribution of labeled protein in the gels was determined.

Results. Of a total of 232 mice immunized with Pap-S-DNPL, 88 (37%) responded with anti-DNP Ab titers of 2 μg protein/ml or greater by the 10th day after the start of the immunization schedule. Most of the responders (75%) showed Ab titers in the ascites fluids in the range from 2 to 5 μg/ml; about 10% gave 5–10 μg/ml, and another 10% 15–25 μg/ml. Two of the mice (1%) developed titers of 50 μg/ml or greater.

Isoelectric focusing in gels with an effectively linear pH gradient from 4 to 8 (Fig. 2*a*) produced a separation of whole mouse gamma globulins into a very large number of sharp bands (Fig. 1*a*). Gel electrophoresis of the light chains prepared from mouse gamma globulins resolved the chains into 6 to 8 bands (Fig. 1*b*). Various control samples were examined on these gels. A sample of pooled mouse anti-DNP Ab, prepared against the conventional antigen DNP-

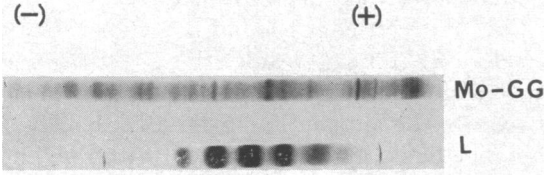

FIG. 1.—(*Top*) Isoelectric focusing in polyacrylamide gel of whole mouse gamma globulins in a pH gradient from about pH 8 (*on the left*) to pH 4 (*on the right*).

(*Bottom*) Electrophoresis in polyacrylamide gel of light chains from pooled mouse anti-DNP Ab elicited by the antigen DNP-bovine gamma globulin. The protein in each gel was stained with coomassie blue.

bovine gamma globulin,[9] and isolated from ascites fluids by the procedure of Thorpe and Singer,[10] was iodinated with ^{125}I and then treated as described in *Materials and Methods*. The isoelectric focusing pattern of the whole Ab is shown in Figure 2*b*, and the gel electrophoresis pattern of the light chains derived from this Ab is plotted in Figure 3. If the Ab was not first reduced, no light-

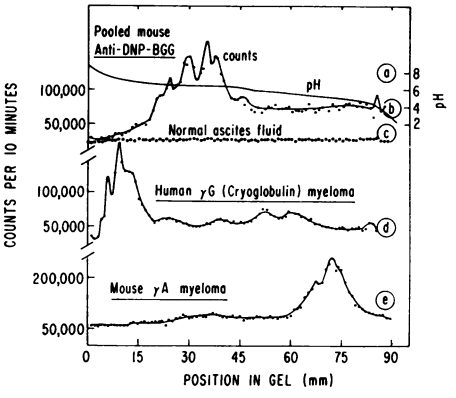

FIG. 2.—Isoelectric experiments in gels with the pH gradient shown in (*a*). The distribution of ^{125}I represents the protein in the different samples.

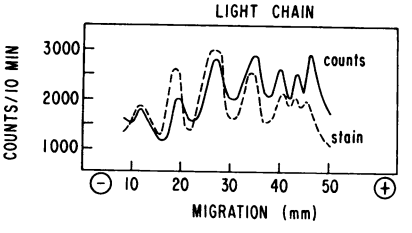

FIG. 3.—Gel electrophoresis of light chains derived from pooled mouse anti-DNP Ab elicited by the antigen DNP-bovine gamma globulin. Close correspondence of the protein distribution as measured by protein staining or by ^{125}I counts is demonstrated.

chain bands appeared. Normal ascites fluids subjected to the entire procedure used to isolate the anti-DNP Ab from the immune fluids showed no components detectable by isoelectric focusing (Fig. 2*c*). Two myeloma proteins, a human IgG cryoglobulin[11] and a mouse IgA with binding activity for DNP ligands,[12] showed two or three closely spaced peaks in isoelectric focusing (Fig. 2*d* and *e*).

The anti-DNP Ab produced in a primary response to the antigen Pap-S-DNPL from 10 representative individual mice are characterized in Figure 4. The isoelectric focusing pattern of the whole Ab (*left*) from an individual mouse and the gel electrophoresis pattern of the light chains (*right*) derived from the same Ab are shown side by side. The Ab samples could be divided into three categories: samples A through E are examples in which predominantly single peaks were observed in both types of electrophoresis experiment; F through H showed two or three peaks in both experiments; and I and J showed multiple peaks in both experiments. In no case was there a markedly different degree of heterogeneity found for the two different experiments with the same Ab. Roughly equal numbers of individual Ab samples were found in each of the three categories shown. The anti-DNP Ab titers of the 10 samples, in μg protein/ml, were 35, 3, 15, 15, 50, 15, 15, 7, 40, and 40, respectively. There did not appear to be any correlation of the degree of electrophoretic heterogeneity with the anti-DNP Ab titer.

Discussion. In a previous report[2] we showed that in about three-fourths of the rabbits responding to immunization with the homogeneous hapten-protein conjugate, Pap-S-DNPL,[3] anti-DNP Ab which had a single predominant light-

chain band in gel electrophoresis were elicited. In extending these studies, we have switched from rabbits to mice for several reasons: (1) Homogeneous myeloma and Bence-Jones proteins can be elicited in mice,[13] and amino acid sequence data[14] and structural information for mouse immunoglobulin chains are accessible; (2) the availability of inbred strains of mice makes genetic studies of the immune response feasible;[15] and (3) the induction of some types of immune responses *in vitro* in cultures of mouse spleen cells has been achieved.[16] The use of an ascites tumor[4] permits reasonable volumes of Ab-containing ascites fluids to be obtained from individual mice.

Our object in these studies was to obtain a statistically significant sample of individual anti-DNP Ab responses in mice to Pap-S-DNPL, and to characterize these Ab with respect to their possible homogeneity. Given the small amounts

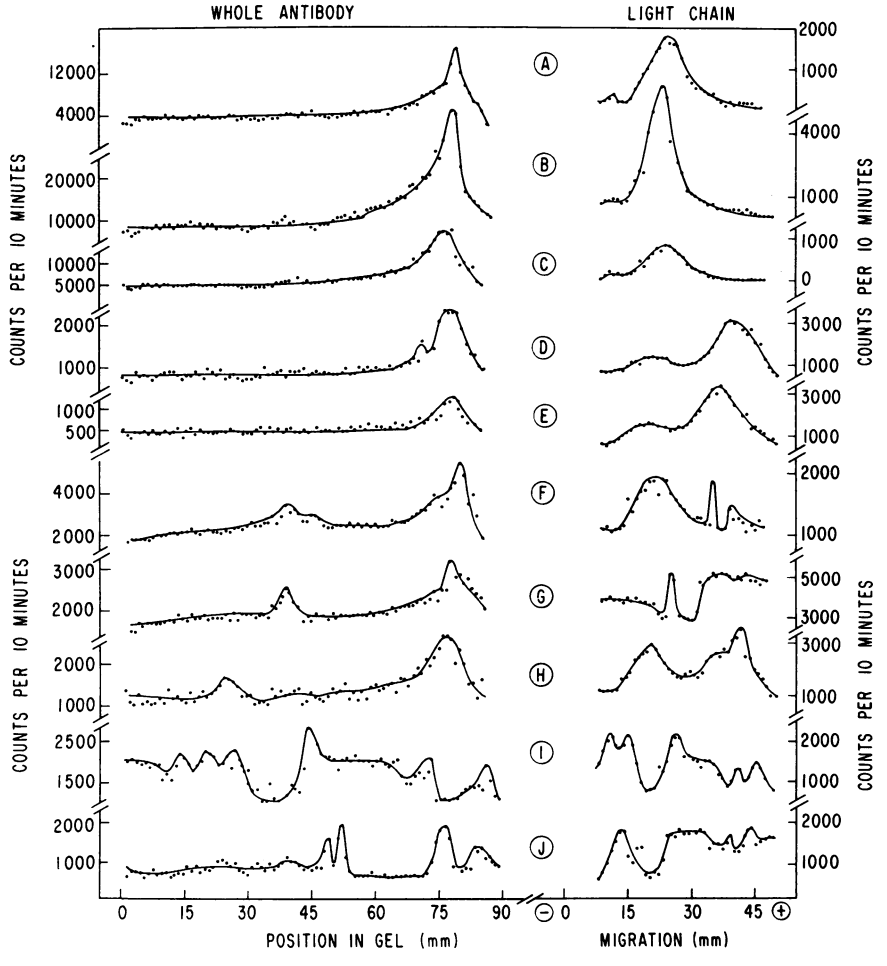

FIG. 4.—Isoelectric focusing patterns of the whole anti-DNP Ab (*left*) and electrophoresis patterns of the light chains derived from that Ab (*right*) from 10 individual mice immunized with Pap-S-DNPL.

of anti-DNP Ab produced in the primary response to Pap-S-DNPL without adjuvant, we sought criteria of molecular homogeneity which would be cogent and at the same time suitable for rapid screening. The most direct criteria would be peptide fingerprints or amino acid sequences of the Ab chains, but such experiments require substantially more Ab than was generally elicited. On the other hand, it has been shown that criteria such as the apparent homogeneity of binding affinity of an Ab for its hapten,[17] or a marked selection of one allotype in an Ab as compared to the normal immunoglobulins of the same heterozygous animal,[18] while certainly demonstrating a restricted heterogeneity of the Ab, are not sufficient evidence for the homogeneity.

The finding of a single light-chain band in gel electrophoresis at around neutral pH[19] clearly demonstrates a marked restriction of the heterogeneity of an individual Ab.[2] However, there is a small but finite probability that such an Ab may contain more than one molecular species of light chains having the same electrophoretic mobility; only about 6–8 bands or electrophoretic mobility classes are observed for grossly heterogeneous rabbit or mouse light chains. Our method of isoelectric focusing in polyacrylamide gels, an adaptation of isoelectric focusing methods used previously,[20, 21] resolves whole molecules of gamma globulins into a very large number of sharp bands (Fig. 1*a*), and a single band in this type of experiment is, therefore, strongly suggestive of Ab homogeneity. In fact, myeloma proteins which are presumably homogeneous in the primary structures of their heavy and light chains are separated into two or three closely spaced bands by this technique (Figs. 2*d* and *e*). This residual degree of heterogeneity may be due to the heterogeneity of their attached oligosaccharides.[22] The *combined* observations of a few closely spaced bands in isoelectric focusing for a sample of whole Ab, *and* of a single electrophoretic band of light chains derived from the same Ab, therefore make it highly probable that the Ab is homogeneous. One would further expect that a particular Ab should exhibit a comparable degree of heterogeneity by both experimental techniques; if only a few molecular species of Ab were present, only a few well-resolved peaks should be observed in isoelectric focusing of the whole Ab, and only two or three light-chain bands should be found; on the other hand, one would expect many bands in isoelectric focusing for a more heterogeneous Ab that exhibited five or six light-chain bands. The expected correlation between the two types of experiments is in fact borne out (Fig. 4).

About a third of the individual primary antisera elicited to Pap-S-DNPL contained essentially homogeneous anti-DNP Ab by these criteria (Fig. 4, *A–E*). This is a remarkably high frequency. Only very rarely is an apparently homogeneous antihapten Ab found in an animal immunized with conventional hapten–protein antigens.[23]

Of special significance is the fact that in all of the Ab samples which were apparently homogeneous or of markedly restricted heterogeneity (Fig. 4, *A–H*), the predominant Ab component had an isoelectric point close to pH 5.0 in the isoelectric focusing gels. On the other hand, pooled mouse anti-DNP Ab produced in ascites fluids against DNP-bovine gamma globulin (Fig. 2*b*) contained many molecular species with a range of isoelectric points from pH 6 to 7. Fur-

thermore, the more heterogeneous anti-DNP elicited by Pap-S-DNPL (Fig. 4 *I* and *J*) contained species with isoelectric points throughout the pH range from 5 to 8. Several explanations of the restriction in isoelectric points are possible. (1) There is a preferred class or subclass of anti-DNP immunoglobulins which is selected by the antigen Pap-S-DNPL that is characterized by a narrow range of isoelectric points around pH 5.0. We have not yet investigated the heavy- and light-chain classes of the anti-DNP Ab. However, it is of interest in this connection that the major protein species of four out of six IgA mouse myelomas with antibodylike activity also focused at a pH near 5.0 in the isoelectric focusing gels. (2) There is a selection for anti-DNP Ab molecules with a large net negative charge, because the carrier protein of the antigen (papain) is relatively positively charged (isoelectric point 8.8).[24] That the net charge of an antigen can influence the charge characteristics of the Ab elicited has been clearly demonstrated by Sela and his colleages.[25, 26] (3) Only a few different anti-DNP molecules with closely similar primary structures of their light chains (including their variable regions) are preferentially elicited by immunization with Pap-S-DNPL in different individual mice. If the last possibility were indeed the case, it would demonstrate that there was a severely limited number of ways in which the *species* could preferentially respond to a truly homogeneous antigen. This would have profound implications for theories of Ab biosynthesis. At the present time, however, we have no information that discriminates among these three or other possible explanations.

The question arises whether the relatively high frequency of homogeneous Ab responses is due to the structural homogeneity of Pap-S-DNPL, or rather to its poor immunogenicity. In the latter case, one might argue that the low probability of an Ab response favors the induction of only one or of a few Ab-producing clones upon immunization. In recent years there have been a number of investigations of the antihapten Ab elicited to chemically defined hapten antigens. Such studies up to 1968 are reviewed by Haber.[27] In some cases,[28, 29] antihapten Ab of uniform binding affinity have been elicited, but light-chain gel electrophoresis was either not performed or, when it was has revealed electrophoretic heterogeneity of the Ab. In several other studies with chemically refined hapten antigens,[30, 31] the antihapten Ab have clearly been heterogeneous. On the other hand, these experiments may not be comparable to the ones we have performed with Pap-S-DNPL because in the former cases Freund's complete adjuvant was used in the immunization procedures. We have previously shown[2] that while the use of this adjuvant increases the anti-DNP Ab titers elicited by Pap-S-DNPL, it also provokes a more heterogeneous Ab response. Recently, we have prepared a defined DNP-conjugate by attaching a DNP-aminocaproyl group to a 21-residue polyalanyl peptide chain which was in turn attached to the protein carrier hemocyanin (E. R. Habicht, Jr., G. N. Trump, and S. J. Singer, to be published). Immunization of mice with this conjugate without adjuvant resulted in anti-DNP Ab titers comparable to those elicited toward Pap-S-DNPL, but by contrast the anti-DNP Ab in each of nine individual responders was grossly heterogeneous in isoelectric focusing. These results suggest, therefore, that it is not the poor immunogenicity of Pap-S-DNPL

that is primarily responsible for the high frequency of homogeneous anti-DNP Ab responses which it elicits but rather its chemical and structural homogeneity.

Electrophoretically homogeneous Ab have been elicited in about 10% of outbred rabbits to the natural carbohydrate antigens of several groups of streptococci.[32] It is remarkable that such Ab are formed in exceedingly high titers. Amino acid sequence analysis at the amino terminals of the light chains of these Ab have been begun,[33] and while they show that some sequence heterogeneity exists, they demonstrate that a very highly restricted population of chains is produced to these apparently homogeneous carbohydrate antigens. With these natural complex antigens, however, the precise chemistry and conformation of the antigenic determinants may be difficult to establish, and in any event, they may be obtained in only a limited number of variations.

In principle, a wide range of haptens and carrier proteins can be systematically utilized to construct homogeneous antigens like Pap-S-DNPL. Proteins such as chymopapain, bromelain, and ficin are structurally related to papain to different degrees, each enzyme having a single reactive cysteine-SH in its active site; IADL, its 2,4,6-trinitrophenyl analogue, or any other analogous hapten derivative[34] can be reacted with these enzymes in the same way that Pap-S-DNPL is prepared. Although the antihapten Ab response at present is of low titer, interesting questions of the role of the carrier, and of the specificity and diversity of the cellular response to such homogeneous antigens, can be explored.

We are grateful for the expert technical assistance provided by DeAnne Kaufman and George Anders.

* This work was supported by grants AI-06659 and GM-12027 from the U.S. Public Health Service.

† U.S. Public Health Service Postdoctoral Fellow, 1967–1969. Present address: Department of Genetics, University of Hawaii.

‡ To whom reprint requests should be addressed.

[1] Singer, S. J., *Immunochem.*, **1**, 15 (1964).

[2] Brenneman, L., and S. J. Singer, these PROCEEDINGS, **60**, 258 (1968).

[3] Brenneman, L., and S. J. Singer, *Ann. N.Y. Acad. Sci.*, **169**, 72 (1970).

[4] Sartorelli, A. C., D. S. Fischer, and W. G. Downs, *J. Immunol.*, **96**, 676 (1966). We are indebted to Dr. M. Cohn for the initial gift of the sarcoma cells.

[5] Greenwood, F., W. Hunter, and J. Glover, *Biochem. J.*, **89**, 114 (1963).

[6] The recipe for the stock solution of acrylamide and ampholines is: 2.00 ml 50% cyanogum (E. C. Apparatus Co., Philadelphia, Pa.), 0.40 ml 8 *M* urea, 1.00 ml 40% ampholines (LKB-Produkter, Bromma 1, Sweden), and 0.40 ml 0.033% riboflavin. To 1.60 ml of the Ab solution in 5 *M* urea was added 0.40 ml of this stock solution.

[7] Fleischman, J. B., R. H. Pain, and R. R. Porter, *Arch. Biochem. Biophys., Suppl. 1*, **174** (1962).

[8] Williams, C., and R. A. Reisfeld, *Ann. N.Y. Acad. Sci.*, **121**, 373 (1964).

[9] Farah, F. S., M. Kern, and H. N. Eisen, *J. Exptl. Med.*, **112**, 1195 (1960).

[10] Thorpe, N. O., and S. J. Singer, *Biochemistry*, **8**, 4523 (1969).

[11] The generous gift of H. Grey.

[12] The generous gift of D. Schubert and M. Cohn.

[13] Potter, M., and C. R. Boyce, *Nature*, **193**, 1086 (1962).

[14] Gray, W. R., W. J. Dreyer, and L. Hood, *Science*, **155**, 465 (1967).

[15] McDevitt, H. O., and M. Sela, *J. Exptl. Med.*, **122**, 517 (1965).

[16] Dutton, R. W., and R. I. Mishell, *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 32 (1967), p. 407.

[17] Pincus, J. H., E. Haber, M. Katz, and A. M. Pappenheimer, Jr., *Science*, **162**, 667 (1968).

[18] Lark, C. A., H. N. Eisen, and S. Dray, *J. Immunol.*, **95**, 404 (1965).
[19] Cohen, S., and R. R. Porter, *Biochem. J.*, **90**, 278 (1964).
[20] Awdeh, Z. L., A. R. Williamson, and B. A. Askonas, *Nature*, **219**, 66 (1968).
[21] Dale, G., and A. L. Latner, *Lancet*, **7547**, 847 (1968).
[22] Melchers, F., E. S. Lennox, and M. Facon, *Biochem. Biophys. Res. Commun.*, **24**, 244 (1966).
[23] Nisonoff, A., S. Zappacosta, and R. Jureziz, *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 32 (1967), p. 89.
[24] Smith, E. L., R. J. Kimmel, and D. M. Brown, *J. Biol. Chem.*, **207**, 533 (1954).
[25] Sela, M., and E. Mozes, these PROCEEDINGS, **55**, 445 (1966).
[26] Rüde, E., E. Mozes, and M. Sela, *Biochemistry*, **7**, 2971 (1968).
[27] Haber, E., *Ann. Rev. Biochem.*, **37**, 497 (1968).
[28] Haber, E., F. F. Richards, J. Spragg, K. F. Austen, M. Valloton, and L. B. Page, *Cold Spring Harbor Symposia on Quantitative Biology*, **32**, 299 (1967).
[29] Wu, W.-H., and J. H. Rockey, *Biochemistry*, **8**, 2719 (1969).
[30] Eisen, H. N., E. S. Simms, J. R. Little, Jr., and L. A. Steiner, *Federation Proc.*, **23**, 559 (1964).
[31] Little, J. R., and R. B. Counts, *Biochemistry*, **8**, 2729 (1969).
[32] Braun, D. G., K. Eichmann, and R. M. Krause, *J. Exptl. Med.*, **129**, 809 (1969).
[33] Hood, L., H. Lackland, K. Eichmann, T. J. Kindt, D. C. Braun, and R. M. Krause, these PROCEEDINGS, **63**, 890 (1969).
[34] Pepe, F. A., and S. J. Singer, *J. Amer. Chem. Soc.*, **78**, 4583 (1956).