Biosynthesis of Antibody Molecules with Similar Properties during Prolonged Immunization*

A. Bruce MacDonald, Lucilla Alescio, and Alfred Nisonoff†

ABSTRACT: Anti-*p*-azobenzoate antibodies of the immunoglobulin G class were purified specifically from pools of serum collected from individual rabbits at intervals of about 6 months. Recombinants were prepared in which heavy chains were derived from the earlier and light chains from the later bleedings of the same rabbit, as well as reciprocal recombinants in which heavy chains were obtained from the later bleedings and light chains from the earlier bleedings. Such recombinants possessed 60–100% as much specific activity as recombinants made from a single pool. Recombinants in which the light chains were derived from nonspecific immunoglobulin G or from anti-*p*-azobenzoate antibodies of other rabbits had much less activity. The results suggest prolonged persistence of cell lines synthesizing antibody molecules of a particular structure. Recovery of activity in both reciprocal recombinants, *i.e.*, with the heavy chains coming from either the earlier or later pool and the light chains from the opposite pool, suggests persistence of both types of polypeptide chain. Since the animals were repeatedly challenged with antigen, it appears that the antigen was largely used to stimulate already committed cells in a hyperimmunized animal, rather than to initiate new clones.

Heavy and light chains isolated from an immunoglobulin of the IgG or IgA class can reassociate through noncovalent interactions to form a product similar in size and physical properties to the original molecule (Roholt *et al.*, 1963; Fougereau and Edelman, 1964; Olins and Edelman, 1964; Grey and Mannik, 1965). Although recombination occurs between H and L chains of different molecules, for example, from a specific antibody and nonspecific IgG (Roholt *et al.*, 1963), different myeloma proteins (Grey and Mannik, 1965), or even immunoglobulins of different species (Fougereau *et al.*, 1964), H and L chains derived from the same molecule in general combine preferentially, as shown by experiments involving competition between homologous and heterologous chains (Grey and Mannik, 1965; Roholt *et al.*, 1967).

Isolated H chains, or recombinants of the H chains of an antibody with nonspecific L chains, retain specific activity (Fleischman *et al.*, 1963; Utsumi and Karush, 1964; Haber and Richards, 1965) but this activity is increased substantially if H and L chains of the same antibody population are allowed to recombine (Edelman *et al.*, 1963; Franek and Nezlin, 1963). The investigations of Roholt *et al.* (1965) with rabbit anti-*p*-azobenzoate antibodies have shown that restoration of a significant amount of hapten binding capacity requires that both the H and L chains be derived from the same rabbit as well as from antibody of the same specificity. In the case of antidinitrophenyl antibodies, which are of relatively high affinity, enhanced activity was observed in recombinants of H and L chains from different rabbits (Zappacosta and Nisonoff, 1967). Here again, however, the homologous recombinants were much more active. In subpopulations of antibodies of the same specificity from a single rabbit, fractionated according to hapten binding affinity, recombinants in which the H and L chains were derived from the same fraction showed maximal activity (Hong and Nisonoff, 1966).

The large difference in activity between homologous and heterologous recombinants of H and L chains of anti-*p*-azobenzoate antibody provides a means of determining whether molecules of antibody synthesized in a single animal over a long period of time are structurally related. This information may be relevant to the question of the persistence of clones of cells during prolonged immunization. The results of such an investigation are presented here.

Materials and Methods

Keynote limpet hemocyanin was obtained from the Mann Research Laboratories and partially purified by passage through Sephadex G-200 at neutral pH; the excluded fraction was used. Nonspecific IgG was prepared from the serum of nonimmunized rabbits by two precipitations with sodium sulfate and passage through DEAE-cellulose, as previously described (Palmer and Nisonoff, 1963). Iodoacetic acid and *p*-iodobenzoic acid were recrystallized from petroleum ether (bp 30–60°) and ethanol, respectively. *p*-Aminobenzoic acid was recrystallized from an ethanol–water mixture. 2-Mercaptoethanol was redistilled and stored at −20° in a number of vials, which were discarded after opening once. *p*-Iodobenzoic acid was labeled with ^{125}I by an exchange reaction and purified according to Blau *et al.* (1958).

An antigen used for immunization was prepared by coupling diazotized *p*-aminobenzoic acid to bovine IgG (Fraction II, Pentex Co.) in the ratio 40 mg/g of protein; the reaction was carried out for 2 hr at pH 9–9.5 in cold NaCl–borate buffer. Unconjugated hapten was removed by extensive dialysis. The antigen used for testing sera and for specific purification of anti-*p*-azobenzoate antibodies was prepared by an

* From the Department of Microbiology, University of Illinois College of Medicine, Chicago, Illinois 60680. *Received January 15, 1969.* This investigation was supported by grants from the National Institutes of Health (AI-06281) and the National Science Foundation (GB-5424).

† Recipient of a research career award (AI-K6-2947) of the National Institutes of Health.

TABLE I: Binding of [^{125}I]*p*-Iodobenzoate by Anti-*p*-azobenzoate Antibodies Purified from Early and Late Sera of Rabbit P3 and by Various Recombinants of Heavy and Light Chains.[a]

Sample[b]	$10^6 \times$ Free Hapten Concn (M) 0.254 $10^6 \times$ Concn Hapten Bound (M)	5.83	$10^{-5} \times K_0$[c]	Sites/ Molecule[d]	a[e]
$P3_1$ (untreated)	0.998 (0.039)	5.67 (0.02)			
$H(P3_1) + L(P3_1)$	0.515 (0.02)	2.45 (0.04)			
$H(P3_1) + L(P3_2)$	0.369 (0.001)	1.96 (0.03)			
$H(P3_1) + L$(pool)	0.096 (0.008)	0.537 (0.022)			
$H(P3_1) + L_N$	0.071 [f]	0.405 (0.068)			
$P3_2$ (untreated)	1.36 (0.01)	7.14 (0.29)	6.5	1.5	0.75
$H(P3_2) + L(P3_2)$	0.468 (0.018)	2.27 (0.13)	4.5	0.70	0.80
$H(P3_2) + L(P3_1)$	0.496 (0.009)	2.73 (0.12)	7.4	0.65	0.95
$H(P3_2) + L$(pool)	0.218 (0.01)	1.05 (0.16)			
$H(P3_2) + L_N$	0.157 (0.007)	0.86 (0.21)			
$H(P3_2) + L(V2_2)$	0.246 (0.017)	1.26 (0.05)			
Pool (untreated)	0.595 (0.008)	4.09 (0.12)			

[a] Equilibrium dialysis was carried out in triplicate except as indicated. Values in parentheses are standard deviations. The concentration of H chains in each sample was 9.4×10^{-6} M. [b] Subscripts 1 and 2 refer to early and late bleedings, respectively. Subscript N indicates nonspecific IgG. Thus, L_N refers to L chains of nonspecific IgG and $P3_1$ to specifically purified antibody of rabbit P3 isolated from the earlier set of bleedings. L(pool) designates light chains isolated from a pool of purified anti-*p*-azobenzoate antibodies from several rabbits, not including P3. [c] Average association constant. [d] For recombinants, this value equals the number of binding sites for each pair of H chains present. [e] Index of heterogeneity. [f] Single determination.

identical procedure from *p*-aminobenzoic acid and hemocyanin.

Specifically Purified Anti-p-azobenzoate Antibodies. A specific precipitate was prepared from antiserum with an optimal amount of the hemocyanin–azobenzoate conjugate. Ethylenediamine tetraacetate (0.01 M) was added to the mixture to minimize uptake of complement. The washed precipitate was dissolved at 37°, with periodic stirring, in 0.3 M *p*-nitrobenzoate (pH 8.5) with approximately 0.5 ml of hapten solution/mg of specific precipitate. Hemocyanin–azobenzoate was removed by passage of the mixture through DEAE-cellulose equilibrated with 0.04 M sodium phosphate buffer (pH 6.9); the same buffer was used for elution. The column volume was 1 cm^3 for each milliliter of the mixture applied. Hemocyanin–*p*-azobenzoate, which is orange, was retained at the top of the column. Antibodies eluted were of the IgG class, as shown by immunoelectrophoresis. Free hapten was separated from the eluted antibody by successive dialyses against cold isotonic NaCl–borate buffer (pH 8) and 0.1 M sodium benzoate, followed by at least 1 week of dialysis against repeated changes of the NaCl–borate buffer.

The number of combining sites per molecule of purified antibody, obtained from hapten binding measurements using [^{125}I]*p*-iodobenzoate, are shown in Tables I and II. This value was not determined for preparation $P3_1$ because of insufficient material.

Recombination of Polypeptide Chains. For separation of H and L chains, reduction and alkylation were first carried out with 2-mercaptoethanol and iodoacetate in 0.3 M Tris-HCl (pH 8.2) according to Fleischman *et al.* (1963). Reduced, alkylated samples of antibodies or nonspecific IgG were dialyzed against cold 1 M propionic acid for 12 hr, then applied to a column of Sephadex G-100 in the cold room. The size of the column was approximately 10 cm^3 for each milligram of protein applied. For recombination studies, each of the reduced preparations was gel filtered through a separate column at the same time. The L chain peaks represented 19–23% of the total absorbancy at 280 mμ, or 22–29% of the total protein on the basis of extinction coefficients reported by Crumpton and Wilkinson (1963), except for pool $P3_1$ for which the L chain peak represented 26% of the absorbancy.

Recombinants were prepared by mixing H and L chains immediately after elution from the Sephadex column in a weight ratio (H/L) of 1.7:1, or an approximate molar ratio of 1:1.3. Mixtures were dialyzed overnight against cold water, then against NaCl–borate buffer (pH 8), ionic strength 0.16. They were concentrated by pervaporation, dialyzed against buffer at pH 8, and adjusted to the desired concentration for equilibrium dialysis. For recombinants, the concentration was 0.8 mg/ml; for other globulin preparations the concentration was 0.7 mg/ml, so that the concentration of heavy chains was approximately the same in each sample. For antibodies or recombinants the extinction coefficient used was 1.50/mg per ml.

Other Methods. Equilibrium dialysis was carried out in NaCl–borate buffer (pH 8) containing [^{125}I]*p*-iodobenzoate (Nisonoff and Pressman, 1958). Samples to be compared were dialyzed simultaneously against a large volume of the hapten solution at a given concentration. After equilibration, concentrations of hapten were determined inside and outside the dialysis bags. For a few samples (Tables I and II) it was possible to obtain five or six point binding curves; values of an

TABLE II: Binding of [^{125}I]*p*-Iodobenzoate by Anti-*p*-azobenzoate Antibodies Purified from Early and Late Sera of Rabbit V2 and by Various Recombinants of Heavy and Light Chains.[a]

Sample[b]	$10^6 \times$ Free Hapten Concn (M) 0.249 $10^6 \times$ Concn Hapten Bound (M)	5.53	$10^{-5} \times K_0$[c]	Sites/ Molecule[d]	a[e]
$V2_1$	2.01 (0.02)	7.66 (0.24)	11.8	2.0	1.0
$V2_1$ reduced, alkylated	2.07 (0.10)	6.82 (0.05)			
$V2_1$ reduced, alkylated, acid[f]	1.91 (0.01)	7.68 (0.10)			
$H(V2_1) + L(V2_1)$	1.38 (0.06)	6.80 (0.08)			
$H(V2_1) + L(V2_2)$	0.811 (0.007)	4.63[g]			
$H(V2_1) + L$(pool)	0.560 (0.002)	2.60 (0.11)			
$H(V2_1) + L_N$	0.521 (0.006)	1.71[g]			
$V2_2$	1.41 (0.07)	6.41 (0.01)	8.1	1.8	1.0
$V2_2$ reduced, alkylated	1.59 (0.18)	6.02 (0.09)			
$V2_2$ reduced, alkylated, acid[f]	1.52 (0.05)	6.99 (0.03)			
$H(V2_2) + L(V2_2)$	0.832 (0.035)	4.97 (0.26)	5.2	1.6	1.0
$H(V2_2) + L(V2_1)$	0.702 (0.021)	4.09 (0.41)			
$H(V2_2) + L$(pool)	0.230 *g*	1.54 (0.00)			
$H(V2_2) + L_N$	0.151 (0.014)	0.35 (0.21)			

[a] For footnotes *b–e* see Table I; however, results in this table are of duplicate determinations, with mean deviations given in parentheses. [f] Reduced, alkylated, dialyzed against cold 1 M propionic acid for 1 day, then against water and neutral buffer. [g] Single determination.

average association constant, K_0, the number of combining sites per molecule, and an index of heterogeneity, a, were estimated from these curves by using the Sips distribution curve (Sips, 1948; Nisonoff and Pressman, 1958).

The index of heterogeneity, a, was calculated with the aid of a computer by determining $1/b$ as a function of $(1/f)^a$, where f and b are free and bound hapten concentrations, for values of a varying by increments of 0.05. A straight line was fitted to each set of results by the method of least squares and the sums of the squares of the deviations of $1/b$ from the line were obtained. The value of a giving the minimum sum was taken as the index of heterogeneity. The total concentration of combining sites, A_t, is given by the intercept of this line on the $1/b$ axis. The average association constant, K_0, was calculated from the slope of the line, which is $1/((K_0)^a A_t)$.

Although a can be estimated directly as the slope of a plot of $\log((A_t/b) - 1)$ *vs.* $\log(1/f)$, the slope is sensitive to small errors in b as b approaches A_t, since $\log((A_t/b) - 1)$ approaches negative infinity. Thus the slope is weighted heavily by the higher values of b. The same characteristic applies to the plot of $\log(r/(n-r))$ against $\log f$, where r is moles of hapten bound per mole of antibody and n is the valence of antibody (Karush, 1962).

Radioactivity was measured in a Packard gamma scintillation spectrometer. A minimum of 3000 counts above background was recorded for each sample.

Specific precipitates made with antibenzoate antibody and the hemocyanin–*p*-azobenzoate test antigen were quantified by dissolving in 0.04 M NaOH and immediately reading the optical density at 280 and 400 mμ. The absorbancy at 400 mμ, which is due to hemocyanin–*p*-azobenzoate, and the ratio of absorbancies of the antigen at 400 and 280 mμ, were used to correct the reading at 280 mμ for the contribution by antigen.

Immunization Schedule. Two rabbits were used to study complementarity of H and L chains of antibodies isolated from the serum of the same rabbit at different times. The allotype of rabbit P3 was a2, a3, b4; that of rabbit V2 was a1, a3, b4. Allotypes present in the specifically purified antibodies of the pool, used as a control, were a1, a2, a3, b4, with a small amount of b5. Each animal was initially inoculated with 10 mg of bovine γ-globulin–*p*-azobenzoate emulsified in complete Freund's adjuvant (Difco Co.). Inoculations were repeated at 2-week intervals. After 3 months 3–5 mg of antigen was injected intravenously into rabbit P3 at 1- or 2-week intervals. The initial pool of antiserum P3 consisted of weekly bleedings taken over a 6-week period after the animal had been immunized for approximately 1-year. The second pool of P3 was collected over a 7-week period starting 5 months after the completion of the first series of bleedings. During each series, intravenous injections of 5 mg of antigen were administered after each bleeding.

For rabbit V2 the schedule was similar; however, the first pool of antiserum was collected over a 7-week period beginning 6 months after the initial immunization. The second pool was taken over a 9-week period starting 4.5 months after the completion of the first series of bleedings. The rabbit was allowed to rest for 2 months just prior to the start of the second series.

The volumes of serum collected and the yields of purified antibody from each series of bleedings were as follows. Subscripts 1 and 2 denote the early and late series, respectively: $P3_1$, 54 ml, 31 mg; $P3_2$, 96 ml, 56 mg; $V2_1$, 132 ml, 58 mg; and $V2_2$, 103 ml, 65 mg. Yields of purified antibody varied from 40 to 69% of the amount present in the serum, as determined by the quantitative precipitin reaction with hemocyanin–*p*-azobenzoate.

Rabbits providing serum for the pool of antibenzoate antibodies were immunized by the schedule described above for periods of 3–10 months. A portion of the resulting large pool of serum of high titer was used.

Results

Tables I and II present data on the hapten-binding activity of recombinants of H and L chains from anti-*p*-azobenzoate antibodies, isolated from the serums of individual rabbits (P3 or V2) at intervals of several months. Data for various heterologous recombinants are also given. In the tables, subscripts 1 and 2 refer to the early and late pools of serum, taken as described under Methods. In each case, reciprocal combinations of H and L chains were made for the two pools. Except for the light chains of nonspecific IgG (L_N) the preparations used were specifically purified antibodies or their polypeptide chains. In some cases enough material was available to permit binding measurements at five or six different hapten concentrations. This permitted calculation of an average association constant, K_0, the number of combining sites per molecule, and the Sips index of heterogeneity, a, which are shown in the last three columns of the tables. For recombinants, which contained a slight molar excess of L chains, the number of molecules assumed to be present for the purpose of calculation was half the number of H chains.

In each instance, light chains derived from the same rabbit were much more effective than heterologous light chains in enhancing the activity of a given preparation of H chains. This was true whether the L chains of an individual rabbit were derived from the same molecules as the H chains (*e.g.*, $H(P3_1) + L(P3_1)$) or from antibody purified from the serum of that rabbit at a different time during the period of immunization (*e.g.*, $H(P3_1) + L(P3_2)$ or $H(P3_2) + L(P3_1)$). The concentration of hapten bound, at a given free hapten concentration, was 1.5–3.5 times as great for recombinants consisting of H and L chains from different bleedings of the same rabbit than for recombinants of the same H chains with L chains from antibenzoate antibody from another source. L chains used in preparing heterologous recombinants were obtained from nonspecific IgG (L_N), from antibenzoate antibodies isolated from a pool of serum from several other immunized rabbits (L(pool)), or from one other rabbit ($H(P3_2) + L(V2_2)$; Table I).

For one recombinant involving early and late bleedings, $H(P3_2) + L(P3_1)$, the activity was as great as that of the homologous recombinant, $H(P3_2) + L(P3_2)$; and for two of the other three such recombinants, $H(P3_1) + L(P3_2)$ and $H(V2_2) + L(V2_1)$, the activity was more than two-thirds as great as that of the corresponding homologous recombinant, $H(P3_1) + L(P3_1)$ or $H(V2_2) + L(V2_2)$.

For one recombinant consisting of chains from early and late pools of serum, $H(P3_2) + L(P3_1)$, a six-point binding curve was obtained. The average association constant, K_0, agreed closely with that of the untreated purified antibody from which the H chains of the recombinant were derived. The decrease in activity was due to loss of active binding sites. Similarly, a comparison of K_0 and number of combining sites per molecule for a recombinant made from a single antibody preparation, $H(P3_2) + L(P3_2)$, with the corresponding values for the untreated antibody, indicates that losses of activity resulted mainly from a decrease in the number of combining sites rather than K_0. It should be noted that the data would not distinguish between loss of activity owing to incorrect pairing or to failure to recombine at all. In either case, the homologous L chains are the most effective in restoring activity. The fact that H chains were rendered completely soluble indicates that most of them had recombined.

For the second series of bleedings of rabbit V2 the number of active combining sites in the homologous recombinant was unusually high, about 90% of that for untreated antibody. Also, reduction, alkylation, and exposure of the purified antibody to propionic acid resulted in no detectable loss of activity. A possible explanation is considered below. Antibodies from rabbit V2 were also relatively homogeneous with respect to binding affinity, with a Sips index of heterogeneity of 1; the coefficients of correlation for linear plots of $1/b$ *vs.* $1/f$ were greater than 0.98 for each preparation.

An additional, separate experiment was performed with the serums from late bleedings of rabbits P3 and V2. Antibenzoate antibodies were purified specifically from each serum. An IgG fraction was prepared from each supernatant after precipitation with the hemocyanin–azobenzoate antigen. The antibodies and IgG were dissociated into H and L chains; recombinants were then prepared and tested for binding capacity at two free hapten concentrations, 0.25×10^{-6} and 5.8×10^{-6} M, with a protein concentration of 0.7 mg/ml. Recombinants in which the H chains and L chains were derived from the same antibody preparation bound 1.8–3.9 times as much hapten as recombinants in which the H chains were derived from antibody and the L chains from nonspecific IgG of the same serum which provided the antibody.

Discussion

Investigations summarized in the introduction have shown that the hapten binding capacity of a recombinant of H and L polypeptide chains may represent a large fraction of that of the native molecule, provided both chains are derived from antibody of the same specificity and from the same animal. In the present study, with specifically purified anti-*p*-azobenzoate antibodies, maximal activity was again observed in recombinants in which the polypeptide chains had been isolated from antibody purified from the serum of a single rabbit. In the heterologous recombinants tested, L chains were derived from nonspecific IgG, from a pool of antibenzoate antibody from other rabbits, or from antibenzoate antibody from an individual rabbit.

Recombinants of H and L chains from two different pools of serum, taken from one rabbit at different times during the course of immunization, had 60–100% as much hapten binding capacity as recombinants prepared from a single serum pool, and were much more active than heterologous recombinants. Similar results were obtained with serums of both rabbits investigated, and with reciprocal recombinants in which H chains were derived from either the earlier or later set of bleedings, and the L chains from the opposite set. The time elapsed between the start of the first and second series of bleedings was about 6 months in each case; from the end of the first series until the beginning of the second it was 4.5–5 months. Thus, molecules with related properties may be found over a long period of time in a hyperimmunized animal. Since the half-life of rabbit IgG is approximately 6 days (Taliaferro and Talmage, 1956; Weigle, 1958), this must be attributed to

continued biosynthesis of closely related or identical molecules. Because activity was recovered in both reciprocal recombinants, *i.e.*, with the H or L chains coming from either the earlier or later pool of serum, persistence of both types of chain is indicated.

The data also suggest that the proportions of at least some of the various species of antibenzoate antibodies, which make up the heterogeneous population in an individual rabbit, did not vary greatly during the period of immunization investigated. If, for example, the proportion of one species of antibenzoate molecule decreased during immunization from, say, 30 to 5% of the total population, insufficient L chains of that species would be available in the later serum for complementation of the appropriate H chains of the early serum. This conclusion is particularly applicable to the antibodies of rabbit P3; L chains of the earlier series of bleedings were equally as effective as those of the later series in enhancing the activity of the H chains from the later series.

Perhaps the simplest hypothesis consistent with the data is that clones of cells, each synthesizing a homogeneous population of antibody molecules, persist during prolonged immunization. In view of evidence suggesting that the life span of most antibody-producing cells does not exceed a few days (Schooley, 1961; Nossal and Makela, 1962; Makinodan and Albright, 1967), such persistence of a clone might be attributable to the stimulation of "memory" cells, which are assumed to be a by-product of the differentiation and multiplication processes leading to cells capable of antibody biosynthesis (Sercarz and Coons, 1962).

An alternative explanation is that H or L chains from a given clone of cells within a single animal are able to interact effectively with chains made by an unrelated clone from the same animal to produce active antibody molecules. Since H and L chains from different animals do not interact effectively in this way, this explanation would imply the existence of amino acid sequences which are peculiar to an individual animal rather than to a clone of cells. This seems rather unlikely.

Recoveries of activity in homologous recombinants from rabbit V2 were exceptionally high, up to 90%. Also, there was little or no loss of activity upon exposure of the reduced antibodies of rabbit V2 to propionic acid. This cannot be attributed to failure to dissociate the H and L chains, since normal yields of L chains were obtained during gel filtration; also, there was much less activity in recombinants made with antibody H chains and nonspecific L chains. It seems likely that the antibodies of this rabbit were of limited heterogeneity. One would expect lower recoveries of activity for a very heterogeneous mixture because of a greater probability of incorrect pairing of H and L chains. The possibility of relative homogeneity is supported by the Sips index, a, which was 1 for both the earlier and later series of bleedings, indicating little heterogeneity with respect to binding affinity. It is of course possible that a considerable degree of heterogeneity can be tolerated without much loss of activity upon dissociation and reassociation of polypeptide chains. Also, the Sips index indicates a small range of K values but does not necessarily provide evidence for molecular homogeneity. Nevertheless, it is noteworthy that the high value of a correlates with retention of activity after exposure of the reduced antibody to propionic acid.

The prolonged presence in serum of apparently identical molecules is observed in pathological conditions such as multiple myeloma or Waldenstrom's macroglobulinemia. It is improbable, however, that this is related to repeated stimulation by antigen, since similar molecules persist after repeated transplantation of the tumor to another animal (Potter *et al.*, 1964) or repeated passage in tissue culture (Cohn, 1967).

It should be of interest to determine whether antibodies from primary and secondary responses are mutually complementary. In the case of rabbit antidinitrophenyl antibod es, the increase in combining affinity during the first few weeks after immunization (Eisen and Siskind, 1964) suggests that the population is undergoing important changes, possibly because of a progressive selection of clones producing antibodies of higher affinity.

References

Blau, M., Johnson, A. C., and Pressman, D. (1958), *Intern. J. Appl. Radiation Isotopes* 3, 217.

Cohn, M. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 211.

Crumpton, M. J., and Wilkinson, J. M. (1963), *Biochem. J. 88*, 228.

Edelman, G. M., Olins, D. E., Gally, J. A., and Zinder, N. D. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 753.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Fleischman, J. B., Porter, R. R., and Press, E. M. (1963), *Biochem. J. 88*, 220.

Fougereau, M., and Edelman, G. M. (1964), *Biochemistry 3*, 1120.

Fougereau, M., Olins, D. E., and Edelman, G. M. (1964), *J. Exptl. Med. 120*, 349.

Franek, F., and Nezlin, R. S. (1963), *Folia Microbiol. 8*, 128.

Grey, H. M., and Mannik, M. (1965), *J. Exptl. Med. 122*, 619.

Haber, E., and Richards F. F. (1966), *Proc. Roy. Soc. (London) B166*, 176.

Hong, R., and Nisonoff, A. (1966), *J. Immunol. 96*, 622.

Karush, F. (1962), *Advan. Immunol. 2*, 1.

Makinodan, T., and Albright, J. F. (1967), *Progr. Allergy 10*, 1.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.

Nossal, G. J. V., and Makela, O. (1962), *J. Exptl. Med. 115*, 209.

Olins, D. E., and Edelman, G. M. (1964), *J. Exptl. Med. 119*, 789.

Palmer, J. L., and Nisonoff, A. (1963), *J. Biol. Chem. 238*, 2393.

Potter, M., Dreyer, W. J., Kuff, E. L., and McIntire, K. R. (1964), *J. Mol. Biol. 8, 814.*

Roholt, O., Onoue, K., and Pressman, D. (1963), *Proc. Natl. Acad. Sci. U. S. 51*, 173.

Roholt, O. A., Radzimski, G., and Pressman D. (1965), *Science 147*, 613.

Roholt, O. A., Radzimski, G., and Pressman, D. (1967), *J. Exptl. Med. 125*, 191.

Schooley, J. C. (1961), *J. Immunol. 86*, 331.

Sercarz, E., and Coons, A. H. (1962), *in* Mechanisms of Immunological Tolerance, Hasek, M., Lengerova, A., and

Vojtiskova, M., Ed., New York, N. Y., Academic, p 73.
Sips, R. (1948) *J. Chem. Phys. 16*, 490.
Taliaferro, W. H., and Talmage, D. W. (1956), *J. Infect. Diseases 99*, 21.
Utsumi, S., and Karush, F. (1964), *Biochemistry 3*, 1329.
Weigle, W. O. (1958), *J. Immunol. 81*, 204.
Zappacosta, S., and Nisonoff, A. (1967), *J. Immunol. 100*, 781.

Steric Effects in the Glyceraldehyde 3-Phosphate Dehydrogenase Catalyzed Hydrolysis of Acyl Phosphates. An Example of Substrate-Induced Cooperativity*

David R. Phillips† and Thomas H. Fife

ABSTRACT: The acyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase has been examined at 25°. This activity can conveniently be divided into three categories depending upon the steric bulk in the acyl group of the substrate, that of: (1) acetyl phosphate which follows apparent second-order kinetics, K_m being too large to measure; (2) propionyl, butyryl, isobutyryl, and isovaleryl phosphate which obey normal Michaelis–Menten kinetics; and (3) the highly branched compounds, trimethylacetyl and 3,3-dimethylbutyryl phosphate, which are not substrates. Arsenate increases the rate of hydrolysis of the compounds in group 2, showing the rate-determining step for those compounds to be deacylation of the acyl enzyme intermediate. Methyl phosphate, however, was found to be a poor catalyst for deacylation of the enzyme. This and other observations support a general base mechanism for deacylation of glyceraldehyde 3-phosphate dehydrogenase.

The highly branched compounds bind to the enzyme since they inhibit acetyl phosphatase activity. In addition, compounds in groups 2 and 3 inhibit the dehydrogenase activity. This inhibition was found to be sigmoidal and was related to a cooperativity effect on binding to the enzyme. A plot of log k_3 (deacylation) *vs.* E_s, the Taft steric effects constants, had a slope of 1.1.

Acyl phosphates are substrates for glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) (Malhotra and Bernhard, 1968; Harting and Velick, 1954). The C–O bond is broken (Park and Koshland, 1958), and a cysteine residue is acylated at the active site of the enzyme (Mathew *et al.*, 1967). This cysteine is also acylated by *p*-nitrophenyl acetate during reaction with the NAD^+-free enzyme (Mathew *et al.*, 1967; Harris *et al.*, 1963). Thus, an acyl enzyme intermediate is formed which is a thiol ester. Histidine has also been implicated in the catalytic process, and mechanisms have been proposed for acylation and deacylation involving cysteine and histidine (Olson and Park, 1964). However, conclusive mechanistic evidence is lacking. Behme and Cordes (1967) found from studies of the esterase activity of the NAD^+-free enzyme that deacylation proceeds much more slowly in D_2O than H_2O and suggested that a general base catalyzed proton transfer may be occurring. There is, of course, recognized ambiguity in the interpretation of D_2O solvent isotope effects in enzymatic reactions (Jencks, 1963).

The study of steric effects in α-chymotrypsin-catalyzed reactions has given results that could be directly related to the mechanism of deacylation of the enzyme (Fife and Milstien, 1967; Milstien and Fife, 1968). It was considered that a similar study of steric effects in glyceraldehyde 3-phosphate dehydrogenase reactions, combined with nonenzymatic studies of steric effects in acyl phosphate and thiol ester hydrolysis, might yield useful information. This paper reports work in which acyl phosphates with varying steric bulk in the acyl group have been examined as substrates for glyceraldehyde 3-phosphate dehydrogenase. With this series, profound differences among the compounds were observed in regard to the kinetics followed and to binding effects which are dependent upon the size of the acyl group.

Experimental Section

Materials. Acetyl phosphate was purchased from Sigma Chemical Co. The remaining acyl phosphates were prepared as previously reported (Phillips and Fife, 1968). The barium salt of DL-glyceraldehyde-3-P diethyl acetal was purchased from Calbiochem. It was converted into the free aldehyde and the concentration of the D isomer determined by the method of Kochman and Rutter (1968). NAD^+ was also purchased from Calbiochem. K & K Laboratories, Inc., supplied the sodium monomethyl phosphate, and its concentration was deter-

* From the Department of Biochemistry, University of Southern California, Los Angeles, California. *Received January 17, 1969.* This work was supported by a research grant from the National Institutes of Health. We also acknowledge support by the Interdepartmental Cancer Research Committee of the University of Southern California. D. R. P. was supported by National Institutes of Health Research Training Grant GM197.

† This study represents part of the work to be submitted by D. R. P. in partial fulfillment of the requirements for the Ph.D. degree, University of Southern California.