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Effects of Traces of *n*-Alcohols on the Acid Denaturation of Horse Ferrihemoglobin*

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ABSTRACT: No binding of ^{14}C-labeled decanol by horse ferrihemoglobin (Hb^+) is detected in equilibrium dialysis experiments. Nevertheless, decanol and other such alcohols (6–12 carbons) at extreme dilution markedly accelerate the rate of acid denaturation without changing the rate–pH profile. Plotting the rate constants of denaturation, k_{obsd}, against the square of the alcohol concentration yields straight lines which show no sign of leveling off. Since Hb^+ is an α,β dimer in the pH region of interest, an average of 1 equiv of alcohol must be bound per subunit to give the observed catalytic effect. The rate constant of the denaturation of the Hb^+ alcohol complex is at least 250 times that of native Hb^+. The catalytic effectiveness increases greatly as the chain length of the alcohol is increased. From this observation it is proposed that hydrophobic interactions play a large part in the determination of the stability of the complex. Plots of log k_{obsd} as a function of $1/T$ are parallel both in the presence and absence of octanol. Thus, the change in the rate constant must be due to a change in the entropy of activation, consistent with the postulated hydrophobic interaction. Preliminary results show that octanol has no catalytic effect on the denaturation of either carbonyl- or oxyhemoglobin. The equilibrium pH profile for the denaturation of the octanol complex is shifted toward greater instability relative to that of Hb^+, and the number of protons required to initiate the denaturation is changed from five in the uncomplexed form to three in the complexed form: *i.e.*, Hb^+ in the presence of octanol shows less cooperativity with respect to hydrogen ion than does Hb^+ in its absence.

The binding of hydrocarbons and hydrocarbon derivatives to proteins has long been of interest to physical biochemists (Kauzmann, 1959). Bovine serum albumin (BSA),[1] for example, has been shown to possess a number of binding sites for simple hydrocarbons (Wishnia, 1962); in addition, 4–5 equiv of the alcohols dodecanol, decanol, and octanol are found to bind to BSA with association constants of 1.5×10^5, 7×10^4, and 3×10^3, respectively (Ray *et al.*, 1966). BSA has also been shown to bind fatty acid and detergent anions (Reynolds *et al.*, 1967; Goodman, 1958). The binding of such ions produces perturbations in the tryptophan, tyrosine, and phenylalanine uv absorption and fluorescence spectra (Bigelow and Sonnenberg, 1962; Polet and Steinhardt, 1968).

With hemoglobin, however, few data are available. Wishnia (1962) showed that butane and pentane were bound to horse oxyhemoglobin. It will be shown here that ferrihemoglobin does not bind detectable amounts of those alcohols which are bound to BSA. Other results, however, indicated that the presence of traces of octanol markedly increased the rate of acid denaturation (Steinhardt *et al.*, 1966). The purpose of this study was, therefore, to examine the seeming paradox that even though Hb^+ did not appear to bind alcohols, the presence of trace amounts of the latter markedly affected the rate of acid denaturation.

Experimental Section

Materials

Horse COHb was prepared from blood of a single animal as described previously (Steinhardt *et al.*, 1966) and stored frozen in 5% solutions. Hb^+ was prepared by oxidizing these solutions with 2 equiv of $K_3Fe(CN)_6$ and then dialyzing against first 0.2 M phosphate buffer (pH 6.8) and then distilled water. The alcohols and diols were obtained from the following sources: 1-dodecanol (Puriss), Fluka; 1-decanol and 1-octanol (Puriss), International Chemical and Nuclear; 1-heptanol, Aldrich; 1-hexanol, Fluka; 1,12-dodecanediol and 1,10-dodecanediol, K & K; 1,8-octanediol, Aldrich. [^{14}C]Decanol was purchased from International Chemical and Nuclear.

Methods

Kinetic runs were performed by following the changes in optical density at 405 nm as in previous work (Polet and Steinhardt, 1969). Runs were carried out either on a Cary 14 spectrophotometer equipped with thermostated cell holders or on a Gibson-Durham stopped-flow apparatus. The temperature was controlled to $\pm 0.1°$. Denaturation was effected by mixing Hb^+ with acetate buffer, pH >3.3 or HCl, pH <3.3. Ionic strength was maintained at 0.02 M with sodium acetate or potassium chloride, respectively.

Solutions of the highest alcohol concentrations were prepared by using lambda micropipets. These solutions were then diluted to give the desired concentrations. Thus, while absolute accuracy of the concentrations is limited by the accuracy of the lambda pipets, the accuracy of the concen-

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[1] Abbreviations used are: BSA, bovine serum albumin; COHb, carbonylhemoglobin; Hb^+, ferrihemoglobin; O_2Hb, oxyhemoglobin.

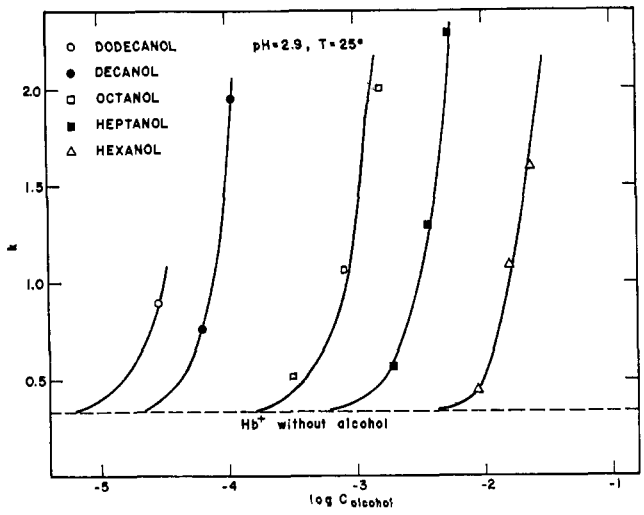

FIGURE 1: The rate constant, k (sec^{-1}), for acid denaturation of Hb^+ (0.01%) in the presence of various alcohols at constant pH (2.92) as a function of log concentration of alcohol. The lines are calculated from eq 2.

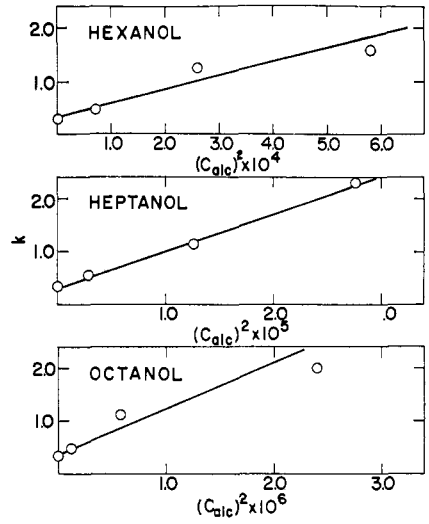

FIGURE 2: The rate constant, k (sec^{-1}), for the acid denaturation of Hb^+ (0.01%) in the presence of hexanol, heptanol, and octanol at constant pH (2.92) as a function of the square of the alcohol concentration.

trations relative to one another is high. Equilibrium data on hemoglobin–octanol were obtained with a Cary 14 spectrophotometer. Solutions were mixed at least 10 half-lives before measurements were made.

Binding measurements with decanol were carried out in Visking dialysis bags at 2° as previously described (Ray *et al.*, 1966). [^{14}C]Decanol (specific activity 7 mCi/mmole) was diluted fivefold with nonradioactive decanol. Control experiments showed that after 48 hr the system had come to equilibrium. Both the inside and outside solutions were counted by adding 0.1 ml of the solutions to 10 ml of Aquasol (New England Nuclear). Analysis showed that mass balance was achieved. Blank experiments showed that the number of counts per second was proportional to the concentration of alcohol when corrected for background.

Results

Kinetic Experiments. When small amounts (final concentration $<10^{-3}$ M) of long-chain alcohols are added to solutions of horse ferrihemoglobin at acid pH, the rate of denaturation is markedly increased. Figure 1, in which the log of concentration of alcohol is plotted as a function of the first-order rate constant for acid denaturation at constant pH (2.92), illustrates the results with several alcohols. The lines are calculated so as to fit eq 2 below. It is apparent that the longer the chain length of the alcohol the lower the concentration that is needed to produce the same degree of instability. The maximum concentration of alcohol in each case is limited by the respective solubilities in water.

Attempts made to fit the data shown in Figure 1 to various kinetic models showed that the best fit is obtained with the equation

$$-\frac{d[Hb^+]}{dt} = (k_0 + k_2K_2[\text{alcohol}]^2)[H^+]^{2.4}[Hb^+] \quad (1)$$

in which k_0 is equal to the rate constant for denaturation of the uncomplexed protein; k_2 and K_2 the rate and association constants for the Hb^+–alcohol$_2$ complex, the justification of which will be discussed later. At constant pH

$$k_{obsd} = k_0 + k_2K_2[\text{alcohol}]^2 \quad (2)$$

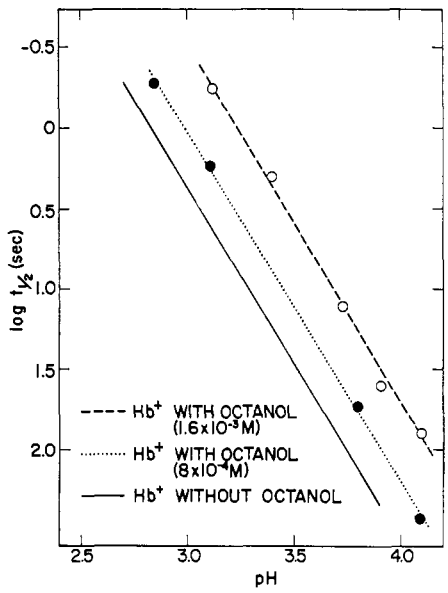

FIGURE 3: The log of the half-life for the acid denaturation of Hb^+ at various octanol concentrations as a function of pH. The results in the absence of octanol are taken from Steinhardt and Hiremath (1967).

The agreement of the data with eq 2 is shown in Figures 1 and 2.[2] The limits of accuracy and the small range of concentrations of alcohol over which measurements were made precluded determination of contributions of any terms other than second order. It is clear that an Hb^+–(alcohol)$_2$ species plays a more important role in the mechanism of denaturation than that of either Hb^+–(alcohol)$_1$ or higher order species.

The pH profile of the log of the acid denaturation rate in the presence and absence of octanol is shown in Figure 3.[3] The plot with octanol is parallel to that obtained without octanol (slope

[2] Formate buffer shifts both the kinetic and equilibrium pH profiles to more acid pH's. The slopes, however, remain the same.

[3] Substitution of the heme by protoporphyrin IX has little effect on the stability.

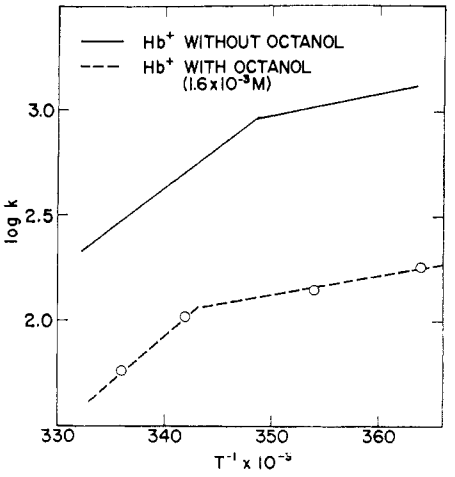

FIGURE 4: The log of the rate constant of acid denaturation of Hb^+ at constant pH (2.92) in the presence of octanol (1.6×10^{-3} M) as a function of T^{-1}. The results for Hb^+ without octanol are extrapolated from data of Allis and Steinhardt (1969) and Zaiser and Steinhardt (1954a).

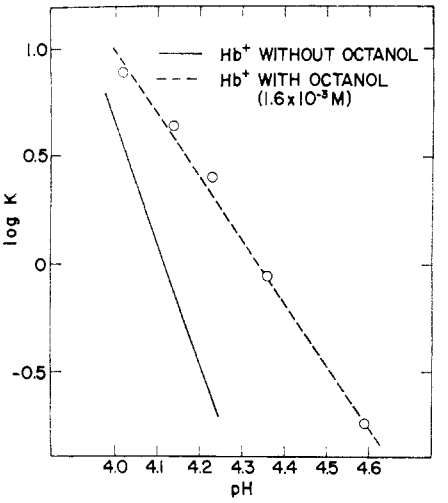

FIGURE 6: Plot of log K (equilibrium) for denaturation as a function of pH for Hb^+ with and without octanol. The slopes are equal to three and five for the plots of Hb^+ with and without alcohol, respectively.

2.4, as in all earlier work). Thus, octanol has no effect on the number of bound protons needed to effect denaturation, regardless of the activation mechanism postulated.

Previous investigators (Zaiser and Steinhardt, 1954b; Allis and Steinhardt, 1969) have found that the dependence of the rate constant on the temperature does not follow the simple Arrhenius equation. Above 15°, plots of log k as a function of $1/T$ give straight lines corresponding to an activation energy of 16 kcal/mole. Below 15°, however, the rate becomes nearly independent of temperature. Plots of log k as a function of $1/T$ (Figure 4) show that Hb^+ in the presence of octanol duplicates this behavior, and, in fact, the lines are nearly parallel. The difference in the temperature corresponding to the transition in slopes is within experimental error.

In an effort to increase the solubility of the hydrocarbon segments, the effect of various diols, 12-carbon and 10-carbon, on the rate of acid denaturation was investigated. In saturated solutions only about a 10% enhancement in rate was seen.

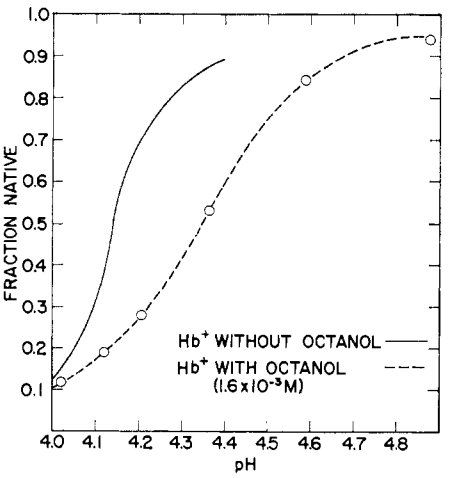

FIGURE 5: The fraction of Hb^+ in the native state as a function of pH for Hb^+ in the presence of octanol (1.6×10^{-3} M) as determined spectrophotometrically. Curve for Hb^+ without octanol is taken from Zaiser and Steinhardt (1954b).

Diols, therefore, lack the catalyzing effect of the corresponding alcohols.

In a few preliminary experiments, the acid denaturation of O_2Hb and COHb in the presence of octanol was investigated. In these cases, there was no enhancement of denaturation rate observed under conditions identical with those in the Hb^+ experiments.

Equilibrium Measurements. The equilibrium between the denatured and native forms was determined as a function of pH by measuring the change in the Soret region of the spectrum. The results are shown in Figure 5, which includes a set for Hb^+ without octanol for comparison. Two features of these data deserve attention: (1) the equilibrium pH profile is shifted toward greater instability, *i.e.*, octanol will bring about denaturation at pH values (greater than 4.4) at which no denaturation occurs in its absence; and (2) the denaturation of Hb^+ by acid in the presence of octanol is less "cooperative" than in the absence of octanol. This is plainly shown in Figure 6 in which log K (equilibrium) is plotted as a function of pH (K is the ratio of the concentrations of denatured to native protein). In the presence of octanol, the slope is equal to 3, without octanol, 5.

Binding Experiments. Attempts to measure the binding of an alcohol to Hb^+ were made by means of equilibrium dialysis with Hb^+ and [^{14}C]decanol at pH 7 in phosphate buffer at an ionic strength of 0.02 M. The concentration of Hb^+ ranged from 0.04 to 0.1%. The concentration of decanol ranged from 1.0×10^{-5} to 2×10^{-4} M. Although the solubility of decanol is small, under the conditions of the experiment the binding of as much as 10% (possibly less) of the total decanol present would have been detected beyond doubt. In all cases, no binding of decanol could be detected. This finding is consistent with the fact that the kinetic curves show that the hemoglobin is far from saturated with alcohol when solubility limits are reached.

If it is assumed that 10% of the amount present represents the maximum amount of alcohol bound, the second-order association constant, K_2, for the reaction

$$2 \text{ alcohol} + Hb^+ \overset{K_2}{\rightleftharpoons} Hb^+\text{-(alcohol)}_2 \qquad (3)$$

has a maximum value of 4×10^6. Based on this value, the rate constant for denaturation *via* the catalyzed path must be at least 250 times that of the uncatalyzed path.

There is no observable difference in either the visible or ultraviolet spectra between Hb^+ in the presence and absence of octanol. This is again an indication that very little protein has bound octanol.

Preliminary results of the binding of decane to human oxyhemoglobin, using the partitioning of [^{14}C]decane between a tridecane phase and aqueous protein indicate that binding does occur in concentrated solutions of hemoglobin (*ca.* 15%). This is consistent with Wishnia's (1962) finding that hemoglobin binds butane and pentane. However, the association constant (first order) appears to be less than 1000 and indicates that under the conditions of the decanol binding experiments, in which the Hb^+ concentration was 0.1%, no binding would have been observed. Octane was found to have no catalytic effect. This result is not unexpected, since octane is about 100 times less soluble than octanol.

Discussion

When long-chain alcohols are added to solutions of Hb^+, a small amount ($\bar{v} < 0.1$) of an alcohol–Hb^+ complex may be formed. This complex is denatured in acid at least 250 times faster than uncomplexed Hb^+. Because of this fortuitous instability of the Hb^+–alcohol complex, we have a sensitive probe to detect the binding of very small amounts of long-chain alcohols which would otherwise be undetected. The nature of the binding can be further elucidated by a consideration of the kinetic mechanism consistent with eq 1. This mechanism is as follows

$$Hb^+ \xrightarrow[n[H^+]]{k_0} \text{denatured products} \qquad (4)$$

$$Hb^+ + 2\ \text{alcohol} \underset{}{\overset{K_2}{\rightleftharpoons}} Hb^+\text{–(alcohol)}_2 \xrightarrow{k_2} \text{denatured products} \qquad (5)$$

where K_2 is an equilibrium constant defined by eq 5 and n is defined by eq 4. That K_2 is independent of pH can be inferred from a comparison of the pH profiles (Figure 3). A comparison of the pH profiles also shows that n is the same for both the Hb^+–alcohol$_2$ complex and "native" protein. The above mechanism is consistent with eq 1 if very little Hb^+–alcohol$_2$ is formed and (alcohol) is in large excess.

At pH's below 4.5, where all of our experiments were conducted, hemoglobin has been shown to exist as an α,β dimer. Since it takes 2 equiv of the alcohol to catalyze the denaturation reaction in question (*cf.* Figure 2), an average of 1 equiv of alcohol/monomer is bound. As the chain length of the alcohol is increased (Figure 1), the alcohol becomes a more effective catalyst: *i.e.*, the concentration of alcohol needed to increase the rate of denaturation by a given amount decreases as the chain length increases (Figure 1). This would be expected if the increasing chain length results in stronger binding between the alcohol and hemoglobin. Such behavior has been previously observed in the binding of alcohols to BSA (Ray *et al.*, 1966; Reynolds *et al.*, 1968). The increase of the binding constant with increasing chain length was attributed to the formation of stronger or more numerous hydrophobic bonds between ligand and BSA. The increase in catalytic effectiveness with increasing chain length could also be due to an arbitrarily postulated increase in k_2 (eq 5) with increasing chain length.

Plots of log k_{obsd} as a function of T^{-1} are parallel in the presence and absence of alcohol: *i.e.*, the enthalpies of activation for the denaturation *via* the catalyzed and uncatalyzed paths are equal. However, the *observed* enthalpy of activation of the Hb^+–alcohol$_2$ complex is the sum of two terms: the actual enthalpy of activation of the complex and the enthalpy of formation of the Hb^+–alcohol$_2$ complex. Since the *observed* enthalpy of activation of the complex is equal to that of Hb^+ itself, the enthalpy of formation is equal to zero, and the actual enthalpy of activation of the complex is equal to that of Hb^+ itself. Hence, the driving force for binding must be largely entropic in nature. This is true for the formation of both hydrophobic and electrostatic bonds. Since the alcohols are neutral molecules, formation of electrostatic bonds is not expected to be important in the formation of complexes. Thus, the hypothesis that hydrophobic complexes are formed is strengthened. Differences in the rate constants for the catalyzed and uncatalyzed paths must be due solely to differences in the entropy of activation.

Comparison of the two equilibrium curves for Hb^+ with and without octanol (Figures 5 and 6) with the kinetic pH profiles (Figure 3) shows clearly that the acid denaturation of Hb^+ is not a two-state system. This is already known from other work (Beychok and Steinhardt, 1960; Steinhardt *et al.*, 1958; Polet and Steinhardt, 1969; Steinhardt and Reynolds, 1969; McGrath and Steinhardt, 1971). With alcohol present, the equilibrium is shifted toward greater instability as might be expected; less expected is the reduction in the apparent number of protons participating in the initiation of the denaturation from five to three (*cf.* Figure 6). In any event, the value of five is irregular in that while the rate law for the acid denaturation yields a value of 2.4 for the exponent of the hydrogen ion concentration, the rate of regeneration is nearly independent of pH (Steinhardt *et al.*, 1958). Hence, a value of three is the more reasonable of the two. Such a value has been found when the equilibrium curve for native Hb^+ in formate buffer (2) is corrected for the formation of irreversible product on denaturation (Steinhardt *et al.*, 1958). The reason for the change in hydrogen ion dependence from the fifth to the third power when alcohols reduce the kinetic stability thus remains obscure.

The answer to the central question—Why the Hb^+–alcohol$_2$ complex is so much less stable than Hb^+ itself?—may lie in the possibility that the Hb^+–alcohol bond is formed at the expense of an internal hydrocarbon–segment interaction within the protein. Since the sacrificed interaction contributes to the overall stability of the protein, its destruction would result in a protein which is more susceptible, both in the kinetic and equilibrium sense, to acid denaturation. Formation of such a complex need not cause a change in the number of "trigger" groups: *i.e.*, the slope of the kinetic pH profile for acid denaturation would remain the same.

The heme pocket is the most hydrophobic region of the molecule. It is well known that the stability of the protein is very sensitive to the nature of the groups within the heme pocket. For example, changing the ligand coordinated to the axial position of the heme iron results in up to a 100-fold change in the rate of acid denaturation at any given pH (Steinhardt *et al.*, 1963; Cassatt and Steinhardt, 1971). Removal of the heme altogether results in a 1000-fold decrease in kinetic stability (Sebring and Steinhardt, 1970) (3). Hence, if the alcohol binding site were in the heme pocket, a large effect on the stability of the protein would be expected, especially if binding of the alcohol displaced the heme, either partially or wholly. The observation that the acid denaturation of COHb

is not catalyzed by the addition of octanol is consistent with this hypothesis. In COHb, the heme would not be expected to be displaced by the alcohol because of the strong covalent bond between the heme iron and proximal imidazole, which remains intact even on denaturation (Geddes and Steinhardt, 1968; Allis and Steinhardt, 1970).

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Alterations of Antibody Binding Properties and Active-Site Dimensions in the Primary and Secondary Immune Response*

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ABSTRACT: Rabbit antibodies specific for the 2,4,6-trinitrophenyl (TNP) determinant group were obtained from the primary and the secondary response and their active sites were studied with two spin-labeled 2,4,6-trinitrophenyl ligands using electron spin resonance spectroscopy and fluorescence quenching. The substitution of the homologous TNP group with a 3-amino- or 3-methylamino-2,2,5,5-tetramethylpyrrolidine nitroxide introduced steric perturbations to the antibody–hapten complex formation. Differences in structure of the combining sites of antibodies produced during the primary or secondary immune response were inferred from the extent and the nature of these perturbations and the binding energy of antibody–hapten complex formation. These results indicate that antibodies produced during primary and secondary immune responses contain distinctive and relatively nonoverlapping subsets with different active-site structures. Antibodies from the primary response: (1) are less tolerant to steric perturbations; (2) form less rigid complexes with haptens; and (3) are more susceptible to organic solvent denaturation. The dimension of the secondary anti-TNP antibody active site parallel to the long axis of the spin-labeled ligand is at least 10 Å.

Many previous studies have documented differences in the ligand binding properties of antibodies produced in primary and secondary immune responses. After the initial exposure to immunogen, a second exposure several weeks or even years later often evokes a more vigorous immune response, and the antibodies produced usually form more stable complexes with antigen than those formed at a comparable time after the first injection (Eisen and Siskind, 1964; Little and Eisen, 1966; Fujio and Karush, 1966; Parker *et al.*, 1967). Antibodies from the secondary response are also more cross-reactive, *i.e.*, less discriminating (Little and Eisen, 1969). In addition, there is evidence of differences in structure between IgG antibody molecules obtained early or later in the immune

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