Volume Changes Accompanying the Antibody–Antigen Reaction*

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ABSTRACT: The volume changes occurring during the antibody–antigen reaction were measured by dilatometry in the following systems: the hapten DNP-lysine, and the macromolecular antigens DNP-bovine γ-globulin, poly Glu[56]-Lys[38]Tyr[6], and poly Glu[60]Lys[40]. The maximal net volume changes were, respectively, 800, 850, 650, and 230 ml per mole of antibody. The magnitude of the volume changes that accompanied the reactions of the anti-DNP antibodies or Fab fragments varied exponentially with the association constant. The addition of the DNP-lysine hapten to the antibody or to the Fab fragment past the point of maximal volume change did not cause any further change. In contrast, the curves representing volume change as a function of the amount of macromolecular antigen resembled precipitin curves: the progressively smaller increase in volume following the maximal volume change corresponded to the antigen excess region of the precipitin curve where soluble antibody–antigen complexes were formed. The volume change following the reaction between the Fab fragment of anti-DNP antibody and DNP-lysine was approximately one-half of that seen with the intact antibody. On the other hand, it was five to ten times greater than the volume change following the reaction of lysozyme with *N*-acetyl-D-glucosamine or the reaction of ribonuclease with cytidine 2′(3′)-monophosphate.

The antibody–antigen reaction has been studied extensively by measurements of the equilibrium constant and subsequent calculation of the free energy, enthalpy, and entropy changes (Epstein *et al.*, 1956; Velick *et al.*, 1960; Karush, 1962). Since the surfaces of molecules are the most directly involved in their interaction, the use of a technique that is very sensitive to changes in the partial specific volume of macromolecules should provide a new and useful approach for investigating the mechanism of the antibody–antigen reaction. The dilatometric technique provides such an approach, since it can measure quite accurately the volume changes associated with the formation of insoluble antibody–antigen aggregates, as well as soluble antibody–hapten complexes. Such changes are not susceptible to measurement by the usual optical and hydrodynamic techniques.

The studies reported here are dilatometric investigations of representative antibody–antigen systems:[1] the hapten DNP-lysine, and the macromolecular antigens DNP-bovine γ-globulin, poly Glu[56]Lys[38]Tyr[6], and poly Glu[60]Lys[40]. For comparison, several enzyme–inhibitor reactions were also studied.

Methods

Preparation and Reagents. The preparation of the antigens has been described previously (Eisen and Siskind, 1964; Friedman *et al.*, 1962). The anti-DNP antibodies for preparations I, II, and III were elicited in separate groups of rabbits by immunizing with 2.5 mg of DNP-bovine γ-globulin in Freund's complete adjuvant to which additional tubercle bacilli were added (final concentration of 3 mg/ml). The emulsion was distributed equally among the hindfoot pads and the back of the neck. The rabbits were bled out after 4 weeks for preparations I and II, and after 8 weeks for preparation III. The antibody for preparation IV was elicited in the same way, except that 5 mg of DNP-bovine γ-globulin was used for immunization; the rabbits were bled out 4 weeks after immunization. The antibody against poly Glu[56]-Lys[38]Tyr[6] was elicited by immunization in the same locations as described above with 10 mg of polypeptide in Freund's complete adjuvant to which additional tubercle bacilli were added. Three weeks later the animals were injected in the same places with 10 mg of the polypeptide dissolved in 0.14 M NaCl + 0.01 M phosphate, pH 7.0, and they were bled out 2 weeks later. The immunization protocol for eliciting antibody to poly Glu[60]Lys[40] was the same, except that 2 mg of antigen was used for each injection and the animals were bled out 1 week after the second injection of antigen. All of the sera were stored frozen until they were used.

In all the experiments, purified specific antibody was prepared according to the method of Robbins *et al.* (1967). The Fab fragment of anti-DNP antibodies was prepared by papain digestion according to the method of Porter (1959), as modified by Putnam *et al.* (1962). The antibodies were completely reactive with their respective antigens, as measured by the precipitin reaction and by quantitative assay with the bromoacetyl cellulose immunoadsorbent

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[1] The nomenclature of the synthetic polypeptides is described in the Tentative Rules for Abbreviated Nomenclature of Synthetic Polypeptides of the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochemistry* 7, 483 (1968)): BγG, bovine γ-globulin; DNP, 2,4-dinitrophenyl; Ag, antigen; Ab, antibody.

using DNP-BγG as the antigen (Gill and Bernard, 1969). The Fab fragments of the anti-DNP antibodies were completely reactive with DNP, as determined by the immunoadsorbent assay. The binding constants for the reaction of the anti-DNP antibodies with DNP-lysine were determined at 27° by the fluorescence quenching technique (Eisen and Siskind, 1964). On acrylamide gel disc electrophoresis at pH 8.9, the antibodies to the synthetic polypeptides showed one band, and the antibodies to DNP-BγG showed one diffuse band in the γ-globulin region and one faint, sharp band in the albumin region, probably a contaminant. Normal γ-globulin for the control reactions was prepared by DEAE-cellulose chromatography (Merler *et al.*, 1963). The molecular weight of poly $Glu^{56}Lys^{38}Tyr^{6}$ (preparation 3) was 94,000 and that of poly $Glu^{60}Lys^{40}$ (preparation 4) was 82,000. The molecular weights were obtained from intrinsic viscosity measurements and the previously established relationship between intrinsic viscosity and molecular weight for this type of polypeptide (Friedman *et al.*, 1962).

The ribonuclease and papain were obtained from Worthington Biochemical Corporation, Freehold, N. J. The ribonuclease was chromatographically prepared ribonuclease A, which was stored frozen (RASE); the preparation was free of aggregates and had an activity >3000 units/mg. The egg white lysozyme was three-times recrystallized and lyophilized, containing >9000 units/mg; it was obtained from Pentex Biochemicals, Kankakee, Ill. The *N*-acetyl-D-glucosamine was from Mann Research Laboratories, New York, N. Y., and the cytidine 2′(3′)-monophosphate was from Miles Laboratories, Elkhart, Ind.

Dilatometry. Volume changes were measured with two kinds of modified Carlsberg dilatometers (Linderstrom-Lang and Lanz, 1938). One of the dilatometers consisted of an inverted, asymmetrical V-tube with the longer arm containing the antibody solution (62 mm long and 13 mm i.d.) and the other arm containing the antigen solution (53 mm long and 13 mm i.d.); a precision bore capillary (300 mm long and 0.23 mm i.d.; 2.685×10^{-4} ml/cm) was attached to the apex of the V-tube through a ground glass joint (10/30 standard taper). The other type of dilatometer had three arms: two short arms (30 mm long and 13 mm i.d.) in the shape of a symmetrical V and a longer arm (100 mm long and 13 mm i.d.) originating at the apex of the V and at approximately 60° to the plane of the V-tube. A precision bore capillary tube was attached at the apex of the V-tube, as described above. The longest arm was used for the antibody solution and the shorter two arms contained the antigen solutions.

All solutions were dissolved in, and dialyzed against, the same buffer (0.14 M NaCl–0.01 M phosphate, pH 7.0), except for DNP-lysine, which was just dissolved in the buffer solution against which the antibody solution had been dialyzed. Therefore, the pH and the ionic strength of all the antibody and antigen solutions were identical. Before pipetting, all solutions were evacuated to remove dissolved air, which could come out of solution and introduce a considerable error during the experiment. The space above the solutions in the dilatometer was filled with kerosene, which was purified by stirring it with several changes of concentrated sulfuric acid for 7–10 days and then with several changes of water; the kerosene was saturated with water. Before fixing the joint to the capillary tube, the tapered part

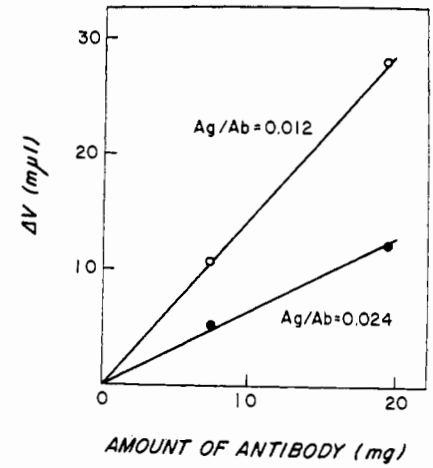

FIGURE 1: The volume change following variation in the amount of antibody at two constant antigen–antibody ratios (by weight). This experiment was carried out with the poly $Glu^{60}Lys^{40}$ system using purified antibody. The total volume of the reactions was held constant at 5 ml.

of the capillary was cooled to 20° lower than the temperature of the V-tube. Because of this cooling, the capillary could be attached to the V-tube very tightly, and there was no leakage through the joint.

The dilatometer was immersed in a well-controlled, double water bath (±0.005°) that had a polyethylene foam box with transparent doors built over the top in order to prevent drafts and to minimize thermal fluctuations. The inner and outer water baths were controlled separately to 30.00 and to 29.0°, respectively, and all measurements were carried out at 30.00°. In this manner a high degree of accuracy was maintained in the regulation of the inner bath temperature. After adjusting the level of the kerosene in the capillary and after thermal equilibration, the solutions were mixed by gently tilting the dilatometer back and forth in the bath. The changes in the level of the kerosene meniscus were followed with a precision cathetometer, and the level of the meniscus was read either when the regulator turned on or off or when the meniscus reached its highest point. The reproducibility of the readings using either method was excellent once thermal equilibrium had been established, and the volume changes could be measured with an accuracy of ±3%. The difference among replicate measurements was usually within ±5%, but occassionally it was as high as ±10%. No volume change occurred when either the antibody solution or the antigen solution was mixed with buffer in the dilatometer.

Calculations. The volume change ΔV was calculated by the formula:

$$\Delta V = \frac{\Delta l\ (\text{cm}) \times 2.685 \times 10^{-4}\ (\text{ml/cm})}{[\text{Ab}]\ (\text{mol})}$$

where Δl is the change in height of the kerosene level in the precision bore capillary following mixing of the reactants; 2.685×10^{-4} ml is the volume change per cm of capillary, which was determined by filling the bore of the capillary with mercury, measuring the length of the column, and

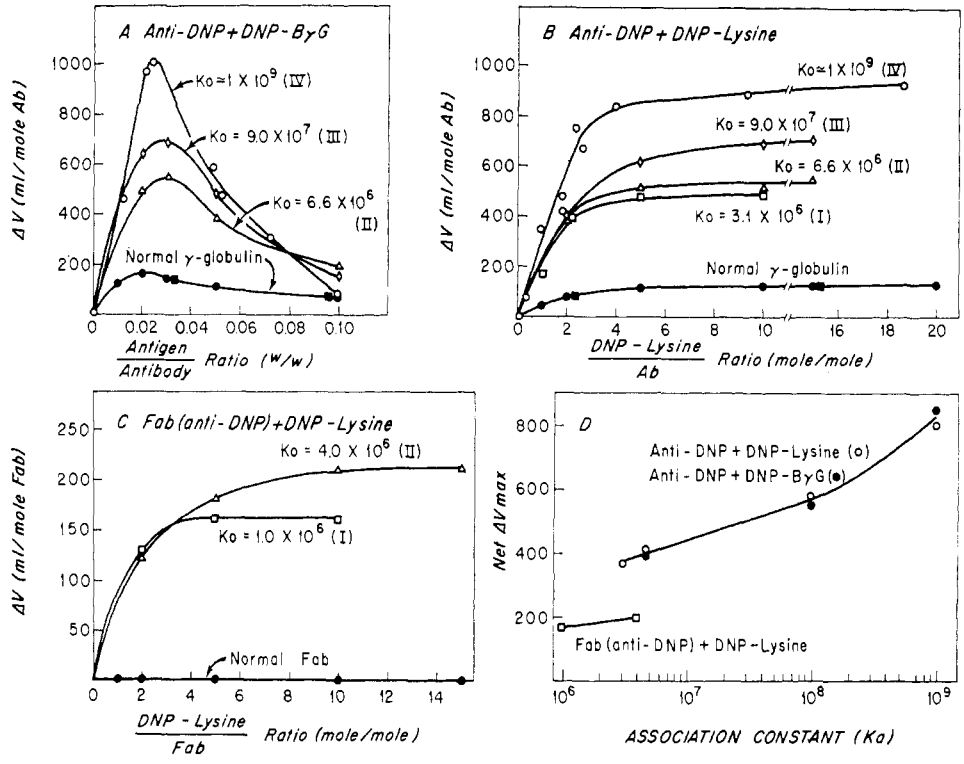

FIGURE 2: (A) Volume changes accompanying the reactions between purified anti-DNP antibody and DNP-bovine γ-globulin. The antibody solution containing 10.3–14.6 mg (3–4 ml) and the appropriate amount of DNP-bovine γ-globulin (0.5–2 ml) were mixed in a two-chamber dilatometer. The amount of antigen added at the maximum of the curve was the same as that added at the maximum of the precipitin curve. The shape of the curve showing ΔV as a function of Ag/Ab ratio was the same as the shape of the precipitin curve. The antibody preparation number is given in parentheses after the association constant. Normal γ-globulin (19 mg) was used in the control reaction. The solid circles and squares represent different preparations of normal γ-globulin. The value of ΔV with normal γ-globulin at Ag/Ab = 0.15 was 63 ml/mol of γ-globulin. (B) The volume change accompanying the reaction between purified anti-DNP antibody and DNP-lysine. DNP-lysine of the appropriate concentration (0.5–2 ml) was added to 3–6 ml of a solution containing 11–12 mg of antibody in a two-chamber dilatometer. The antibody preparation number is given in parentheses after the association constant. Normal γ-globulin (19 mg) was used in the control reaction. The solid circles and squares represent different preparations of normal γ-globulin. (C) The volume change accompanying the reaction between the Fab fragment of purified anti-DNP antibody and DNP-lysine. DNP-lysine of the appropriate concentration (2 ml) was added to 3 ml of a solution containing 8–15 mg of the Fab fragment in a two-chamber dilatometer. The number of the antibody preparation from which the Fab fragment was prepared is given in parentheses after the association constant. The Fab fragment prepared from normal γ-globulin (15 mg) was used in the control reaction. The value of ΔV with the normal Fab fragment at a molar ratio of 20 was also 0. In addition, mixing the normal Fab fragment and DNP-BγG over the Ag/Fab (w/w) range 0–0.15 did not cause any volume change. (D) The net maximal volume change as a function of the association constant for the reactions between anti-DNP antibody and DNP-lysine or DNP-BγG and for the reaction between the Fab fragment of anti-DNP antibody and DNP-lysine. The net maximal volume change was calculated by subtracting the volume change in the control reaction with normal γ-globulin from the value for the antibody–antigen reaction at the point of maximal volume change for the latter reaction.

determining the weight of the mercury; and [Ab] is the number of moles of antibody or Fab fragment used in the experiment. The concentrations of antibody and of Fab fragment were calculated from the extinction coefficients $E^{1\%}_{1\,cm}$ 13.8 and 15.1, respectively. The molecular weight of the antibody was taken as 155,000 and that of the Fab fragment as 52,000. Since the measured volume change is normalized by dividing by the concentration of antibody, the experiments shown in Figure 1 were done in order to demonstrate the linear dependence of the magnitude of the volume change on the amount of antibody used over the concentration ranges studied. In addition, the linear dependence was demonstrated at two different antigen–antibody ratios, so it holds true over the range of mixtures most commonly employed in this study.

Antibody–Antigen Reactions. When the anti-DNP antibody was mixed with DNP-bovine γ-globulin in different ratios, the volume change increased to a maximal value and then decreased (Figure 2A). The magnitude of the volume increase was greater as the association constant of the antibody increased. There was also some reaction between normal γ-globulin and DNP-bovine γ-globulin. On the other hand, the reaction between anti-DNP antibody and DNP-lysine showed an increase in volume, whose magnitude depended upon the association constant of the antibody, but there was no further change in volume after the maximal change was attained (Figure 2B). There was also some reaction between normal γ-globulin and DNP-lysine.

The reaction between the Fab fragment of anti-DNP antibody and DNP-lysine showed a volume increase which was approximately one-half of that seen with the intact antibody. The magnitude of this increase depended upon

TABLE I: Volume Changes Occurring during the Antibody–Antigen and Enzyme–Inhibitor Reactions.

Antibody	Preparation	Antigen	Association Constant (M^{-1})	Net ΔV_{max} (ml/mol)
DNP	I	DNP-lysine	3.1×10^6	365
	II		6.6×10^6	410
	III		9.0×10^7	580
	IV		$\sim 1 \times 10^9$	800
DNP	II	DNP-BγG	6.6×10^6	400
	III		9.0×10^7	550
	IV		$\sim 1 \times 10^9$	850
Fab (DNP)	I	DNP-lysine	1.0×10^6	162
	II		4.0×10^6	196
Poly $Glu^{56}Lys^{38}Tyr^6$		Poly $Glu^{56}Lys^{38}Tyr^6$		650
Poly $Glu^{60}Lys^{40}$		Poly $Glu^{60}Lys^{40}$		230
Enzyme		**Inhibitor**		
Lysozyme		*N*-Acetyl-D-glucosamine	20–50[a]	48
Ribonuclease		Cytidine 2′(3′)-monophosphate	$2–3 \times 10^3$[b]	23

[a] Chipman and Sharon, 1969. [b] Hummel *et al.*, 1961; Hammes and Schimmel, 1965.

the association constant. There was no reaction between the Fab fragment from normal γ-globulin and DNP-lysine. The data are plotted in Figure 2C.

The relationship between the magnitude of the net maximal volume increase and the association constant of the antibody or the Fab fragment is graphically displayed in Figure 2D.

The volume change in the synthetic polypeptide systems (Figure 3) followed the same pattern as that seen in the reaction of anti-DNP antibody with DNP-bovine γ-globulin: there was an increase to a maximal value and then a decrease. The maximal volume change with poly $Glu^{56}Lys^{38}Tyr^6$ (650 ml/mol of Ab) was considerably larger than that seen with poly $Glu^{60}Lys^{40}$ (230 ml/mol of Ab); neither polypeptide reacted with normal γ-globulin.

The values of the maximal net volume increase for all of the antibody–antigen systems are summarized in Table I.

Enzyme–Inhibitor Reaction. The reactions between enzymes and small molecular weight inhibitors were studied for comparison with the reaction between the Fab fragment of anti-DNP antibody and DNP-lysine. The interactions of lysozyme and of ribonuclease with inhibitors are shown in Figure 4, and the maximal volume changes are listed in Table I.

Discussion

The magnitude of the volume change occurring during the antibody–antigen reaction is probably not significantly affected by precipitation of the antibody–antigen complexes, since there was no significant difference in the volume change up to the maximal value for the reaction between anti-DNP antibody and DNP-bovine γ-globulin or DNP-lysine. Several subsidiary lines of evidence also support this conclusion: (a) the method of Linderstrom-Lang, which was used in this study, is valid for volume changes in solution or in suspensions of solids (Krivacic and Rupley, 1968); (b) the density of proteins is the same whether the protein is dissolved or precipitated (Cox and Schumaker, 1961); and (c) there is a direct relationship between volume changes measured by densitometry and by dilatometry (Krausz and Kauzmann, 1965).

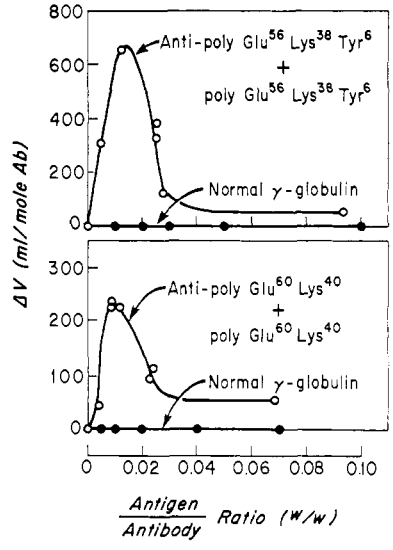

FIGURE 3: The volume change accompanying the reactions os purified antibodies with synthetic polypeptide antigens. In the studier on the poly $Glu^{56}Lys^{38}Tyr^6$ system, two separate aliquots of 0.4 of 0.5 ml of the appropriate dilution of antigen were added to 14.0–15.6 mg of antibody in 3–5 ml of solution. In the poly $Glu^{60}Lys^{40}$ system, 7.0–19.0 mg of purified antibody in 5 ml was mixed with two separate aliquots of 0.5 ml of the appropriate dilution of antigen. The double addition of antigen was possible because of the use of a three-chamber dilatometer. The amount of antigen added at the maximum of each curve was approximately the same as that added at the maximum of the respective precipitin curve. Normal γ-globulin (15 mg) was used in the control reactions.

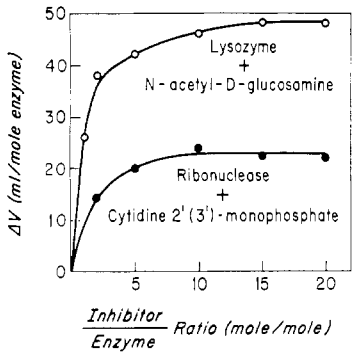

FIGURE 4: The volume change accompanying the reaction between an enzyme and a small molecular weight inhibitor. These studies were done for comparison with the reaction between the Fab fragment from anti-DNP antibody and the hapten DNP-lysine. The appropriate concentration of inhibitor (1 or 2 ml) was added to 3 or 4 ml of a solution containing 12–15 mg of enzyme in a two-chamber dilatometer. Control experiments in which either the inhibitors or the enzymes alone were mixed with buffer in the dilatometer showed no volume change.

The volume changes accompanying the antibody–antigen reaction (Table I) are large in comparison to those seen in most other reactions: thus, the partial specific volume of the antibody–antigen complex is considerably greater than that of its components. The volume changes occurring in the reaction between the Fab fragment of anti-DNP antibody and DNP-lysine are approximately one-half as large as those seen with the intact antibody. Therefore, the major, if not exclusive, source of the volume change must lie within the Fab fragment; presumably, the areas involved are in or near the combining site. The direct correlation between the magnitude of the volume change and the association constant provides further evidence for this suggestion. The magnitude of the volume change accompanying the reaction of the Fab fragment with DNP-lysine is comparable with that seen with the polymerization of flagellin (200 ml/mol at 30°) (Gerber and Noguchi, 1967) or of G-actin (391 ml/mol at 25°) (Ikkai *et al.*, 1966), but it is much larger than the volume increase occurring with enzyme–inhibitor reactions (Table I). Finally, since the normal Fab fragment does not show any nonspecific reactions with DNP-lysine or with DNP-bovine γ-globulin, whereas normal γ-globulin does, the site of the nonspecific interaction probably is on the Fc portion of the γ-globulin molecule.

The volume increase occurring during the antibody–antigen reaction could arise from several factors: conformational change in the Fab portion of the molecule, charge neutralization, or intermolecular hydrophobic interactions. Any contribution by conformational change would most likely be small, since Cathou *et al.* (1968) were unable to measure by circular dichroism any conformational changes in the Fab fragment of anti-DNP antibody when it reacted with DNP-lysine. Also, the volume changes associated with conformational alterations are rather small and often cause a decrease in volume; for example, disruption of a bond causes a decrease of *ca.* −2 ml/mol (Kauzmann, 1959; Noguchi and Yang, 1963). One transition causing an increase in volume is the coil to helix transition, which is associated with a change of only 0.5–1.0 ml per mol of amino acid residue (Noguchi and Yang, 1963). Hence, alterations in the structure of the Fab fragment large enough to be detected by optical methods would have to occur in order to account for a significant portion of the measured volume changes. It seems unlikely that electrostatic interactions between charged residues on the antibody and those on the macromolecular antigen DNP-BγG can be important, since the reactions of anti-DNP antibody with DNP-BγG or with DNP-lysine showed essentially the same maximal volume change (Table I). Hence, it appears that hydrophobic interactions, which are associated with an increase in volume (6–23 ml/mol) (Kauzmann, 1959; Nemethy and Scheraga, 1962a,b), play a significant role in the antibody–antigen reaction. Further evidence for this suggestion comes from the finding that there was a greater volume change associated with the antibody–antigen reaction of poly $Glu^{56}Lys^{38}Tyr^{6}$, where tyrosine is an important part of the antigenic site (Gill *et al.*, 1963), compared to that seen with poly $Glu^{60}Lys^{40}$, where only charged amino acid residues are present.[2]

When the DNP-lysine hapten reacted with anti-DNP antibody, the volume increased to a maximum when all of the combining sites were occupied, and it did not change thereafter. With the macromolecular antigens, however, the volume increased up to the antigen concentration corresponding to the precipitin maximum and then decreased. The progressively smaller volume increase in the antigen excess region occurred without any change in the number of antibody–antigen bonds (the maximal number was formed at equivalence and remained constant thereafter); therefore, it must be governed by factors reflecting the structure of the antibody–antigen aggregate. This structure changes continuously as the proportion of the two reactants changes (Marrack, 1938; Pauling, 1940), and the most striking feature of the antigen excess zone is the formation of soluble antibody–antigen complexes. Thus, the progressively smaller volume increase in the antigen excess region may reflect the difference between the maximal volume increase which occurred in the equivalence zone and a superimposed, competing process which caused a decrease in volume. This competing process could be a change in the partial specific volume of the antibody–antigen aggregates when they pass from the solid phase into soluble complexes.

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[2] The magnitude of the difference in the volume change due to the reaction at the combining sites may not be quite so large as suggested by the measurements in the poly $Glu^{56}Lys^{38}Tyr^{6}$ and the poly Glu^{60}-Lys^{40} systems. In the equivalence zone, poly $Glu^{56}Lys^{38}Tyr^{6}$ is completely precipitated, whereas 50% of poly $Glu^{60}Lys^{40}$ remains in solution, presumably as soluble complexes (Gill and Doty, 1961). Thus, the maximal volume change that is measured in the poly $Glu^{60}Lys^{40}$ system is probably too low due to the formation of soluble complexes. Nonetheless, it would still be smaller than the volume change occurring in the antibody–antigen reaction involving poly $Glu^{56}Lys^{38}Tyr^{5}$.

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Biosynthesis of 3-Hydroxy-3-methylglutarate and Mevalonate by Rat Liver Homogenates *in Vitro**

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ABSTRACT: Methods have been developed for studying regulation of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG–CoA) and mevalonate biosynthesis in fractionated rat liver homogenates. Evaluation of individual steps or combinations of steps between acetate and mevalonate, including HMG–CoA condensing enzyme and HMG–CoA reductase, are described. The requirements for each reaction, including optimal concentrations of substrate and cofactors, have been defined, and simplified methods for separating and purifying HMG and mevalonate have been developed. A method for determining the rate of synthesis of HMG and mevalonate, as contrasted with ^{14}C incorporation from labeled precursors, is described. Very little of the HMG formed is bound to protein, and data supporting the role of HMG–CoA as an intermediate in mevalonate synthesis are presented. Using methods described here, accurate delineation of early regulatory points in the cholesterol biosynthetic pathway may be performed. The validity of previous studies, the precautions required, and the usefulness and importance of these methods are discussed.

Biosynthesis of cholesterol from 2-carbon fragments proceeds by well-known pathways. Recently, interest has been shown in control of these pathways and attempts have been made to localize a single rate-limiting step at which regulation occurs. Based on theoretical considerations as well as experimental observations, emphasis has been placed on the early steps in cholesterol formation involving mevalonate synthesis (Rudney, 1963), and it has been suggested that control is exerted on the step catalyzed by 3-hydroxy-3-methylglutaryl–CoA[1] reductase (mevalonate:NADP oxidoreductase, E.C. 1.1.1.34) (Bucher *et al.*, 1960; Siperstein and Fagan, 1964). This reaction, involving TPNH-dependent reduction of HMG–CoA to form mevalonic acid, is considered of importance in cholesterol synthesis because quantitatively significant alternative pathways exist prior to, but not beyond, mevalonic acid formation. By analogy to bacterial systems, its position beyond a branch point on the direct pathway to

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[1] The following abbreviations are used throughout the text: HMG and HMG–CoA for 3-hydroxy-3-methylglutaric acid and its CoA ester; Ac and Ac–CoA for acetate and its CoA ester; AcAc and AcAc–CoA for acetoacetate and its CoA ester; ACP for acyl carrier protein.