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# Thermal stability of hepatitis B surface antigen S proteins

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**Thermal stability of hepatitis B surface antigen (HBsAg) has been studied by analyzing alterations in the native secondary structure and the antigenic activity. After heating for 19 h, circular dichroism showed a cooperative transition with a midpoint at 49°C. The conformational changes induced by temperature reduced the helical content of HBsAg S proteins from 49% at 23°C to 26% at 60°C and abolished the antigenic activity, as measured by binding to polyclonal antibodies. Furthermore, the six different antigenic determinants recognized by our panel of monoclonal antibodies were also shown to be dependent on the native structure of HBsAg proteins. Hence, it can be inferred that these epitopes are conformation-dependent. Binding of monoclonal antibodies to HBsAg protected the native structure of the corresponding antigenic determinant from thermal denaturation. In fact, binding of one of the monoclonals tested resulted not only in protection of the corresponding epitope, but also in a consistent increase of antibody binding with increasing temperature. Such an increase in antibody binding occurred simultaneously with an increase in the fluidity of surface lipid regions, as monitored by fluorescence depolarization of 1-(trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene. This correlation, along with the observation that lipids play an important role in maintaining the structure and antigenic activity of HBsAg (Gavilanes et al. (1990) Biochem. J. 265, 857–864), allow to speculate that certain epitopes of HBsAg which are close to the lipid-protein interface, are dependent on the fluidity of the surface lipid regions. Thus, any change in the physical state of the lipids could confer a different degree of exposure to the antigenic determinants.**

## Introduction

Hepatitis B surface antigen (HBsAg), the envelope of the hepatitis B virus (HBV), is a complex macromolecular structure composed of proteins (75% by weight), carbohydrates (in the form of glycoproteins) and host derived lipids (25%) [1]. During infection, hepatocytes synthesize and secrete HBsAg in excess, mainly in the form of lipoprotein particles of 20 nm diameter [2,3]. SDS-polyacrylamide gel electrophoresis of HBsAg reveals the existence of two proteins, designated S and gS, which account for more than 90% of the protein content. These two proteins have identical amino acid sequences and differ only by the presence of carbohydrates attached to gS at position Asn-146 [4,5].

HBsAg is also antigenically complex. All HBsAg have a group-specific determinant designated 'a', which has been demonstrated to be composed of at least three different antigenic sites [6]. In addition, there are two sets of normally mutually exclusive subtype-specific determinants designated d/y and w/r. Thus, there are four major antigenic subtypes of HBsAg, adw, ayw, adr and ayr [7]. Additional complexities have led to the recognition of 10 different serotypes of HBsAg [8].

Of the different components of HBsAg, it is the proteins which are responsible for the induction of antibodies; both group- and subtype-specific antigenic determinants are also located within the proteins [9–12]. However, lipids play an important role in maintaining the structure and antigenic activity of HBsAg [13]. Moreover, the dependence of antibody binding on the

Abbreviations: HBsAg, hepatitis B surface antigen; CD, circular dichroism; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene.

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lipid content seems to indicate a greater involvement of the lipid-protein interaction for some of the epitopes than for others [13].

Chemical modification studies support the idea that S-related antigenic determinants are critically dependent on the conformation of HBsAg proteins. Thus, reduction and alkylation of disulfide bridges resulted in complete loss of antigenic activity [14–16]. A similar conclusion has been drawn from Western immunoblotting studies since proteins separated by SDS-gels and electrotransferred to nitrocellulose membranes fail to react with anti-HBs antibodies obtained from human subjects either after a natural HBV infection or after active immunization with a licensed vaccine [17]. Moreover, the fact that reduced and alkylated HBsAg fails to elicit antibody formation against native antigen supports the contention that the molecule is a conformational antigen dependent upon the integrity of the disulfide bridges [15].

Heat inactivation is one of the procedures used for sterilizing HBsAg for vaccine use [18]. Lelie et al. [19] reported that such vaccine was highly efficacious even for patients on maintenance hemodialysis for whom vaccine inactivated with urea, formalin and pepsin was not so efficacious [20]. Heat treatment can affect antigenic activity to a varying extent. Thus, it has been reported that heating at 100°C for 1.5 min has no appreciable effect on the antigenic activity of 'a' determinants, and that heating at 100°C for 1.5 min and then at 60°C for 10 h, preserved more than half of the 'a' activity [21]. Millman et al. [22] found that treatment at 85 and 100°C for 1 h, destroyed the immunologic reactivity of HBsAg although the morphology of the particles was not affected. De Flora [23] reported that inactivation of HBsAg was linear over the range from 20 to 98°C with a half-life of 6 min for the immunologic reactivity of HBsAg at 98°C. On the other hand, heat treatment has also been used to augment the immunogenicity of HBsAg [24]. Although treatment of HBsAg, partly contaminated with plasma proteins, for 1.5 min at 103°C reduced the content of HBsAg, as determined by RIA, to 50–65% and caused aggregation, the immunogenicity of HBsAg was significantly enhanced.

In this paper we describe the effect of temperature on the secondary structure and antigenic activity of HBsAg using both polyclonal and monoclonal antibodies directed against six different antigenic sites.

## Materials and Methods

### *Materials*

1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(trimethylammonio-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were from Molecular Probes; electrophoresis reagents were obtained from Bio-Rad. Ausria II-125 and Ausab kits for measuring antigenic activity were purchased from Abbott Laboratories.

### *Purification of HBsAg*

HBsAg was purified from the plasma of high titre chronic carriers of HBsAg, adw subtype, following previously described procedures [4].

Protein concentration was determined either from the absorbance spectrum, using the value of 3.726 as the absorbance at 280 nm of a 0.1% solution of HBsAg [25] or by amino acid analysis in which aliquots of protein (10–20 $\mu$g) were hydrolized in sealed evacuated tubes with constant boiling HCl for 24 h at 110°C. Amino acid analysis was performed with a Durrum D-500 amino acid analyzer.

### *Fluorescence polarization measurements*

DPH and TMA-DPH were used as probes of different lipid regions. They were dissolved at 1 mM concentration in tetrahydrofuran and methanol, respectively, and added to HBsAg solutions at a ratio of 1 molecule of fluorophore for every 500 phospholipid molecules. HBsAg concentration was maintained between 20–40 $\mu$M in lipid phosphorus. In this range fluorescence polarization (P) was not dependent upon concentration and/or light scattering [26]. Fluorescence polarization was measured within the 10–50°C temperature range in a Perkin-Elmer MPF 44-E spectrofluorimeter equipped with polarizers in both excitation and emission beams. $P$ was calculated according to:

$$P = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + G \cdot I_{VH}}$$

where $I_{VV}$ and $I_{VH}$ are, respectively, the vertical and horizontal fluorescence intensities when the sample is excited with the excitation polarizer oriented vertically. The emission grating factor was calculated as $G = I_{HV}/I_{HH}$. Temperature was controlled by a Lauda circulating water bath. The probes were excited at 365 nm and the emission was measured at 425 nm.

### *Circular dichroism studies*

CD spectra were obtained on a Jobin Yvon Mark III dichrograph using a 0.1 cm path-length quartz cell and 60–120 $\mu$g/ml of protein. For every sample a minimum of three scans were collected. The values of mean residue weight ellipticities were calculated on the basis of 110 as the average molecular weight per residue and they are reported in terms of $\Theta_{MRW}$ (degrees $\times$ cm$^2$ $\times$ dmol$^{-1}$). The secondary structure estimations were performed by computer fit according to ellipticity reference values of Bolotina et al. [27] which are based on proteins of known three-dimensional structure. The

fitting is accomplished by the analytical method based on matrix formulation previously described [28].

*Antigenic activity assays*

The antigenic activity of HBsAg was determined using the Ausria II-125 kit from Abbott Laboratories. This is a 'sandwich' solid-phase radioimmunoassay which uses polyclonal (guinea pig) anti-HBsAg coated onto beads, to capture any antigen in a sample incubated with the beads. Bound antigen is then detected by incubating the beads with $^{125}$I-labelled polyclonal (human) anti-HBsAg. The incubation conditions were those recommended by the manufacturers. After the final washing, the radioactivity bound to the beads was determined by using a Beckman Gamma 5500 counter. All the assays were done in duplicate.

The antigenic activity was also determined using monoclonal antibodies and an inhibition radioimmunoassay based on the standard Ausab kit from Abbott Laboratories, modified as follows: a fixed amount of each monoclonal antibody was mixed with HBsAg heated for 19 h at different temperatures, prepared in normal human serum (NHS), as well as with a control lacking antigen. Three HBsAg concentrations (5000, 500 and 50 ng/ml) were used for each monoclonal tested. After incubation for 2 h at room temperature, an Ausab bead (coated with HBsAg) was added to each sample and incubated for 18 h at room temperature. Thus, the monoclonal which has not reacted with HBsAg in the initial mixture will bind to the bead. The amount of monoclonal bound to the beads is then detected by incubation with $^{125}$I-labelled HBsAg. After the final washing, the radioactivity was determined as indicated above. The results are expressed as the percent inhibition by the corresponding HBsAg of the monoclonal ability to bind to HBsAg-coated beads, using the expression [29]:

$$\text{Percent inhibition} = \frac{(\text{MC anti-HBs} + \text{NHS}) - (\text{MC anti-HBs} + \text{HBsAg})}{(\text{MC anti-HBs} + \text{NHS}) - \text{assay negative control}}$$

where (MC anti-HBs + NHS) is the radioactivity bound when the monoclonal is incubated with NHS alone, (MC anti-HBs + HBsAg) is that obtained when the monoclonal is preincubated with HBsAg and the negative control gives the amount of $^{125}$I-labelled antigen which binds nonspecifically to the beads, in an assay which lacks both the monoclonal and HBsAg. The inhibition assays were also performed as follows: HBsAg was incubated with each monoclonal for 19 h at different temperatures and then an Ausab bead was added to each sample and incubated for 18 h at room temperature. From here the assays were performed as indicated above. All the assays were done in duplicate.

Monoclonals were prepared and characterized as previously described [6]. The six monoclonals are all directed against the HBsAg protein and have the following specificities: H35C16, H5C14 and H166C63 are directed against the group specific 'a' determinant but not against the same site since they do not compete with each other; H95C46 has the 'd' specificity and H12C237 and H53C160 cannot be assigned to determinants classifiable by their reactions against the accepted NIH panel of subtypes [6].

## Results

*Thermal stability studies*

The effect of temperature on the secondary structure and antigenicity of HBsAg proteins was measured

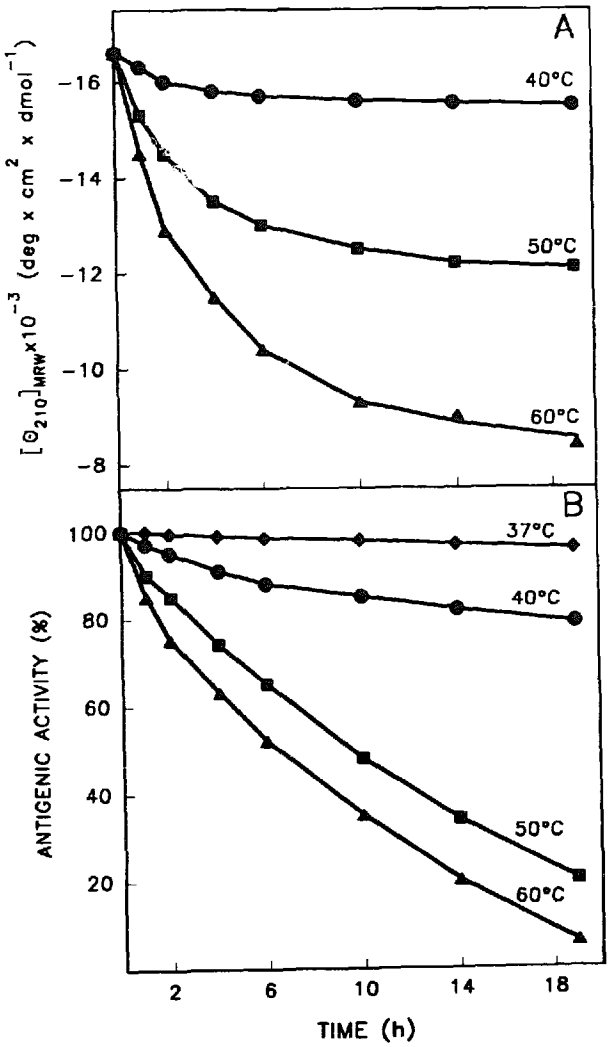


Fig. 1. Effect of temperature on structure and antigenic activity of HBsAg. Variation of (A) ellipticity at 210 nm and (B) antigenic activity. HBsAg, at a protein concentration of 60–80 μg/ml, was incubated at temperatures indicated; at different time intervals, aliquots were withdrawn and CD spectrum and antigenic activity were measured at room temperature. The antigenic activity was measured against guinea pig anti-HBs antibodies as described in Materials and Methods.

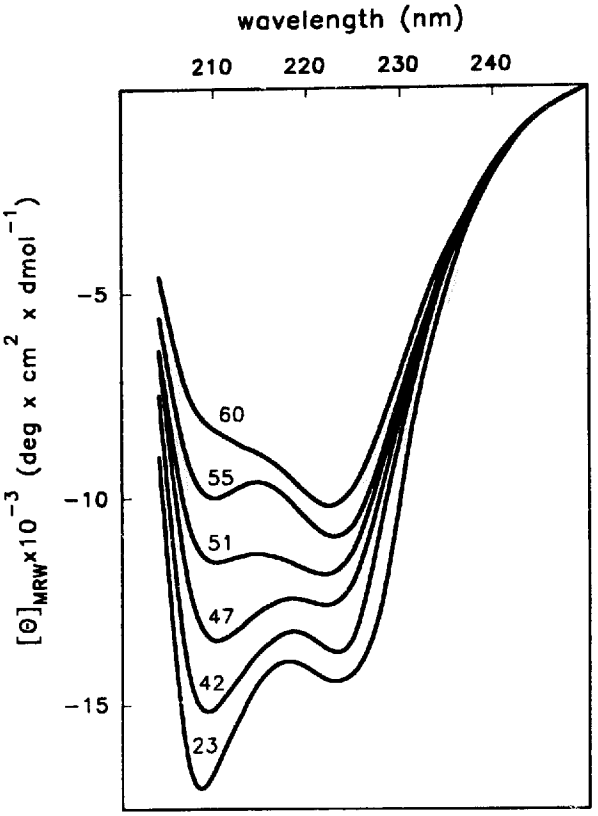


Fig. 2. CD spectrum of HBsAg at different temperatures. HBsAg, at 60–80 $\mu$g/ml of protein, was incubated for 19 h at the temperatures indicated above each spectrum, and then the CD spectrum was recorded at room temperature.

by circular dichroism techniques and binding to both polyclonal and monoclonal antibodies, respectively.

Temperature-induced conformational changes on HBsAg structure are reflected in alterations of the CD spectrum. Fig. 1A shows the variation of ellipticity at 210 nm when HBsAg was heated at the temperatures indicated for different time intervals. At 40°C, $\Theta_{210}$ increased from −16650 to −15500 (7%) while the change observed at 60°C was much more pronounced being $\Theta_{210}$ −8400 (50%). The conformational changes became stable after 19 h since no additional changes were observed after this time. Thermal denaturation also had an effect on the antigenic activity. As shown in Fig. 1B binding of HBsAg to guinea pig polyclonal anti-HBs decreased when the temperature was raised. However, the kinetic behaviour was different at every temperature. Thus, while after 19 h at 60°C the activity was almost negligible there was almost 80% remaining antigenic activity at 40°C.

Additional studies were undertaken after incubation of HBsAg for 19 h at different temperatures since, as indicated above, no further structural changes were observed after this time. Fig. 2 shows the CD spectra of HBsAg heated for 19 h at different temperatures. Since spectra were recorded at 20°C after incubation, it can be concluded that the thermal induced conformational changes are non-reversible. The CD spectrum remained unchanged up to 37°C. Heating at higher temperatures, however, produced changes in both magnitude and shape of the spectrum. Thus $\Theta_{210}$ increased from −16650 to −8400, and the ratio $\Theta_{210}/\Theta_{222}$ decreased from 1.14 at 23°C to 0.82 at 60°C. As a measure of the change induced by temperature, the secondary structure was calculated from the CD spectra (Table I). In the temperature range 23–60°C, the helical content of HBsAg decreased from 49% to 26%, increasing the aperiodic structure content in a similar percentage.

TABLE I

*Secondary structure of HBsAg calculated from CD spectra*

The values were calculated using the parameters of Bolotina et al. [27] and are representative of those obtained for three different preparations.

| Temperature (°C) | Conformation (% of total) | | | |
|---|---|---|---|---|
| | α-helix | β-sheet | β-turn | aperiodic |
| 23 | 49 | 11 | 17 | 23 |
| 42 | 44 | 17 | 17 | 22 |
| 47 | 40 | 19 | 18 | 23 |
| 51 | 35 | 16 | 15 | 34 |
| 55 | 29 | 14 | 13 | 44 |
| 60 | 26 | 15 | 14 | 45 |

Fig. 3 shows the relationship of both ellipticity at 210 nm and antigenic activity (as measured by binding to polyclonal antibodies) with temperature. The changes in secondary structure induced by temperature start taking place after 37°C with an abrupt change beyond this point. The variation indicates a cooperative transition with a midpoint at 49°C. The antigenic activity remained practically constant up to 37°C and from here is almost abolished at 60°C. The sigmoidal curve indicates a cooperative transition with a midpoint at 45°C, a value close to that obtained for $\Theta_{210}$.

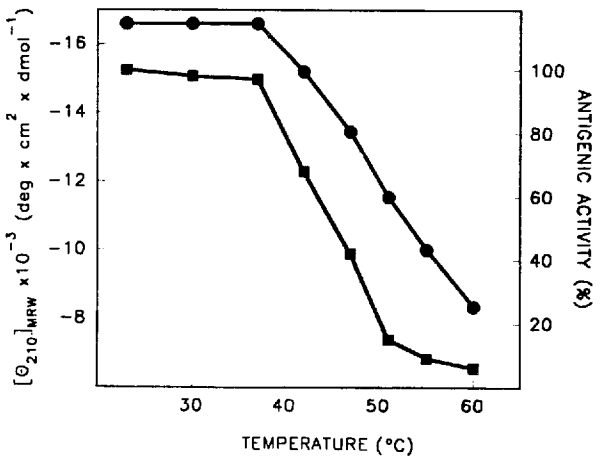


Fig. 3. Thermal denaturation of HBsAg. $\Theta_{210}$ (-●-) and remaining antigenic activity (-■-) are plotted as a function of temperature. Experimental conditions were those of Fig. 2.

When the data of antigenic activity and $\alpha$-helix content are plotted together, Fig. 4 is obtained. Loss of 15% $\alpha$-helix caused a decrease of almost 85% antigenic activity, while the additional decrease of 10% $\alpha$-helix practically abolished binding of anti-HBs.

Since the antigenicity was measured with a polyclonal antibody and temperature could affect differently the antigenic determinants, we have used a panel of monoclonal antibodies which recognize either group or subtype-specific determinants [6]. Fig. 5 shows the results of an inhibition assay on which HBsAg was heated for 19 h at different temperatures and then incubated for 2 h with the monoclonal antibodies. Since the conformational changes induced by temperature are non-reversible, the effect of such changes on the epitopes recognized by the monoclonals could be measured. It is clear that all the antigenic determinants recognized by the monoclonals decreased their ability to bind the corresponding antibody as temperature was raised, although to different extents. Thus, from 23 to 65°C there was a 60–70% decrease of inhibition for monoclonals H35, H95 and H53, while for H5, H12 and H166 the decrease was 40–45%. From the values in Fig. 5, a midpoint for thermal induced antigenic loss can be calculated. For all monoclonals this value lies between 48–52°C. As indicated above, the midpoint of the conformational change determined by CD was 49°C. Thus, it seems clear that the antigenic determinants are dependent on the native structure.

In order to check whether binding of monoclonals protected their corresponding epitopes against thermal denaturation, the inhibition assays were performed incubating native HBsAg with the monoclonals for 19 h from 4 to 47°C. No higher temperatures were attempted to avoid denaturation of the monoclonal antibodies (CD shows that the monoclonal structure is not

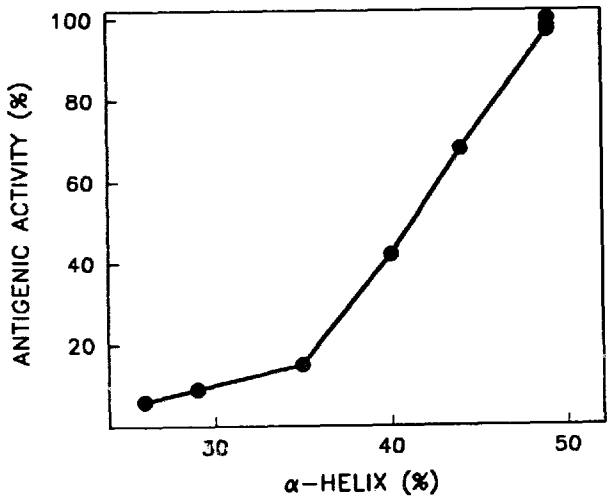


Fig. 4. Relationship between antigenic activity and helical content of HBsAg. The values of % of $\alpha$-helix were calculated for the CD spectra of Fig. 2 using the parameters of Bolotina et al. [27]. Values of antigenic activity are those of Fig. 3.

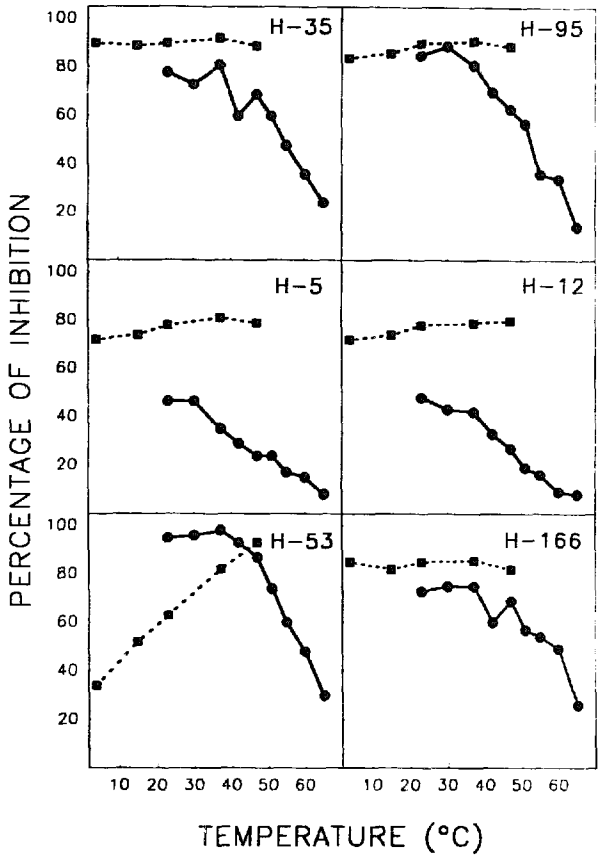


Fig. 5. Effect of temperature on antigenic activity of HBsAg. (-●-) HBsAg, heated for 19 h at the temperatures indicated, was incubated with the monoclonals for 2 h. The concentration of antigen was 500 ng/ml except for H-53 and H-166 which was 5000 ng/ml. (-■-) HBsAg at 500 ng/ml, except 5000 ng/ml for H-166, was incubated for 19 h with the monoclonals at the temperature indicated. The values were obtained according to the method described in Materials ans Methods and are representative of those obtained for three different preparations.

changed upon incubation up to 47°C, data not shown). The results are depicted in Fig. 5. As can be seen, except for H53 the % inhibition remained virtually constant in the temperature range studied. At 47°C, the midpoint of the transition for the six monoclonals, the percentage of inhibition is almost the same as that at 4°C. This could indicate a protection of the antigenic determinant by the binding of the corresponding monoclonal. However, for H53, an increase in antibody binding at increasing temperatures is observed, that could be due to a different degree of exposure of the antigenic determinant induced by temperature. The same pattern of results were obtained by incubating HBsAg at concentrations lower than that indicated in Fig. 5.

*Fluorescence depolarization studies*

DPH and TMA-DPH have been shown to probe different membrane regions. DPH is assumed to be aligned with the phospholipid acyl chains [30] while

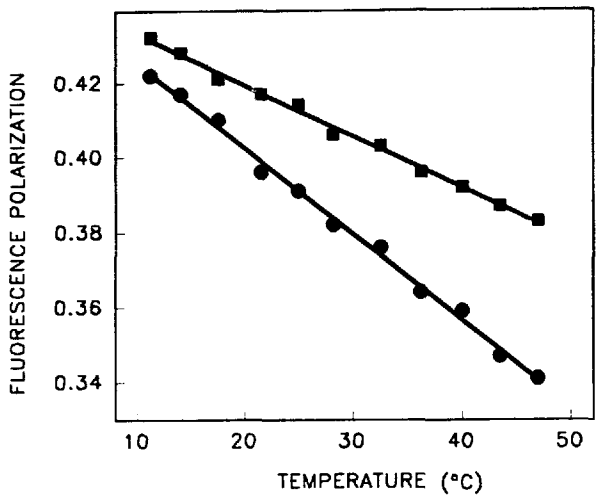


Fig. 6. Temperature-dependence of fluorescence polarization of DPH (-●-) and TMA-DPH (-■-) labelled HBsAg. Fluorescence polarization was measured at each temperature after equilibration for 10 min.

TMA-DPH interacts, apparently, with the phospholipid polar head groups and the fatty acyl chain region, probably as far down as C8-C10 [31]. Thus, DPH partitions into deep core regions while TMA-DPH partitions into surface regions.

The temperature-dependence of the fluorescence polarization of DPH and TMA-DPH incorporated into the hydrophobic matrix of HBsAg is shown in Fig. 6. *P* values were higher for TMA-DPH than DPH. Since both probes have the same limiting polarization values it can be concluded that the surface domains probed by the former are more rigid. The linearity of the plots indicated the absence of a discrete phase transition within the range of temperatures studied, as expected from the heterogeneous lipid composition of HBsAg [1]. As the temperature was raised from 10 to 50°C the fluidity of both regions increased. However, the effect was different for both probes with a higher diminution observed for DPH.

## Discussion

Heat treatment is one of the procedures used to sterilize plasma-derived vaccine [18,19] because HBsAg proteins are fairly resistant to thermal denaturation. Indeed, the CD spectrum of HBsAg revealed the existence of 26% $\alpha$-helix after incubation at 60°C for 19 h. Both, the structure and antigenicity, as measured by binding to polyclonal antibodies, remained practically unchanged when the antigen was heated for 19 h up to 37°C. Above this temperature, the decrease of antigenic activity paralleled the CD-observed conformational changes induced by temperature. The fact that the transition temperature determined for antigenic activity is 4°C lower than that observed for $\Theta_{210}$ could be due to a change in the overall structure of HBsAg, not affecting secondary structure but altering certain antigenic determinants. As shown in Fig. 4, loss of 15% $\alpha$-helix is accompanied by a decrease of almost 85% antigenic activity; the remaining 15% could represent less conformation-dependent epitopes. Thus, one would conclude that the antigenic determinants are dependent on the native structure of HBsAg proteins. Similar conclusions have been drawn from chemical modification studies [14–16].

The effect of temperature on discrete antigenic determinants was measured with six different monoclonal antibodies in an inhibition assay. These monoclonals recognized either group or subtype-specific determinants [6]. Although to a different extent, temperature also induced a diminution of binding of the monoclonal to HBsAg. Moreover, the transition temperature was between 48–52°C, and that observed by circular dichroism was 49°C. Thus, it seems clear that the antigenic determinants, at least those recognized by our panel of monoclonals, are conformation-dependent and any change on the structure will cause a change on binding to the corresponding antibody.

On the other hand, the inhibition assays were performed by incubating HBsAg with the same panel of monoclonals at temperatures up to 47°C. Under these conditions, binding of H35, H95, H5, H12 and H166 to HBsAg was not dependent on temperature. As indicated above, the transition temperature of the conformational changes, which alter the secondary structure of the antigenic determinants and abolish binding of the monoclonals, is around 47°C. Thus, it can be concluded that binding of the monoclonal inhibits the overall conformational changes or, presumably, has only a local effect on HBsAg structure, which should result in protection of the native structure of the antigenic determinant from denaturation. Such effects on protein conformation have been assigned to antibody binding. For instance, protection from thermal denaturation has been demonstrated for $\beta$-galactosidase from *E. coli* [32]. In the case of H53, the inhibition increased from 35 to 93% in the temperature range studied. The antigenic determinant recognized by this monoclonal is also dependent on the native structure and becomes denatured when HBsAg alone is heated. Another parameter that can also be altered by increasing temperatures is lipid fluidity. As indicated by fluorescence depolarization studies, the fluidity of both internal and surface domains increased as the temperature was raised. Thus, the increased binding of H53 to HBsAg could be due to the higher membrane fluidity causing the antigenic determinant to become more exposed. From solubilization studies we have shown that there is a dependence of antibody binding on the lipid content which could indicate a greater involvement of the lipid–protein interaction for some of the epitopes than for other [13]. In fact, binding of H53 to HBsAg is

remarkably affected by removal of 55% of the lipids. Perhaps this is one of the antigenic determinants closest to the lipid-protein interface. Then, it is reasonable to assume that there are epitopes, like the one recognized by H53, which may have a different degree of freedom depending on the fluidity of the surface region. Hence, the physical state of the lipids may alter antigen-antibody complex formation as well as antigenic presentation of HBsAg. The enhanced immunogenicity observed with partly purified HBsAg after heat treatment [24] could be interpreted as due to the protecting effect of residual plasma proteins and, simultaneously, to the different degree of exposure of some antigenic determinants induced by the higher lipid fluidity caused by temperature.

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