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ANTIBODY-MEDIATED THERMAL STABILIZATION OF HUMAN HEXOSAMINIDASES

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Abstract—N-Acetyl-hexosaminidase (Hex) exists in various human tissues in two major isozymic forms—Hex A and Hex B. The main difference between the two forms is the lability of Hex A to both heat and acid as compared to the stability of Hex B. When heated to 50°C for 2–3 hr Hex A loses its entire enzymatic activity.

In the present study we demonstrate that specific antiserum stabilizes Hex A to heat inactivation. When maintained at 50°C for 3 hr in the presence of antibodies the enzyme retains up to 80 per cent of its original activity. This phenomenon is dependent on antibody concentration, and reaches its maximal value at the equivalence zone of Ag–Ab interaction. The inactivation is temperature-dependent; a shift of 12°C was observed in the midpoint of heat inactivation between the native and antibody-bound enzyme. A similar shift was observed for Hex B. Hex A was stabilized also by Hex A-specific antibodies which do not cross-react with Hex B.

These findings indicate that the antibodies act as stabilizers of the active conformation of hexosaminidases.

INTRODUCTION

The interaction of enzymes with their specific antibodies generally leads to a reduction in their enzymatic activity. The mechanism of this inhibition is rarely a direct combination of the antibodies with the catalytic site, but is rather due to steric hindrance, namely, barring the access to the active site. In several systems, however, the mechanism of antibody effect is by conformational changes which it induces on the enzyme. In these cases the interaction with the antibodies may result either in the inhibition or, in contrast, in the enhancement or stabilization of the enzymatic activity (Arnon, 1974).

Indeed, several cases have been reported in which enzymes were rendered more active by interaction with their antibodies. With some enzymes, mainly in cases where the enzyme was assayed under non-optimal conditions (an inactive mutant or poor substrate) antibodies brought about a drastic enhancement in the activity. This phenomenon has been described by Rotman and Celada (1968) and Messer and Melchers (1969) for inactive mutants of β-galactosidase, and by Lehman (1970) for glutamic dehydrogenase.

It has also been demonstrated that antibodies are capable of affecting the conformational structure of enzymes so as to stabilize a naturally labile form. This phenomenon was exemplified by the results of Michaeli *et al.* (1969) regarding the stabilization of acetylcholinesterase towards heat inactivation and by the findings of Feinstein *et al.* (1971) that antibodies to wild type catalase provide a marked stability to a mutant mouse strain enzyme which is abnormally sensitive to heat and to mild alkalinity. Essentially the same phenomenon was encountered in the case of unstable human catalase which appears in patients with Swiss type acatalasemia (Shapira *et al.*, 1973). Mutants of β-galactosidase, activatable to β-galactosidase activity by antibodies to the wild type enzyme, were also found to exhibit an increased stability toward heat denaturation when complexed with antibodies (Melchers and Messer, 1970).

In the present study we have investigated the effect of specific antibodies on the enzyme hexosaminidase (β-2-acetamido-2-deoxy-D-glucosideacetamidodeoxyglucohydrolase, EC. 3.2.1.30). This enzyme (abbreviated Hex), appears in various tissues in several isozymic forms (Robinson *et al.*, 1972; Hayase *et al.*, 1973). In the human it exists in two main forms which differ in their electrophoretic mobility, Hex A being the more acidic isozyme, and Hex B the more basic one (Robinson and Stirling, 1968). Both isozymes catalyze the hydrolysis of N-acetyl glucosamine and N-acetyl galactosamine from adjacent sugar moieties present in various substrates, mainly glucosphingolipids (Sandhoff *et al.*, 1971; Tallman *et al.*, 1972). Their activity may be assayed using chromogenic or fluorogenic synthetic substrates (Leaback and Walker, 1965; Dance *et al.*, 1969; Sandhoff and Wässle, 1971).

The two isozymes show similar substrate specificity and kinetic parameters when assayed with the synthetic low mol. wt substrates (Wenger *et al.*, 1972; Sandhoff and Wassle, 1971; Geiger *et al.*, 1974). Conflicting results were reported, however, as for their activity on the natural ganglioside substrate (Sandhoff, 1970; Tallman *et al.*, 1972; Tallman *et al.*, 1974; Li *et al.*, 1973; Wenger *et al.*, 1972). In their antigenic properties the two isozymes show a high degree of

cross-reactivity (Carroll and Robinson, 1973), but there are indications (Srivastava and Beutler, 1972) that specific antigenic determinants are present on Hex A, of which Hex B is deficient.

The main difference between the two hexosaminidase isozymes is their resistance to heat inactivation. Whereas Hex B retains most of its activity when exposed for several hours to a temperature up to 50° C, Hex A loses its activity within less than 2 hr (O'Brien *et al.*, 1970).

We wish to report herewith a stabilizing effect of the antibodies on the heat labile Hex A form. In this stabilization both specific anti-Hex A antibodies, and antibodies cross-reactive with the B form are effective. The latter type of antibodies stabilize Hex B as well.

MATERIALS AND METHODS

Cyanogen bromide was purchased from Fluka A. G. (Switzerland), DEAE-cellulose (DE-52) and CM-Cellulose (CM-52) were from Whatman (USA). 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide and methylumbelliferone were from Pierce Chemical Co. (USA). Naphthol-AS-BI-N-acetyl-β-D-glucosaminide and Diazo Blue B (o-dianisidine) were obtained from Sigma Chemical Co. (USA). Sepharose 4B and Sephadex G-200 were from Pharmacia (Sweden). All the reagents used were analytically pure or best grade available.

Purification of human hexosaminidases

The enzyme was prepared from human brain liver and placenta according to general procedures (Sandhoff and Wässle, 1971; Carroll and Robinson, 1973; Srivastava and Beutler, 1972) modified as follows: Tissues were homogenized in a Waring blender with 4 vol of 0·01 *M* phosphate buffer pH 6. The homogenate was centrifuged in the cold at 20,000 **g** for 30 min and ammonium sulfate was added to the supernatant to yield 65 per cent saturation (Dixon, 1953). The precipitate was spun down, dissolved in the original phosphate buffer and dialyzed against the same buffer for 72 hr (six changes). This solution was chromatographed on DEAE-cellulose and the fractions were monitored for enzymatic activity. The B form of the enzyme did not adsorb to the column whereas the A form adsorbed and was subsequently eluted by salt gradient (0–0·2 *M* NaCl) in 0·01 *M* phosphate buffer pH 6. The material under each enzyme peak was pooled, concentrated by vacuum ultrafiltration and chromatographed on Sephadex G-200. Hex A was further purified by rechromatography on DEAE-cellulose and Hex B by chromatography on CM-cellulose (in 0·01 *M* phosphate buffer pH 5 with a linear gradient of NaCl ranging from 0–0·9 *M*). These preparations were used throughout the experiments.

Immunization

Rabbits were immunized by intradermal injections at multiple sites with each of the two enzyme preparations containing about 1 mg protein emulsified in an equal volume of Freund's complete adjuvant (Difco, USA). Two weeks later the animals were boosted by a similar procedure. Antibodies were found in the sera of the immunized rabbit about 14 days following the last injection.

Enzymatic assay

Enzyme preparations were diluted with 0·04 *M* citrate buffer pH 4·4 to an enzyme concentration of 0·1–0·6 munits/ml. One unit is defined as the amount of enzyme liberating 1 μmole methylumbelliferone per minute. The diluted enzyme (0·1 ml) was incubated at 37° C for 10 min with 0·2 ml of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide solution (0·2 m*M*, final concentration) in citrate buffer containing 0·1% bovine serum albumin (Calbiochem, USA). The reaction was terminated by the addition of 3 ml of 0·2 *M* glycine NaOH buffer pH 10·4 and the fluorescence was measured in a Turner fluorometer model No. 110 with filter setting for maximal excitation at 365 nm and maximal emission at 450 nm. Standard solutions of methylumbelliferone containing up to 0·5 nmole were tested simultaneously and used for calibration.

Immunochemical methods

Antibody activity in the sera of the immunized rabbits was demonstrated by double diffusion in agar (Special Noble Agar Difco, USA). This method had also been used to test the tissue specificity and isozyme specificity, using pretested active antisera. Enzyme-containing precipitin lines were stained for activity using naphthol AS-BI-β-D-N-Acetyl glucosaminide and Diazo Blue B (0·4 and 4 mg/ml, respectively) in 0·04 *M* citrate buffer pH 4·4.

Enzyme antibody interaction in liquid medium was performed at pH 7 in phosphate buffered saline. Increasing amounts of antiserum were added to a fixed amount of enzyme (determined by activity). Controls were treated similarly with normal rabbit sera instead of anti-hexosaminidase antisera. Following 30 min at room temperature and about 2 hr at 0° C the precipitates were spun down and the activity determined.

IgG fraction was prepared from anti-Hex A serum by precipitation at 40 per cent saturation of $(NH_4)_2SO_4$. Fab fragments were prepared from this IgG fraction by digestion with papain according to Porter (1958).

Preparation of specific anti-hexosaminidase A antiserum

Hex B was conjugated to cyanogen bromide-activated Sepharose 4B according to Porath *et al.* (1967). Hex B solution containing 3·2 g protein was bound to 80 g Sepharose with efficiency of 95 per cent. Anti-Hex A antiserum was passed through this column. The fraction which did not adsorb to the column contained specific anti-Hex A antibodies which did not cross-react (as measured by gel diffusion) with Hex B. It should be noted that a parallel procedure, namely adsorption of anti-Hex B on Sepharose-Hex A column removed all antibody activity. Similarly, no antibodies could be detected following adsorption on homologous Hex-Sepharose conjugates.

Heat inactivation

Enzyme preparations have been incubated at 50° C or other temperatures for various periods of time, following which the samples were transferred to an ice-bath, diluted with 0·04 *M* citrate buffer pH 4·4 to the optimal concentration and tested for enzymatic activity. It was separately ascertained that the reaction conditions (pH 4·4 at 37° C) do not cause significant dissociation of the immune precipitate (less than 10 per cent).

RESULTS

Immunochemical analysis of hexosaminidase isozymes from different organs

Enzyme preparations isolated from brain, placenta and liver were characterized by double gel diffusion

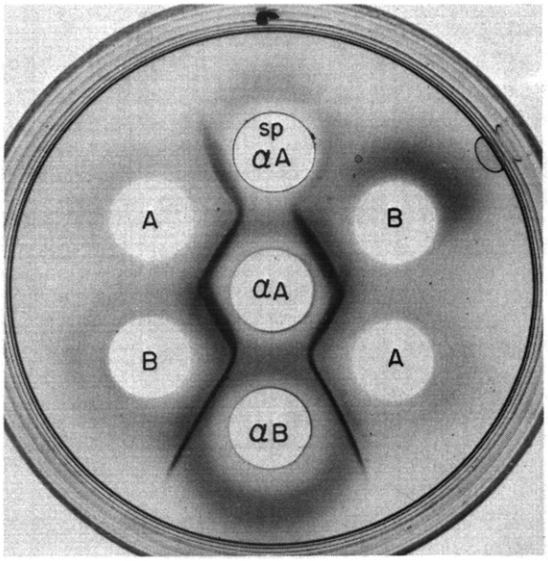

Fig. 1. Double diffusion in agar of hexosaminidase A (A) and hexosaminidase B (B) with antisera against Hex A (αA), against Hex B (αB) and specific anti-Hex A (Sp · αA).

with antisera against the brain enzymes. The Hex A preparations from the different organs all gave a precipitin band of identity when tested either with anti-Hex A or anti-Hex B. Similarly, the various Hex B preparations reacted identically with the two antisera. However, when tested with anti-Hex A each of the Hex A preparations showed a spur over the precipitin band obtained with Hex B. Absorption of anti-Hex A with Hex B yielded a specific anti-Hex A preparation which did not react with Hex B but still precipitated Hex A (Fig. 1).

Effect of antibodies on the enzymatic activity

The addition of antiserum brought about precipitation of hexosaminidases as is shown in the precipitation curve in Fig. 2. The precipitation, however, did not impair the enzymatic activity. All the activity originally present in the enzyme sample could be found in the mixture of enzyme and antiserum or in the isolated and washed immune precipitate. The figure describes the interaction between anti-brain Hex A and Hex A from placenta, but similar results were obtained also with other combinations of enzymes from the various tissues and the various antisera.

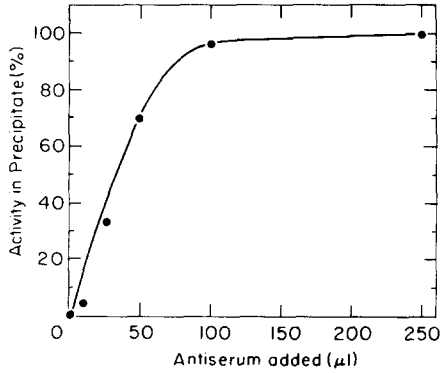

Fig. 2. Precipitation of placental hexosaminidase A by anti-brain Hex A antiserum.

Effect of increasing amounts of anti-hexosaminidase A on heat stability of the enzyme

Most of the heat stability experiments have been carried out on placental enzymes with antibodies prepared against brain Hex A. This antiserum was chosen because of its high titer and its clear-cut distinction between Hex A and Hex B.

Samples of Hex A were reacted with anti-brain Hex A under the conditions described in Fig. 2. The reaction mixtures were maintained at 50°C for 1 hr or 3 hr, following which aliquots were assayed for enzymatic activity. The results are shown in Fig. 3. Whereas in the presence of antiserum the enzyme retained about 80 per cent of its original activity, in the controls, when reacted with normal rabbit serum, all its activity was abolished. Similar samples with either immune or normal sera, maintained at 0°C, retained all the original enzymatic activity.

Fab fragment prepared from the IgG fraction of anti-Hex A serum brought about an identical stabilizing effect to that caused by the whole antiserum (Fig. 3). The stabilization is, therefore, a result of the enzyme–antibody interaction *per se*, and not due to the aggregation process.

Effect of anti-hexosaminidase A on the kinetics of heat inactivation

Hex A was incubated with 200 μl of either immune or normal (non-specific) rabbit serum as described above. The mixtures were incubated at 50°C and, at various times up to 3 hr, samples were withdrawn and chilled in ice. After the last sample was withdrawn all the samples were diluted and assayed for enzymatic

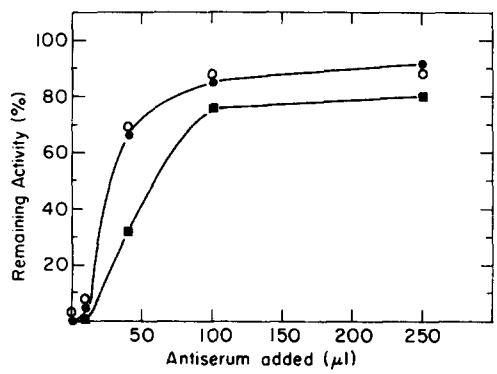

Fig. 3. Remaining activity of placental hexosaminidase A incubated with various amounts of anti-brain Hex A, at 50°C for 1 (●) or 3 hr (■). Remaining activity after incubation with Fab fragment of anti-Hex A (○).

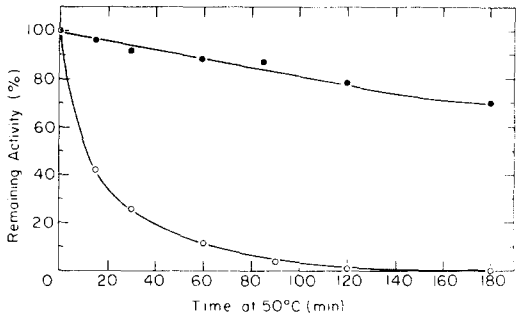

Fig. 4. Remaining activity of placental hexosaminidase A incubated with anti-brain Hex A antiserum (●) or normal rabbit serum (○) for various periods of time at 50°C.

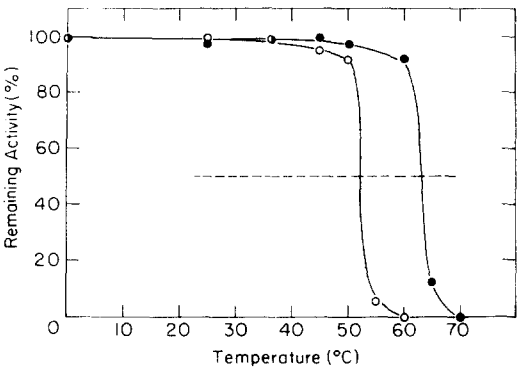

Fig. 6. Temperature-dependent inactivation of placental hexosaminidase B in the presence of anti-brain Hex A (●) or normal rabbit serum (○).

activity. Figure 4 demonstrates that the enzyme treated with non-specific serum was readily inactivated, losing almost all its activity following 90–120 min of incubation at 50°C. In contrast, the antibody-treated enzyme is relatively stable retaining about 70 per cent of the original activity after 3 hr heating at 50°C.

Degree of stabilization of hexosaminidases A and B by anti-hexosaminidase A antiserum

In the presence of non-specific serum at pH 7 at 50°C Hex A rapidly loses its activity whereas Hex B remains unaffected. At higher temperatures Hex B is inactivated as well. Both isozymes (from placenta) were incubated with anti-brain Hex A (which cross-reacts with Hex B) in concentration corresponding to the maximal precipitation zone. The reaction mixtures were incubated at various temperatures for 2 hr. Figures 5 and 6 depict the effect of the antibodies on the temperature-dependent inactivation of Hex A and Hex B, respectively. It can readily be seen that both isozymes are stabilized; thus a shift of 12 degrees was observed in the temperature causing 50 per cent inactivation of either isozyme under these conditions.

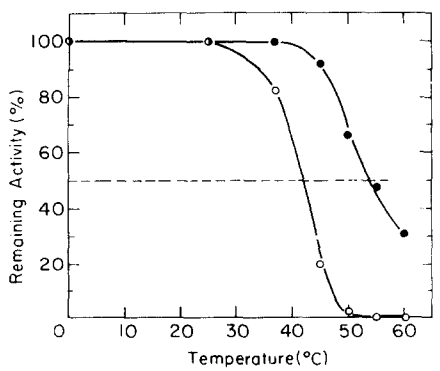

Fig. 5. Temperature-dependent inactivation of placental hexosaminidase A in the presence of anti-brain Hex A antiserum (●) or normal rabbit serum (○).

Stabilizing effect of anti-Hex B and Hex A-specific antiserum on the heat stability

The experiments described hitherto were performed with antiserum against Hex A, which is cross-reactive with Hex B (see Fig. 1). When the experiments were repeated with anti-Hex B they yielded identical results to those obtained with anti-Hex A. Stabilizing effect was achieved also with anti-Hex A which was previously adsorbed with Hex B. This serum is reactive only with Hex A and not with Hex B (Fig. 1). As expected it had a stabilizing effect on Hex A exclusively. The results are summarized in Table 1.

Interaction of heat inactivated Hex A with anti-Hex A antiserum

The following experiment was performed in order to test whether heat inactivated Hex A may regain enzymatic activity by interaction with antibodies directed against the native enzyme. Hex A was heat inactivated at 50°C for 3 hr, following which no enzymatic activity could be detected. To these preparations anti-Hex A antibodies were added at optimum concentration (deduced from precipitation of the same amount of native enzyme). No enzymatic activity was restored due to this treatment. This result could be explained by either antigenic dissimilarity between the native and heat-denatured enzyme, or by actual incapability of

Table 1. Effect of anti-Hex A, anti-Hex B and specific anti-Hex A on the temperature of 50% inactivation of Hex A and Hex B

	Midpoint of inactivation °C	
Serum added	Hex A	Hex B
Normal rabbit serum	42	52
Anti-brain Hex A	54	63
Anti-brain Hex B	54	64
Specific anti-brain Hex A	55	52

Table 2. Competition [a] between active and inactive placental hexosaminidase A in a precipitin test with anti-brain Hex A.

Ratio inactive Hex A / native Hex A	Activity in precipitin	Efficiency of competition[b] (%)
0	100	—
0·2	88	75
0·4	80	72
0·6	73	71
0·8	68	72
1·0	64	72
2·0	41	72

[a] The antigenic similarity between active and inactive Hex A was studied by the inhibition of precipitation of a constant amount of active enzyme by increasing amounts of inactive Hex A.

[b] The efficiency of competition is calculated as the per cent of observed inhibition of precipitation of activity, assuming the expected inhibition by theoretical immunologically identical inactive enzyme as being 100 per cent.

the antibodies to induce recovery of catalytic activity. To test which of these two possibilities were effective, an inhibition experiment was carried out. The results, shown in Table 2, demonstrate that heat denatured Hex A competes with the active enzyme in the precipitin test with the respective antibodies. Thus it seems that although capable of binding to it, the antibodies do not bring about reactivation of the already denatured enzyme.

DISCUSSION

One of the first observations in this investigation was the close similarity between hexosaminidases of various organs in the reaction with the antibodies. Antisera were prepared against both Hex A and Hex B from either brain, liver or placenta. All the antisera gave reaction of identity with the A form of the enzyme from the various organs. Similarly, the B form obtained from these tissues also showed immunological identity. According to these findings the antisera used in the various experiments were chosen according to their titer and not according to the enzyme source. This phenomenon of almost complete identity between enzymes originating from different organs of the same species is not unique for hexosaminidases, but has been reported for several other enzymes (Arnon, 1974). For example, cathespin D isolated from liver, spleen, heart kidney, testis, brain and limb bones were all found to be identical according to their reaction with specific antiserum to liver cathespin D (Weston, 1969; Dingle *et al.*, 1971). Identity was observed between citrate synthetase from rat heart and liver (Moriyama and Srere, 1971) and also in asparaginases isolated from guinea-pig serum and liver (Suld and Herbut, 1970). In such cases it is reasonable to assume that isofunctional enzymes of the same species, namely on the same evolutionary level, are practically identical. In the case of hexosaminidase this assumption of identity is corroborated by the findings that in physiological disorders involving lack of one or both of the hexosaminidase forms, the enzyme is missing in every organ tested (Sandhoff *et al.*, 1971).

In contrast to the identity between the same isozyme of different organs a distinct immunological difference was observed between Hex A and Hex B regardless of the tissue they originate from. Although a strong cross-reactivity is observed between the two isozymes, which led in the past to the erroneous conclusion that they are immunologically identical (Carroll and Robinson, 1972, 1973), a clear-cut spur could be demonstrated by our findings (Fig. 1). The existence of antigenic difference between Hex A and Hex B was mentioned also by Srivastava and Beutler (1973) who claimed that specific absorption of anti-Hex A by purified Hex B may lead to a specific anti-Hex A serum. A similar procedure was used in the present study, where specific anti-Hex A serum was prepared by absorbing anti-Hex A on Hex B immunoadsorbent (Fig. 1). It is of interest that anti-Hex B cross-reacts completely with Hex A.

The antibodies raised by Hex A or Hex B precipitated the two isozymes, but did not inhibit their catalytic activity. The enzyme preparations used for immunization were not pure, and thus the immune precipitate contained the immune complexes of the contaminating proteins as well. However, all the enzymatic activity added to the reaction mixture could be quantitated in the washed precipitate (Fig. 2), and no activity could be demonstrated in the supernatant at antibody excess.

The interaction of enzymes with their antibodies usually leads to total or partial inactivation. However, a few examples have been reported in the literature where no such inactivation takes place, e.g. acetylcholinesterase (Michaeli *et al.*, 1969*a*) and β-galactosidase (Rotman and Celada, 1968). It should be borne in mind that both these enzymes and hexosaminidases were assayed on low mol. wt substrates In view of the relation between the extent of inactivation by antibodies and the size of substrate, one cannot exclude the possibility that inactivation might have been observed if high molecular substrate were used for the enzymatic assay of hexosaminidase.

Stabilization of enzymes by their specific antibodies is a phenomenon that can be explained by the capacity of the antibodies to 'lock' the enzyme in an active conformation and prevent changes induced by the environment. It is usually observed, therefore, mainly in cases of enzymes which are normally unstable to either heat or other denaturating conditions. The present findings demonstrate clearly that the relative lability of the enzymes is not an essential factor. Both heat labile Hex A and stable Hex B show the same shift of 12°C in the transition temperature upon interaction with the antibodies (Figs. 5 and 6). In this stabilization, antibodies cross-reactive with both A and B forms are

effective on both isozymes. However, specific anti-Hex A antibodies, which are reactive with Hex A exclusively, do not have any stabilizing effect on Hex B, while being as efficient as the total anti-A antibodies in stabilizing Hex A. These specific anti-Hex A antibodies are directed towards a relatively limited number of antigenic determinants on the molecule. The finding that, nevertheless, they induce a stabilizing effect, imply either that they are reactive with a crucial region in the molecule, or alternatively, that the A-specific antigenic determinants are distributed over several regions in the molecular structure of the enzyme.

The stabilizing antibodies are not capable, however, of reviving the activity of Hex A which had been previously inactivated by heat. These results should be compared with the data of Michaeli *et al.* (1969*b*) who reported partial reactivation of heat-inactivated acetylcholinesterase. About 12 per cent of the enzymatic activity was restored by the interaction with the antibodies. In the case of Hex A no significant reactivation was observed.

The mechanism whereby the antibody confers upon an enzyme increased stability is not clear. The findings that even the limited population of specific anti-Hex A, which is probably not directed towards the enzyme catalytic center, is also effective supports the notion that the antibodies do not provide protection to the enzyme active site proper, but rather exert their protective effect by stabilizing an overall conformation.

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