Immunochemistry, 1976, Vol. 13, pp. 485–490. Pergamon Press. Printed in Great Britain

IMMUNOLOGICAL RELATIONSHIPS AMONG HEXOSAMINIDASES OF DIFFERENT SPECIES

BENJAMIN GEIGER, YOAV BEN-YOSEPH and RUTH ARNON

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

(*First received* 10 *November* 1975; *in revised form* 8 *December* 1975)

Abstract—The immunological inter-relationships between hexosaminidases* of several mammals were established. Qualitative immunochemical analysis indicated similarity between the enzymes derived from man, Rhesus monkey, Iris maccaque, bovine, pig, goat and guinea-pig. Quantitation of the extent of antigenic similarity among the various species, using antigen binding assay and radioimmunoassay, correlated well with the phylogenetic relationships between the species investigated. The evolutionary distance between the species used as sources of enzyme and the immunized animal affects the capacity to distinguish between closely related enzymes. Thus, the use of closer animals for immunization enables the detection of subtle differences between the tested enzymes, whereas a phylogenetically remote animal recognizes better their similarity.

INTRODUCTION

In the course of evolutionary process, many changes and variations occur in the primary sequence of both structural and biologically active proteins, which are manifested in variations among isofunctional homologous proteins. Several approaches have been proposed for the investigation of such phylogenetic interrelationships, including the application of immunochemical methods. The high specificity of immunological techniques as well as their high sensitivity offers an accurate tool for the comparison of protein molecules from different species. Thus, for example, extensive immunochemical studies on cytochromes *c* of as many as 25 species, revealed that a rough correlation exists betweeen the number and nature of sequence differences and the antigenic cross-reactivity of the various cytochromes *c* (Margoliash *et al.*, 1970); Nisonoff *et al.*, 1970; Reichlin *et al.*, 1970). Similarly, comparison of lysozymes from 16 different birds using anti-hen egg white lysozyme antibodies (Prager & Wilson, 1971*a*; 1971*b*), also pointed to a good agreement between structural and antigenic similarities. In the case of trypsins derived from several species it was again demonstrated that the order of similarity between them, according to immunological criteria, correlated well with their phylogenetic relationship (Arnon & Neurath, 1969). An immunological approach was applied to the study of many other enzymes, yielding similar information (reviewed by Arnon, 1973). As a general rule in such studies it was observed that the preservation of structure is greater, the more crucial the enzymes are for survival. In the present study we report on the immunochemical relationships among hexosaminidases of various species.

The enzyme *N*-acetyl β-D-hexosaminidase exists in a large variety of species, including mammals, lower vertebrates, invertebrates, plants, etc. (Walker, 1966). This enzyme is involved in the degradation pathway of various glycoprotcins and glycolipids in which it cleaves the terminal β-linked *N*-acetyl glucosamine or *N*-acetyl galactosamine. Purification and characterization of hexosaminidases from different animals, including rat (Sellinger *et al.*, 1973; Robinson *et al.*, 1967) bovine (Robinson *et al.*,1972; Frohwein & Gatt, 1967; Hayase *et al.*, 1973), sheep (Winchester, 1971), pig (Findlay & Levvy, 1960), mouse (Hunter & Milson, 1966) and man (Robinson & Stirling, 1968; Sandhoff & Wässle, 1971; Srivastava *et al.*, 1974), indicated that these enzymes exist in the various tissues in several isozymic forms, which are possibly immunologically similar. At least in the case of the human isozymes a strong immunological cross-reactivity was observed between the two major isozymes A and B. Thus, it was reported that although the A isozyme has some antigenic determinants which are not present on the B counterpart (a finding which enables the preparation of antisera which react specifically with hexosaminidase A), the total antiserum prepared against the A isozyme reacts fully with both isozymes and can serve as an equally reliable reagent for their quantitation (Geiger *et al.*, 1975).

In the present study antisera prepared in rabbits or goats against human and bovine hexosaminidase A served for immunochemical investigation of hexosaminidases of various species.

MATERIALS AND METHODS

Materials

Sephadex G-200 was purchased from Pharmacia (Sweden). DEAE-cellulose (DE-52) was from Whatman (U.S.A.). Agarose was from L'Industrie Biologique Francaise (France). 4-Methylumbelliferyl *N*-acetyl-β-D glucosaminide and methylumbelliferone were obtained from Pierce Chemical Co. (U.S.A.). Naphthol AS-Bi-*N*-Acetyl β-D glucosaminide, fast garnet BBC (o-amino azo toluene diazonium salt) and lactoperoxidase were from Sigma (U.S.A.). $Na^{125}I$ for protein iodination was bought from The Chemical Centre, Amersham (U.K.).

Enzyme preparations used for immunizations

1. Human placental hexosaminidases A and B were purified to a homogenous state as described elsewhere (Geiger

* Hexosaminidase: β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30.

et al., 1975). The purity was confirmed by gel electrophoresis and analytical ultracentrifugation. Hexosaminidase A of human liver was purified similarly.

2. Bovine liver hexosaminidases were partially purified by precipitation of the enzyme containing fraction from the crude homogenate with neutral $(NH_4)_2SO_4$ (25–60% saturation), gel chromatography on Sephadex G-200, and separation on DEAE-cellulose into two isozymes—acidic and basic, which are referred to as bovine hexosaminidase A and B respectively. Both isozymes were used for immunization and were shown to be highly cross reactive (unpublished data). The overall purification of bovine hexosaminidase A was 340-fold.

Tissues used as enzyme source for immunological comparison

Human placentae for the purification of hexosaminidase were obtained from the maternity ward of Kaplan Hospital, Rehovot. Human liver samples were taken in autopsy. Livers of rabbit, rat, mouse, guinea-pig, and Rhesus monkey (Maccaca mulata) were obtained from the Experimental Animal Unit of the Institute and kept at −20°C until tested. Freeze dried liver of Iris maccaque (Maccaca iris) was a gift from Dr. A. C. Wilson, Department of Biochemistry, Berkeley. Bovine, porcine and chicken livers were frozen immediately after slaughter and kept at −20°C until tested.

Preparation of homogenates

Livers were homogenized in 3 vol of ice-cold water in an omni-mixer (Sorvall, U.S.A.). Homogenization proceeded for 3 min at top speed. The homogenates were clarified by two successive centrifugations at 27,000 *g* at 4°C. The clear supernatants were taken, avoiding the upper lipid layer. Centrifugation was repeated whenever necessary.

Antisera

Antisera against highly purified human placental hexosaminidases A and B were prepared in goats by two weekly injections of 1 mg of purified enzyme emulsified in complete Freund's adjuvant.

Rabbit antisera to human liver hexosaminidase A and to bovine liver hexosaminidase A were prepared by injecting purified enzyme preparations containing about 1 or 10 mg of protein, respectively, with complete Freund's adjuvant. Blood was collected from the immunized animals, starting two weeks following the last injection.

Enzymatic assay

Enzymatic activity was determined as described elsewhere, using 4-methylumbelliferyl *N*-acetyl-β-D glucosaminide as a substrate, by monitoring the fluorescence of the released methylumbelliferone at pH 10.4 in a Turner model 110 fluorometer, with filter setting yielding maximal excitation at 360 nm (Ben Yoseph *et al.*, 1975). One unit of enzyme was defined as the amount of enzyme liberating 1 μmole of methylumbelliferone per minute in the assay system.

Immunological assays

(1) *Double immunodiffusion in agar.* Double immunodiffusion studies were carried out in 1.5% agarose gels, following the general procedure of Ouchterlony (1948). Agarose solution (10 ml) poured on 4.5 × 9.5 cm plates. Holes (7 mm) were punched in the gel, and test samples introduced into these. Usually enzyme samples from various species containing the same amount of enzymatic activity (5×10^{-2} units/ml) were pipetted into the peripheral wells whereas the central well was filled with antiserum. Diffusion was allowed to proceed at 4°C for 48 hr after which the plates were rinsed for 48 hr in phosphate-buffered saline (PBS), pre-equilibrated with 0.1 *M* citrate buffer pH 4.4, and stained for enzyme activity with Naphthol AS-Bi-*N*-acetyl β-D-glucosaminide and fast garnet GBC.

(2) *Antigen binding assay.* The capacity of different antisera to bind hexosaminidase was determined in the following manner: to 50 μl of antiserum in proper dilution, increasing amounts of the enzyme preparations (purified enzyme or tissue homogenates) were added in a volume ranging from 10–500 μl. The volume was made up to 550 μl with PBS in all the tubes. After 30 min incubation at 37°C, antiserum against the particular antibodies used in the assay was added to each tube (i.e. goat anti-rabbit IgG was added to tubes containing rabbit antiserum against hexosaminidase, and rabbit anti-goat IgG was employed when goat antiserum was used in the system). This second antiserum was added in an amount, sufficient to precipitate all the anti-hexosaminidase antibodies present. After 30 min incubation at 37°C and additional 16 hr at 4°C, the precipitate was spun down 3 times and washed with ice-cold PBS. Finally, it was suspended in 0.04 *M* citrate buffer pH 4.4, diluted to a suitable concentration and the enzymatic activity determined as described above. Preliminary studies proved that none of the antisera had an inhibitory capacity on the different enzymes tested, and the total enzymatic activity was retained in the immune precipitates (Ben Yoseph *et al.*, 1975).

(3) *Radioimmunoassay.* Radioimmunoassay using I^{125} labelled human hexosaminidase A was performed as described elsewhere (Geiger *et al.*, 1975). Hexosaminidase A (15 μg) were iodinated according to the method of Marchalonis (1969). Radioactively labeled (~10^4counts/min) enzyme (50 μl), diluted in PBS containing 0.1% gelatin, was mixed with various dilutions of antisera, and incubated at 37°C for 30 min (antisera were diluted in a 1:10 solution of the respective normal serum in PBS). Antisera against the anti-hexosaminidase antibodies were added as described in the previous binding assay. The precipitates formed were washed twice with cold PBS and dissolved in 1 ml 0.1 *M* NaOH. Radioactivity was measured in an autogamma Packard, scintillation spectrometer model 3002. Competition studies were carried out in a similar manner, but the radioactive hexosaminidase A was mixed with the tested enzyme solution prior to the addition of the antibodies. About 15% of the radioactivity was non-specifically bound when tested with either goat or rabbit normal sera. These values were subtracted in the calculations of percent binding.

RESULTS

The immunological cross-reactivity among hexosaminidases of various species was demonstrated by using several techniques. Qualitative results were obtained by *double immunodiffusion in agar*, which revealed the cross-reactions with several antisera, including goat anti-human hexosaminidase A, rabbit anti-human hexosaminidase A and rabbit anti-bovine hexosaminidase A. The results, minifested as enzyme-containing precipitin bands, are shown in Fig. 1.

As shown, high degree of cross-reactivity exists between the enzyme derived from either of two monkeys (Maccaca iris and Maccaca mulata), and the human enzyme, when tested with either goat or rabbit anti-human hexosaminidase. With the rabbit antiserum the enzyme of the three sources shows bands of identity, and only the goat antiserum detects the specific human determinants by a distinct spur. None of the other species tested, including bovine, porcine, rabbit, guinea-pig, rat, mouse and chicken, gave any cross-reaction with the two anti-human hexosaminidase sera. Broader spectrum of cross-reactivity with other species was observed with the antiserum against bovine hexosaminidase. In this case a strong cross-reactivity is observed between the bovine and the goat enzymes. A reaction is observed also with the

enzymes from pig, guinea-pig, monkeys and human. However, the extent of cross-reactivity here is apparently lower, as indicated both by the low intensity of the band and by the finding that the bands are located close to the antiserum well, suggesting that only a relatively small proportion of the antibodies in the serum are cross-reactive. The human and the monkeys enzymes give identity bands, but both the pig and the guinea-pig show spur formation over them indicating that they share more antigenic determinants with the bovine enzyme. On the other hand, no cross-reactivity is detected in this assay with hexosaminidase from rabbit, mouse or chicken.

Similar results, but on a quantitative basis were obtained in the *antigen-binding assay*. No external labeling of the antigen was required, since the enzyme retained its catalytic activity in the immuno-precipitate and could thus be monitored at a very high sensitivity. The results are shown in Figs. 2–4. When the assay was carried out with goat antiserum to human

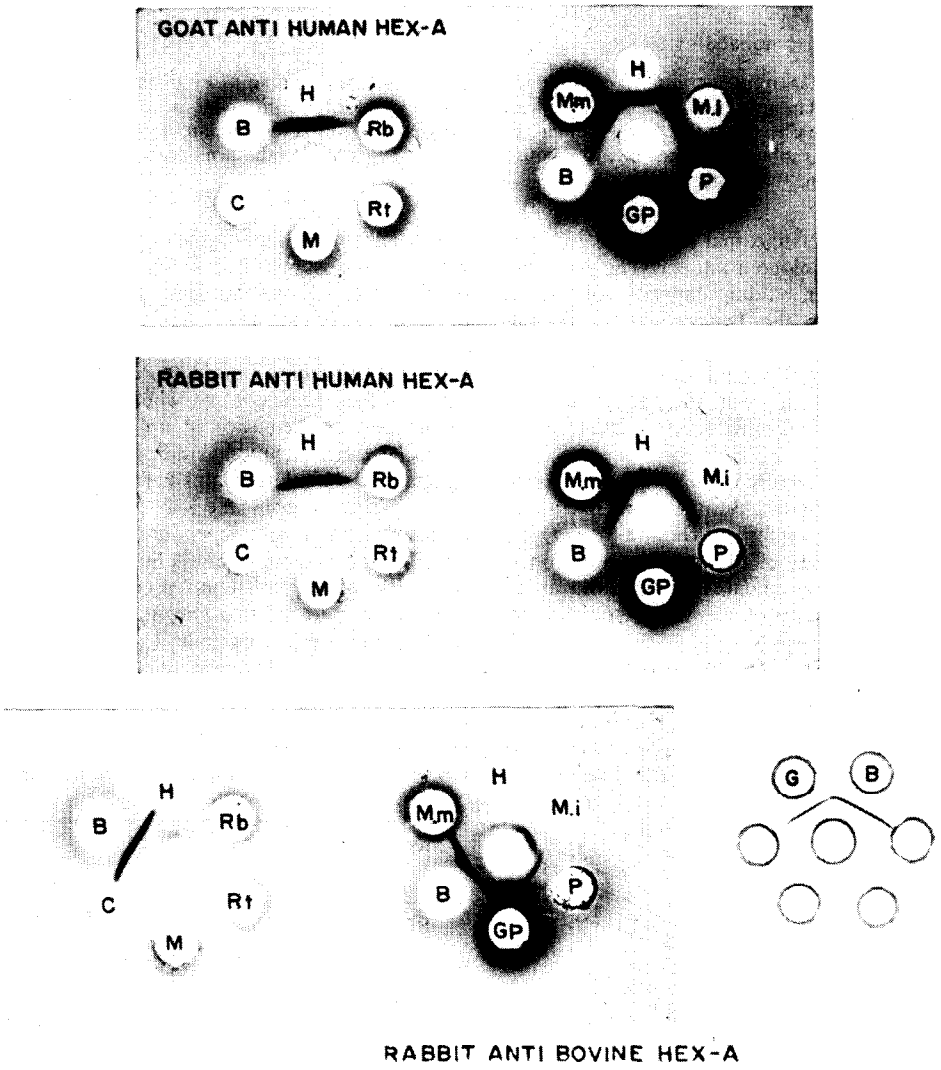

Fig. 1. Double immunodiffusion of hexosaminidases in agar gels. The various antisera were placed in the central well as indicated in the figure. Enzyme extracts with the same enzymatic activity were placed in the peripheral wells. H, human; M.i., Maccaca iris; P, pig; GP, guinea-pig; B, bovine; M.m., Maccaca mulata; Rb, rabbit; Rt, rat; M, mouse; C, chicken; G, goat.

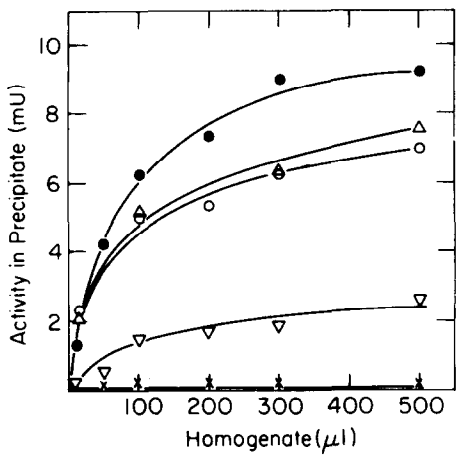

Fig. 2. Antigen binding assay of the various hexosaminidases with goat anti-human hexosaminidase A. All enzyme solutions were of the same enzymatic activity. Human, ●; M. mulata, △; M.iris, ○; rabbit, ▽; bovine, pig, rat, mouse, chicken, guinea-pig, X.

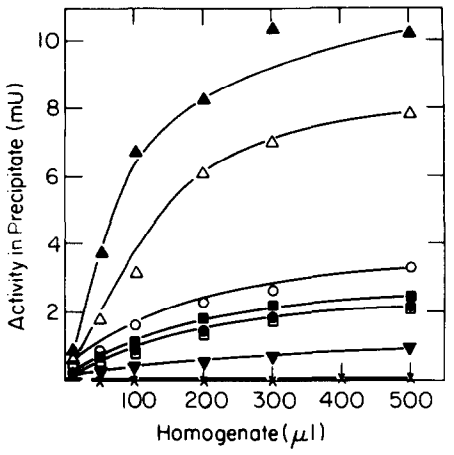

Fig. 4. Antigen binding assay of the various hexosaminidases with rabbit anti-bovine hexosaminidase A. Bovine, ▲; goat, △; M.iris, ○; human, ●; guinea-pig, ■; pig, □; rat, ▼; rabbit, mouse, chicken, X.

hexosaminidase (Fig. 2), maximal binding of 85% was obtained with the homologous antigen. The goat antibodies exhibited lower binding capacity toward the enzymes derived from the two monkeys, reaching about 75% of the values obtained with the human enzyme. A weak, but significant binding was obtained in this system with the rabbit enzyme, whereas enzymes from all other species tested showed no detectable binding.

A slightly different picture is obtained with rabbit anti-human hexosaminidase serum (Fig. 3). In this case, both monkeys' enzymes were bound to an extent almost identical to that of the homologous human enzyme. Rabbit hexosaminidase did not bind at all, and the binding capacity of the serum towards pig and guinea-pig serum was very low.

A somewhat wider range of cross-reaction was observed when enzymes of the various species were tested with antiserum against bovine hexosaminidase (Fig. 4). In this system, as expected, the highest binding (75–80%) was obtained with the homologous bovine enzyme. A high degree of binding was observed with goat hexosaminidase, reaching about 80% of that of the homologous antigen. Lower binding was observed with enzymes from several other species namely, human, the two monkeys, pig and guinea-pig. Rat hexosaminidase bound to less than 10% of the extent of the bovine control, whereas tissues of rabbit, mouse and chicken showed no binding whatsoever.

In the third technique, radioimmunoassay, giving insight into the relationships between hexosaminidase of the various species, the sole parameter effective is antigenic similarity, independently of enzymatic activity. The labelled enzyme was human hexosaminidase regardless of the antiserum used. Hence, the extent of its binding to the two anti-human hexosaminidase sera, namely, rabbit and goat, was quite high—65 and 75% respectively. However, the binding to the anti-bovine hexosaminidase serum was rather weak—15% (Fig. 5). This low degree of binding reflects the limited cross-reactivity between the human and bovine enzymes. The radioimmunoassay, which was carried

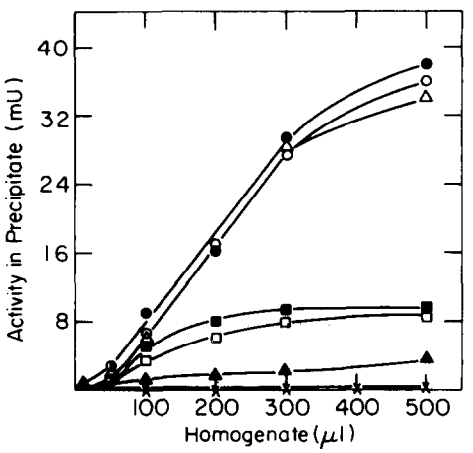

Fig. 3. Antigen binding assay of the various hexosaminidases with rabbit anti-human hexosaminidase A. Human, ●; M.mulata, △; M.iris, ○; guinea-pig, ■; pig, □; bovine, ▲; rat, rabbit, mouse, chicken, X.

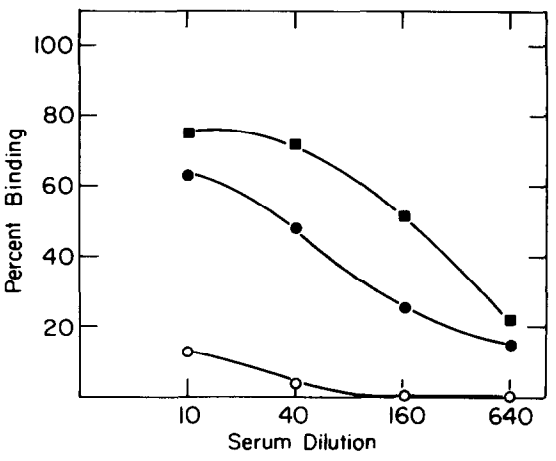

Fig. 5. Binding of I^{125}-labelled human hexosaminidase A by the various antisera. Goat anti-human hexosaminidase A, ■; rabbit anti human hexosaminidase A, ●; rabbit anti *bovine* hexosaminidase A, ○.

Table 1. Radioimmunoassay with various anti-hexosaminidase antisera*

Competing, unlabelled hexosaminidase	Percent inhibition of binding		
	Goat anti-human/ hexosaminidase A	Rabbit anti-human hexosaminidase A	Rabbit anti-bovine hexosaminidase A
Human	77.0	72.2	73.9
M. iris	63.2	65.6	48.9
M. mulatta	61.6		
Bovine	2.8	6.1	70.1
Pig	10.1	12.3	11.4
Guinea-pig	30.9	10.5	8.0
Rabbit	26.3	0	−3.1
Rat	0	0.5	26.4
Mouse	0.6	5.7	6.3
Chicken	0	−4.0	0

* The numbers indicate the extent of inhibition of binding of labelled human hexosaminidase A by the tissue extracts of the various species. The enzymatic activity in all the extracts tested was identical—0.88 units per ml. The dilution of the antibody preparations was 1:80 for the goat anti-human hexosaminidase A and 1:60 for the rabbit anti-human and the rabbit anti-bovine hexosaminidase A.

out in optimal antiserum concentration, measured the capacity of hexosaminidases of various species to compete with the binding of the labeled human enzyme. The results are presented in Table 1. With both goat and rabbit antisera to human hexosaminidase, the highest extent of competition was obtained, as expected, with 'cold' human hexosaminidase, but the two monkey enzymes showed considerable cross-reactivity with the human enzyme. Lower cross-reactivity was observed with pig and guinea-pig hexosaminidases and is even lower—with bovine. Rabbit enzyme exhibited significant cross-reactivity, but only with goat antiserum; no competition was obtained when rabbit antiserum was used in the assay. In the system using the antiserum against bovine hexosaminidase similar data were obtained except for one major difference—the bovine enzyme showed competition capacity as high as that of the human enzyme, higher than that manifested by the monkeys. This must be due to the fact that in this system, only the antigenic determinants common to the human and the bovine enzymes are reflected and hence the two enzymes are reactive in it to the same extent.

DISCUSSION

In the present investigation the immunological relationship was established among hexosaminidases of various mammals, including man, monkeys, cattle, pig, goat, rabbit and guinea-pig. Quantitation of their antigenic similarity is in accordance with the evolutionary and phylogenetic relationship among the species investigated.

The experimental data were obtained by three techniques, qualitative double diffusion in agar gels, and quantitative antigen-binding assay and radioimmunoassay respectively. All three methods are highly sensitive; in the double diffusion and the antigen-binding methods, advantage is taken of the enzymatic activity in order to detect minute amounts of the enzyme in immune complexes, whereas in the radioimmunoassay the high sensitivity is achieved by the high specific labeling of the purified enzyme. The use of enzymatic activity as a handle for monitoring presence of enzyme is feasible, in view of our findings in previous studies that the enzymatic activity of hexosaminidase is unimpaired by the formation of immune complexes or immune precipitates (Ben Yoseph *et al.* 1975). The synthetic substrate used in the present study allows the determination of hexosaminidase in the binding assay as low as concentration of 50 ng/ml. The antigen-binding assay used here has therefore two advantages—it requires no external labeling of the antigen and is somewhat more sensitive than the commonly used antigen-binding assays.

The enzyme samples of the various species used throughout this study were crude liver homogenates. The use of homogenate without further purification is permissible, since the assay methods assure a selective and exclusive determination of the enzyme. This selectivity stems from the use of monospecific antisera and of purified hexosaminidase A for labeling, in the radioimmunoassay, and in the other assays by the use of enzymatic reaction for monitoring of the enzyme. In our previous experiments with human and bovine hexosaminidase obtained from different organs (including liver, brain, kidney, spleen and placenta) two observations were made; first enzymes of the various organs of the same species were shown to be immunologically identical. This is in agreement with the findings with other enzyme systems—e.g. human Cathepsin D (Weston, 1969; Dingle *et al.*, 1971), guinea-pig asparaginases (Suld & Herbut, 1970) and rat citrate synthetase (Moriyama & Srere, 1971). The second observation was that the isolated A and B isozymes of hexosaminidase of either human or bovine origin are highly cross-reactive. In view of these two observations, the liver homogenate was considered as a representative tissue and the hexosaminidase in it was regarded here as a single enzymatic entity regardless of its distribution into different isozymes.

The liver homogenates of the various species were prepared in a similar manner, so as to contain approximately the same level of enzymic activity. The activity served as the only parameter for estimation of the enzyme, since a total purification, which might

allow comparison on a weight basis, is extremely laborious.

Comparison of the results obtained in the double diffusion in agar and the antigen-binding assay reveals a very good agreement between the two methods. In those cases where antigenic identity between samples was indicated by double diffusion, the extent of antigen binding was similarly high, for example the reaction between the human and the two monkey hexosaminidases with rabbit antiserum. On the other hand, when the goat antiserum was used, which could differentiate between the human and the monkeys enzymes (a spur in double diffusion) a difference was also observed in the quantitative binding test—the human enzyme bound better than either of the monkey enzymes.

This finding sheds light on an additional aspect which is of significance in immunochemical studies of enzymes, namely, the influence of the phylogenetic distance between the species serving as the source of the antigen and the immunized animal, respectively. It appears that the use of more closely related animal for immunization enables the detection of small and subtle differences between the enzymes tested. Whereas, when an evolutionarily more remote animal is immunized it recognizes the similarity between related enzymes.

In the radioimmunoassay, one set of results (third column of Table 1) was obtained using labeled *human* enzyme as the antigen and anti-*bovine* hexosaminidase as the antiserum. In this case attention was focused on the similarity of the enzymes from various species with respect to a limited number of antigenic determinants—those determinants which are common to human and bovine hexosaminidase A. As expected, both human and bovine enzymes exhibited an almost identical capacity to compete in this system, since both contain these common determinants.

Noteworthy is also the finding that rabbit antiserum against human hexosaminidase did not show any cross-reactivity with the rabbit enzyme whereas the goat antiserum reacted with it to a significant extent. This phenomenon might be explained by assuming that the rabbit does not produce antibodies to those antigenic determinants which are present on its own hexosaminidase. It should be mentioned, however, that in the case of cytochrome-*c* (Nisonoff *et al.*, 1970), the rabbit protein was capable of displacing labeled human cytochrome-*c* in the reaction with its specific rabbit antiserum.

Acknowledgement—The able technical assistance of Mrs. Ronit Hirsch is gratefully acknowledged.

REFERENCES

Arnon R. (1973) in *The Antigens* (Edited by Sela M.), Vol. 1, p. 87. Academic Press, N.Y.
Arnon R. & Neurath H. (1969) *Proc. natn Acad. Sci. U.S.A.* **64**, 1323.
Ben-Yoseph Y., Geiger B. & Arnon R. (1975) *Immunochemistry* **12**, 221.
Dingle J. J., Barrett A. J. & Weston P. D. (1971) *Biochem. J.* **123**, 1.
Findlay J. & Levvy G. A. (1960) *Biochem. J.* **77**, 170.
Frohwein Y. Z. & Gatt S. (1967) *Biochemistry* **6**, 2775.
Geiger B., Navon R., Ben-Yoseph Y. & Arnon R. (1975) *Eur. J. Biochem.* **56**, 311.
Hayase K., Reisher S. R. & Kritchevsky D. (1973) *Proc. Soc. exp. biol. Med.* **142**, 466.
Hunter G. D. & Millson G. L. (1966) *J. Neurochem.* **13**, 375.
Marchalonis J. J. (1969) *Biochem. J.* **113**, 299.
Margoliash E., Nisonoff & Reichlin M. (1970) *J. biol. Chem.* **245**, 931.
Moriyama T. & Srere P. A. (1971) *J. biol. Chem.* **246**, 3217.
Nisonoff A., Reichlin M. & Margoliash E. (1970) *J. biol. Chem.* **245**, 940.
Ouchterlony Ö. (1948), *Acta path. microbiol. scand.* **25**, 186.
Prager E. M. & Wilson A. C. (1971) *J. biol. Chem.* **246**, 7010.
Prager E. M. & Wilson A. C. (1971) *J. biol. Chem.* **246**, 5978.
Reichlin M., Nisonoff A. & Margoliash E. (1970) *J. biol. Chem.* **245**, 947.
Robinson D., Jordan T. W. & Horsburgh T. (1972) *J. Neurochem.* **19**, 1975.
Robinson D., Price R. G. & Dance N. (1967) *Biochem. J.* **102**, 525.
Robinson D. & Stirling J. L. (1968) *Biochem. J.* **107**, 321.
Sandhoff K. & Wäsale W. (1971) *Hoppe-Seyler's Z. physiol. Chem.* **352**, 1119.
Sellinger O. Z., Santiago J. C., Sands M. A. & Furinsloat B. (1973) *Biochem. biophys. Acta* **315**, 128.
Srivastava S. K., Awasthi Y. C., Yoshida A. & Beutler E. (1974) *J. biol. Chem.* **249**, 2043.
Suld H. M. & Herbut D. A. (1970) *J. biol. Chem.* **245**, 2802.
Walker P. G. (1966) in *The Amino Sugars* (Edited by Balaz & Jeanloz), Vol. **IIB**, Academic Press, N.Y.
Weston P. D. (1969) *Immunology* **17**, 421.
Winchester B. G. (1971) *Biochem. J.* **124**, 929.