Immunologic Analyses of Mouse Cystathionase in Normal and Leukemic Cells†

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ABSTRACT: Rabbit antisera have been raised against mouse liver cystathionase and shown to possess enzyme neutralizing activity. Agar gel double immunodiffusion analyses demonstrated that both mouse liver cystathionase and rat liver cystathionase react with the antisera, the latter enzyme being completely cross-reactive with the former. Following radioiodination of the purified rat liver enzyme, a double antibody radioimmunoassay was developed in which greater than 90% of the labeled protein could be specifically precipitated with the anti-mouse cystathionase antibodies. In this test the purified rat liver and mouse liver enzymes were virtually indistinguishable, generating superimposable competition displacement curves on a protein mass basis. These results indicate that both enzymes are immunologically identical, thus validating the use of the rat in lieu of the murine liver enzyme as radiolabeled tracer in an assay for mouse cystathionase. In addition, competition radioimmunoassays demonstrated that the immunological reactivities of both the purified rat liver and mouse liver enzymes were equally heat sensitive. The sensitivity of the assay was determined to be 1 ng of enzyme protein/0.22 mL of assay mixture, and the assay could be used to detect the presence of enzyme protein in tissue homogenates of single mouse organs. Mouse or rat cross-reactivity with human liver cystathionase was incomplete, but, with the exception of heart and spleen, parallel radioimmunoassay competition displacement curves were obtained for cystathionase from different mouse organs including thymus. Extracts of 7-, 9-, and 10-month-old spontaneous AKR mouse thymomas were tested in the radioimmunoassay along with extracts of age-matched thymuses which were grossly tumor free. A reaction of nonidentity was observed for all of the tumor extracts while a reaction identical with that of the pure liver enzyme was found with all of the normal thymus extracts. Analogous results were obtained with a murine type C virus-induced thymoma compared to age-matched normal thymus from a Balb/c mouse. Thus, the previously reported enzyme depletion in type C virus-induced thymic tumors is reflected in the lack of one or more of the antigenic determinants of the normal thymus enzyme.

A variety of mammalian leukemic cell lines and fresh leukemic cell preparations have been shown to be cysteine auxotrophic relative to their nonleukemic counterparts (Lazarus et al., 1974; Livingston et al., 1976; Iglehart et al., 1977). Coupled to this phenotypic characteristic is the finding that activity levels of the enzyme cystathionase, the last enzyme in the mammalian cysteine biosynthetic pathway, are reduced in a variety of human and rodent lymphoid and myeloid leukemic cell lines relative to levels in comparable cells derived from healthy donors (Lazarus et al., 1974; Livingston et al., 1976; Iglehart et al., 1977). Similarly, extracts of spontaneous AKR and Moloney type C virus induced thymic lymphomas are characterized by lower levels of cystathionase activity than are present in extracts of thymocytes from tumor-free mice (Livingston et al., 1976). These results made cystathionase activity levels a potentially useful marker for the transformed cell phenotype.

The biochemical mechanism underlying this enzyme depletion phenomenon is not understood. Recent, indirect evidence obtained with human cell lines suggests that the decrease in intracellular cystathionase activity may be accompanied by a reduction in the concentration of active enzyme (Iglehart et al., 1977). In order to assess this possibility in a number of cell and animal systems, a measurement of enzyme protein is essential. We, therefore, set out to develop a specific radioimmunoassay for cystathionase, a prerequisite for which is the availability of either pure enzyme for radiolabeling or monospecific antisera. In a previous report (Bikel et al., 1978), the purification to apparent homogeneity of the enzyme from mouse liver—the richest source of the enzyme in this animal—was described. The apparent molecular weight of the enzyme was found to be 160 000, and the enzyme appears to consist of four subunits of equal size. The development of a specific radioimmunoassay and its validation as an accurate measure of this protein, as well as of the rat enzyme, is described here. Measurements of cystathionase protein concentrations in a number of tissue homogenates, including normal and spontaneous AKR tumored thymuses as well as normal and Moloney type C virus induced Balb/c tumored thymus, also constitute a subject of this report. For reasons to be discussed below, purified rat liver cystathionase (Uren et al., 1978) was used as radioactive tracer in the radioimmunoasssay for the mouse enzyme.

Experimental Procedures

B6D2F1 mice (C57 Bl/6 ♀ × DBA/2 ♂), AKR mice, and Balb/c mice were obtained from the Jackson Laboratory, Bar Harbor, Me.; Bur/Cr rats were obtained from Tulane University School of Medicine, New Orleans, La.; goat anti-rabbit γ-globulin was obtained from Sera Source, Bradford, N.H.; sucrose density gradient purified AKR virus, lot 727-30-10, was obtained from ElectroNucleonics Laboratories, Inc., through the courtesy of the Office of Program Resources and Logistics of the National Cancer Institute.

Extraction of Organs and Solubilization of Proteins.

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Mouse, rat, or human livers were homogenized at half-maximal speed for 2 min in an ice-cold Sorvall Omni-Mixer with 2 vol of 10 mM KH_2PO_4, 20 μM PLP,[1] 1 mM DTE, 10 mM EDTA adjusted to pH 7.7 with NaOH. Following centrifugation at 10 000g for 30 min the pellet was discarded. The supernatant was used in enzyme and protein assays and in competition radioimmunoassays.

Other mouse organs were homogenized in the same buffer in a Kontes glass–glass tissue grinder and then centrifuged as described above.

Electrophoresis. Analytical polyacrylamide gel electrophoresis under denaturing conditions and in the presence of reducing agents (Weber & Osborn, 1969) was performed as previously described (Bikel et al., 1978).

Enzyme and Protein Assays. Cystathionase from both mouse and rat was assayed with L-cystathionine as substrate as previously described (Bikel et al., 1978). Protein concentrations were determined by the method of Lowry et al. (1951).

Preparation of Antibody. Antisera against hydroxylapatite-purified cystathionase (Bikel et al., 1978) was prepared in rabbits, which were prebled prior to the first immunization. Rabbit no. 1 was injected intradermally at multiple sites in the back with 0.18 mg of protein, emusified with an equal volume of Freund's complete adjuvant. Booster injections were given at 25, 47, and 65 days after the primary immunization.

Rabbit no. 2 was injected with 0.36 mg of protein (1:1 in complete Freund's adjuvant) intramuscularly in the two hind legs, as well as subcutaneously in the back. Subsequently, it was injected as above with a total of 0.18 mg of protein, similarly emulsified. Booster injections were given at 25 and at 50 days after the primary immunization. Rabbits were bled from the ear vein into plastic tubes, which were incubated at 25 °C for 1 h. After 16 h at 4 °C, sera were collected by centrifuging the clotted blood at 1500g for 10 min and then at 3000g for 20 min. The antisera, as well as preimmune sera, were subaliquoted and stored at −70 °C, and used in both immunodiffusion studies and in radioimmunoassays after heating at 56 °C for 30 min.

Enzyme Neutralization Assay. The antisera employed in these experiments were not heat-treated before use.

Constant amounts of enzyme with increasing dilutions of sera were incubated at 37 °C for 30 min in a total volume of 100 μL of 10 mM KH_2PO_4, 20 μM PLP, 1 mM DTE, 1 mM EDTA, 5% glycerol, adjusted to pH 7.0 with NaOH. The tubes were then transferred to an ice–water bath and 100 μL of enzyme reaction mixture (Bikel et al., 1978) and 100 μL of substrate were added. The mixtures were further incubated at 37 °C for 30 min. Enzyme activity was measured without centrifugation.

Immunodiffusion. Double-diffusion was carried out in 2% Difco special Noble Agar in 1 M glycine, 0.15 M NaCl (pH 7.2). Diamter of Wells and center to center distance between wells were 1 and 7 mm, respectively. Plates were incubated in a humid atmosphere at 25 °C for 1–3 days at room temperature, washed in a Tris–saline solution (25 mM Tris-HCl, pH 7.8, 0.15M NaCl) for 4 days, with several changes of buffer, and then stained with Coomassie brilliant blue and destained as previously described for polyacrylamide gel electrophoresis (Bikel et al., 1978).

Iodination. Purified rat liver cystathionase (Uren et al., 1978) was dialyzed against 0.25 M potassium phosphate buffer, pH 7.6, then labeled with ^{125}I using the iodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Pierce Chemical Co., Rockford, Ill.). Iodogen, dissolved in chloroform, was delivered into 10 × 75 mm siliconized glass tubes such that each tube contained 10–20 μg of the reagent. The chloroform was then evaporated under a stream of nitrogen, and the tubes were stored at −20 °C until used. In a typical labeling preparation 24 μg of purified rat liver cystathionase in a volume of 40 μL was made 1% with respect to NP40 (Particle Data Laboratories, Elmhurst, Ill.) by adding 5 μL of a 10% solution of NP40 in water. This solution was added to an iodogen tube, followed by approximately 0.5 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.; 11–17 mCi/μg). After a brief period (1–2 min) at room temperature, the reaction mixture was added to a column (1 × 13 cm) of Sephadex G-25 (Pharmacia) equilibrated with RIA buffer (20 mM Na_2HPO_4, 0.15 M NaCl, 0.01% NaN_3, 5 mM EDTA, 0.4% NP40, pH 7.7) and run at a flow rate of 7 mL/h. Fractions (0.18 mL) were collected. The specific activity of [^{125}I]cystathionase peak fractions, eluted in the void volume of the column, was approximately 10 μCi/μg of protein (1–1.5 μCi/pmol of cystathionase, assuming 80% recovery). The labeled enzyme was further purified on an identical size Sephacryl S-200 superfine (Pharmacia) column, equilibrated in RIA buffer and run at a flow rate of 3 mL/h. A peak of [^{125}I]cystathionase, eluting in the column volume corresponding to enzyme activity elution volume, was collected. Approximately 95% of the radioactivity could be precipitated with 8% (v/v) trichloroacetic acid.

Radioimmunoassay. Rabbit anti-mouse cystathionase was titrated by serial dilutions in a final volume of 100 μL of RIA buffer in 12 × 75 mm Falcon polystyrene tubes. Twenty microliters of [^{125}I]cystathionase (25 000 cpm) was added and the mixture incubated for 1 h at 37 °C, followed by an 18-h incubation at 4 °C. Ten microliters of normal rabbit serum (previously diluted 1:1 in RIA buffer) was then added, followed by 40 μL of goat anti-rabbit IgG (previously titrated to ensure optimal precipitation). Following incubation at 4 °C for 6–24 h, the reaction mixture was centrifuged at 3000g for 5 min. RIA buffer (0.5 mL) was added to each tube, and centrifugation was resumed at 3000g for 20 min, after which the supernatant was aspirated and the precipitate counted in an Autogamma (Nuclear Chicago) spectrometer. Background values due to normal rabbit serum alone were in the range of 2–10%. Percent [^{125}I]cystathionase precipitated was computed by dividing the radioactivity precipitated with the immune serum minus the radioactivity precipitated with nonimmune serum by the total Cl_3CCOOH-precipitable radioactivity added. In competition assays, samples tested for the presence of cystathionase were diluted in RIA buffer in a final volume of 50 μL. One hundred microliters of specific antiserum at a dilution sufficient to bind 75% of the tracer was added and the mixture incubated for 15 min at 37 °C. Following addition of 20 μL of [^{125}I]cystathionase standard and further incubation for 1 h at 37 °C and 18 h at 4 °C, antigen–antibody complexes were precipitated by the addition of normal rabbit serum and of goat anti-rabbit IgG as described above.

Results

Immunization of Rabbits. Production of Enzyme-Neutralizing and Precipitating Antibodies. In preparing mouse liver cystathionase for immunization, we have purified the enzyme through the hydroxylapatite step of our published procedure (Bikel et al., 1978), omitting the final Sephadex G-200 gel filtration. Electrophoretic analysis under denaturing conditions of the preparation used to immunize rabbits is shown in Figure 1. Clearly the immunogen used was highly purified, although not homogeneous.

[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; DTE, dithioerythritol; NP40, Nonidet P40; RIA, radioimmunoassay.

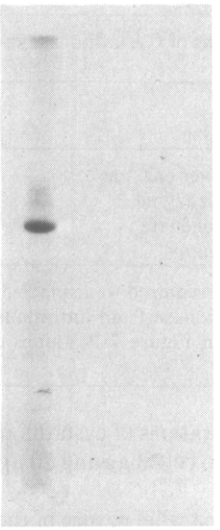

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of mouse liver cystathionase. A 7.5% gel was used. Note that purification is through the first hydroxylapatite step only, and that two DEAE-cellulose columns have been employed here. For further details of the purification, see Bikel et al. (1978). Protein (14.5 μg) was applied to the gel.

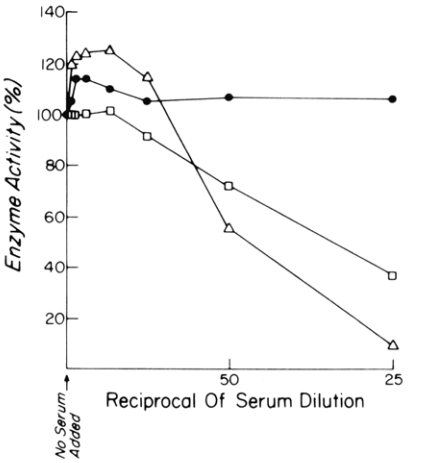

FIGURE 2: Effect of rabbit sera on mouse liver cystathionase activity (purified through the hydroxylapatite step). Details are given in Experimental Procedures. (●) Normal rabbit serum; (□) rabbit no. 1 antiserum 7–21 (40 days post-primary immunization); (△) rabbit no. 2 antiserum 7–21 (40 days post-primary immunization).

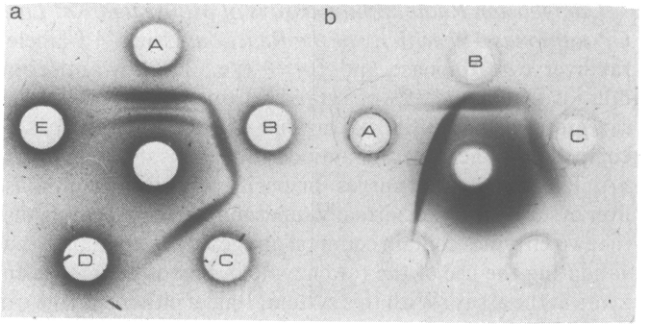

FIGURE 3: (a) Double immunodiffusion analysis of mouse liver cystathionase. Rabbit no. 2 antiserum 7–21 (40 days post-primary immunization) was placed in center well. (A) hydroxylapatite-purified cystathionase (cf. Figure 1); (B and C) Sephadex G-200 purified cystathionase (Bikel et al., 1978); (D and E) pre-immune serum. The antiserum in this experiment was not heat-treated before use. (b) Double immunodiffusion analysis of mouse and rat liver cystathionase. Rabbit no. 2 antiserum 7–21 was placed in the center well. (A) Purified rat liver cystathionase (Uren et al., 1978), 0.6 mg/mL; (B) purified mouse liver cystathionase (Bikel et al., 1978), 0.03 mg/mL; (C) same as A but diluted 1:5.

In Figure 2 are depicted results which demonstrate that our antisera contained specific enzyme-neutralizing antibodies. Employing 0.7 unit of enzyme (approximately 2 μg of enzyme protein), 1/25 dilution of rabbits no. 1 and 2 sera was found to inhibit cystathionase activity by 60% and 90%, respectively (Figure 2). Nonimmune serum, on the other hand, did not affect the catalytic activity of the enzyme under these conditions. Thus, anti-cystathionase antibodies were present in both sera by this criterion.

As can be seen in Figure 3a, three precipitin lines are observed between the antiserum and the preparation used for immunization (well A). However, only a single precipitin line is seen (wells B and C) between the antiserum and mouse liver cystathionase purified to homogeneity through the entire seven-step purification procedure (Biel et al., 1978). No precipitin lines were detected between hydroxylapatite-purified cystathionase (well A) or Sephadex G-200 purified cystathionase (well C) and preimmune serum (wells E and D). Careful inpection of the immunodiffusion plate failed to reveal a reaction of even partial antigenic identity among any of the heterologous species present in well A and the mouse liver enzyme (well B), for no more than one precipitin line of well A could be seen to fuse with the immune precipitate of the purified enzyme (well B). These results demonstrate that, although the antiserum generated to mouse liver cystathionase contained antibodies of multiple specificities, only one immunoprecipitable species is present in our preparation of purified mouse liver cystathionase, previously demonstrated to be homogeneous by biochemical means. Similar results were obtained with antisera from rabbit no. 1.

Demonstration of Antibodies to Cystathionase-Radioimmune Precipitation. Iodination of purified mouse liver cystathionase with $Na^{125}I$ by either the Chloramine-T method (Greenwood et al., 1963) or with the iodogen reagent (cf. Experimental Procedures) resulted in a radioactive protein that, although coeluting with enzymatic activity upon Sephacryl S-200 superfine gel filtration chromatography, reacted with the antiserum to a very limited and variable extent (0–30% precipitations in several iodination experiments). However, the same antiserum precipitated the enzyme in Ouchterlony plates (Figure 3a). The most likely explanations for these seemingly disparate results are that the immunoreactivity of the enzyme was impaired by iodination, or that the difference in extent of precipitability of the unlabeled and labeled enzyme can be accounted for by the differential sensitivity of the two methods used (i.e., Ouchterlony double diffusion in agar gel vs. radioimmune precipitation). Rather than utilize an alternative labeling procedure, we elected to attempt to utilize the rat liver enzyme as tracer. This protein has been purified to homogeneity previously (Uren et al., 1978).

As shown in the double-diffusion test in Figure 3b, the precipitin line formed between the purified rat enzyme (well A) and anti-mouse cystathionase antibodies (center well) completely fused with the precipitin line formed between the mouse enzyme (well B) and the (homologous) antibodies, whereas the latter precipitin showed slight spurring. This indicates that, in addition to antigenic determinants common to the two enzymes, the antisera also recognized one or more antigenic determinants which are present in mouse cystathionase but not in rat cystathionase.

When examined for its ability to react with antiserum, greater than 90% of the labeled enzyme was precipitated at a

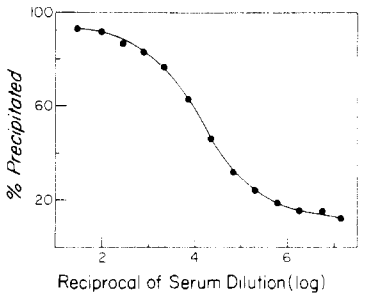

FIGURE 4: Titration of rabbit no. 2 antiserum 7-21 by precipitation of ^{125}I-labeled, purified rat liver cystathionase, in the double-antibody radioimmunoassay. Details are given in Experimental Procedures.

TABLE I: Concentrations of Cystathionase in Mouse and in Rat Livers.

preparation	immunoreactivity (ng of protein)[a]
purified mouse liver enzyme	3.55[b]
purified rat liver enzyme	2.82[b]
mouse liver homogenate	825[c]
rat liver homogenate	141[c]

[a] Amount of protein required to displace 50% of ^{125}I-labeled purified rat liver cystathionase from anti-mouse liver cystathionase. [b] Values obtained from Figure 7. [c] Values obtained from Figure 10.

dilution of $10^{-1.5}$ of rabbit no. 2 antiserum 7-21 (Figure 4); approximately 10% of the labeled protein was precipitated at a dilution of $10^{-5.8}$. These results demonstrate the preservation of immunoreactivity of rat liver cystathionase following ^{125}I-labeling with the iodogen reagent and suggest that this molecule could be tried as probe in a radioimmunoassay for the mouse enzyme. Rabbit no. 2 antiserum 7-21 was used in all subsequent experiments.

Competition Radioimmunoassays of Mouse and Rat Liver Cystathionase-Specificity of the Reaction. Using ^{125}I-labeled rat liver cystathionase and $10^{-3.3}$ (i.e., 1:2000) antiserum dilution that bound 75% of a standard amount of the iodinated rat protein, the ability of various enzyme preparations to compete with the tracer for antibody binding is shown in Figure 5A. The competition curves for purified rat liver and mouse liver cystathionase are virtually superimposable, indicating that the two enzymes contain common antigenic determinants, thus validating the use of the rat enzyme in lieu of the mouse enzyme in the assay. With this system, 1 ng of either enzyme can be readily detected.

Also shown in Figure 5A is a competition curve for mouse liver cystathionase purified through the 33-63% ethanol precipitation step (Bikel et al., 1978). The slope of the displacement curve appears similar, if not identical, to those of the purified mouse and rat enzyme curves.

A comparison of the immunoreactivities of soluble rat, mouse and human liver homogenates is shown in Figure 5B. As expected, the concentration-dependent slopes of the rat and mouse liver radioimmunoassay competition curves are virtually indistinguishable. The amount of protein required to displace 50% of the ^{125}I-labeled probe is approximately 5.6-fold greater for the mouse liver enzyme as compared with the rat liver enzyme indicating that, on the basis of liver protein mass, the latter liver possesses 5.6 times more enzyme than the former. A comparison of the competition displacement curves of liver homogenates and purified enzymes (Table I) allowed us to calculate the concentrations of cystathionase protein in mouse liver and in rat liver to be 4.3 μg and 20 μg/mg of liver protein, respectively.

In contrast to the parallel nature of the mouse and rat liver competition curves, human liver cystathionase reacted in a distinctly nonparallel fashion and to an extent of approximately 40% (Figure 5B). Thus, human liver cystathionase is only partially related to the mouse and rat liver proteins as defined by these immunological criteria.

In order to further ascertain the specificity of the assay for mouse liver cystathionase we wished to compare the specific enzymatic and immunoreactivities of a single preparation. The ethanol preparation of mouse liver cystathionase (1.4 mL; 7.3 mg of protein) was therefore applied to a DEAE-cellulose column (1.5 × 5 cm) equilibrated and eluted in buffer B (Bikel et al., 1978). Individual fractions (1 mL) were collected and assayed for both enzyme activity and protein content. In Figure 6 (insert) are shown the enzyme activity and protein profiles obtained. The radioimmunoassay competition curves for fractions 8 and 11 are also shown in Figure 6, and the comparison of enzyme specific activity and immunoreactivity for these two fractions is summarized in Table II. The two column fractions yield radioimmunoassay competition curves of identical slopes; there was excellent agreement between specific enzymatic and immunologic activities: 20-fold more protein in fraction 11 was required to displace the same amount of antibody-bound radioactivity as was required of fraction 8, which, in turn, contained 20-fold more enzyme than fraction 11. These results further demonstrate the validity of using the rat enzyme and anti-mouse cystathionase antibodies for a specific radioimmunoassay of the mouse enzyme. Furthermore, as expected, it would appear that this assay could be used to monitor the purification of the mouse liver enzyme.

Heat inactivation experiments, designed to further test the specificity of the radioimmunoassay, were performed with purified mouse and rat cystathionase preparations. Various

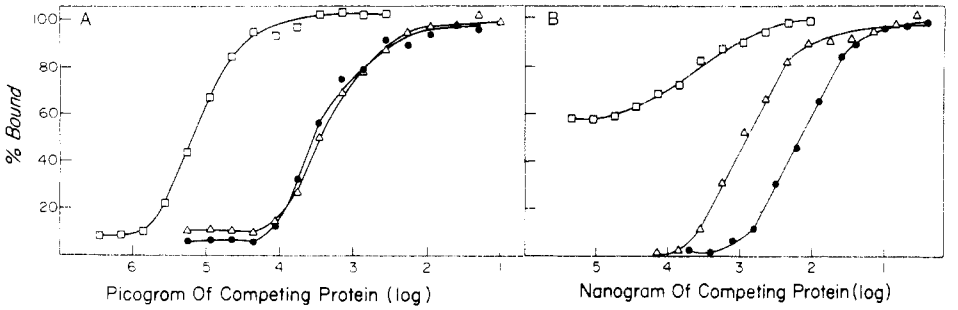

FIGURE 5: Competition of various preparations with ^{125}I-labeled rat liver cystathionase for rabbit no. 2 antiserum 7-21, diluted 1:2000. Details of double-antibody radioimmunoassay are given in Experimental Procedures. (A) Competition of purified mouse liver cystathionase (●), purified rat liver cystathionase (△), and mouse liver cystathionase purified through the 33-63% ethanol precipitation step (Bikel et al., 1978) (□). (B) Competition of supernatant fluids from homogenates of rat liver (●), B6D2F1 mouse liver (△), and human liver (□).

TABLE II: Enzyme Activity and Immunoreactivity of Mouse Liver Cystathionase in Fractions 8 and 11 from DEAE-Cellulose Column.[a]

fraction	enz act. (units/mL)	protein (mg)	sp act. (units/mg)	fraction 8/ fraction 11	immunoreact. (ng of protein)[b]	fraction 11/ fraction 8
8	3.66	0.80	4.57	19.87	63	20
11	0.20	0.87	0.23		1260	

[a] Figure 8, insert. [b] Amount of protein required to displace 50% of ^{125}I-labeled purified rat liver cystathionase from anti-mouse liver cystathionase.

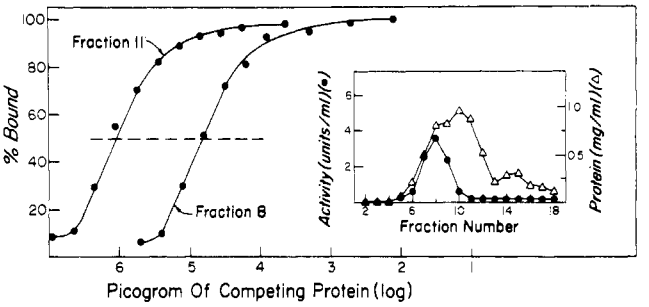

FIGURE 6: Double-antibody competition radioimmunoassays of fractions 8 and 11 from DEAE-cellulose (DE-52) column chromatography (insert). Details of DEAE-cellulose chromatography are given in the text. Enzyme activity and protein content were measured as described in Experimental Procedures.

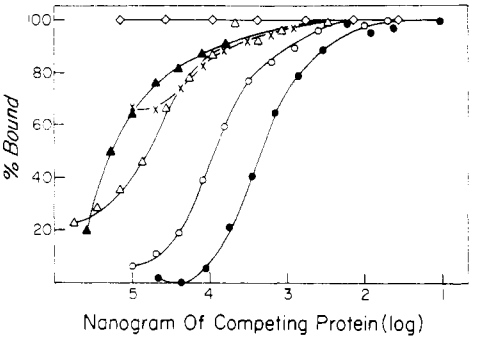

FIGURE 7: Double-antibody competition radioimmunoassays of supernatant fluids from homogenates of B6D2F1 mouse livers (●), kidneys (○), spleens (△), thymuses (▲), and hearts (X). (◇) Purified AKR virus.

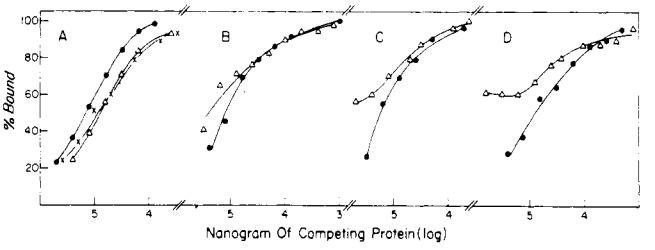

FIGURE 8: Double-antibody competition radioimmunoassays of supernatant fluids from homogenates of AKR mouse thymuses and thymic tumors. (A) Two-month-old AKR thymus (●); 4-month-old AKR thymus (△); 5-month-old AKR thymus (X); (B) 7-month-old AKR thymus (●); 7-month-old AKR spontaneous thymoma (△); (C) 9-month-old AKR thymus (●); 9-month-old AKR spontaneous thymoma (△); (D) 10-month-old AKR thymus (●); 10-month-old AKR spontaneous thymoma (△).

dilutions of these proteins were made, and aliquots of them were placed in a 63 °C water bath for a period of 2.5 h, while duplicates were held at 4 °C for the same period of time. The tubes held at 63 °C were then chilled, and antiserum ($10^{-3.3}$ dilution) was added to all. Incubation at 37 °C for 15 min ensured, followed by the addition of the ^{125}I-labeled tracer, and the standard assay performed exactly as described in Experimental Procedures. Competition displacement curves with slopes identical with those shown in Figure 5A were obtained for the two enzymes held at 4 °C, whereas curves with reduced slopes were obtained for the enzymes held at 63 °C. A linear least-square regression analysis of the data points (all points taken from the linear portion of the competition displacement curves) demonstrated similar slopes for the two enzymes in each category: The enzymes held at 4 °C had slopes of 64 for the rat enzyme (r = 0.96) and 57 for the mouse enzyme (r = 0.98); the enzymes held at 63 °C had slopes of 19 for the rat enzyme (r = 0.99) and 22 for the mouse enzyme (r = 0.95). [r values represent the correlation of the fit which, in turn, are the square roots of the calculated coefficients of determination (r^2)]. These results indicate that the two enzymes are not grossly dissimilar, antigenically, at least by this assay with this serum.

Tissue Distribution of Cystathionase. In Figure 7 are shown the results of competition radioimmunoassays for five mouse organs, as well as for purified endogenous ecotropic AKR type C virus [a representative of a family of endogenous murine viruses whose proteins are often found in a variety of AKR normal and leukemic tissues (Rowe & Pincus, 1972)]. Liver, thymus, and kidney cystathionase competed with high efficiency, whereas the spleen enzyme competed less efficiently, and the heart enzyme did not compete more than 35% in the immunoassay. AKR virus is, as expected, completely nonreactive in the assay. Based on the competition displacement curves, the ratio 4:1 is obtained here for cystathionase concentration in mouse liver and kidney. Interestingly, by enzyme activity measurements in rats, Mudd et al. (1965) obtained a ratio of 2.7:1 for these organs. Thus, the ratios of immunoreactivity (of the mouse enzyme) and catalytic activity (of the rat enzyme) are in good agreement with each other.

Immunoreactivity of Cystathionase in Thymuses of Normal and Leukemic AKR Mice. AKR mice are characterized by an incidence of lymphoma of approximately 90% by 12 months of age. These mice show gross pathological evidence of neoplastic transformation, occurring first in the thymus (Gross, 1970; Kaplan, 1974), and beginning at 7–8 months of age. Most of these animals subsequently die of the disease at 9–10 months of age (compared with a mean life span of 22 months for C57Bl/6 mice, a low virus and low leukemia incidence strain).

We wished to determine whether the reduced levels of cystathionase activity in spontaneous thymic lymphomas of AKR mice (Livingston et al., 1976) are due to lower levels of enzyme protein or could possibly be due to the presence of structurally altered enzyme or both. In Figure 8 are shown the results of competition radioimmunoassays for cystathionase in thymuses from healthy and from leukemic AKR mice (thymuses from the latter mice were grossly enlarged). The competition displacement curves generated by the thymoma extracts show distinct nonparallelism with the curves obtained with age-matched normal thymus extract, indicating that, with age, the

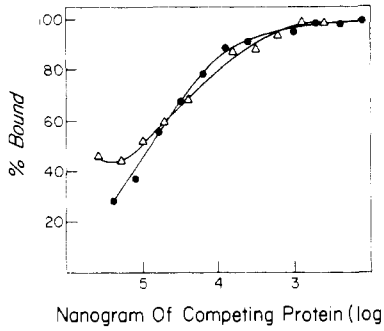

FIGURE 9: Double-antibody competition radioimmunoassays of supernatant fluids from a homogenate of 9-week-old Balb/c mouse thymus (●) and 9-week-old Moloney type C virus induced Balb/C mouse thymoma (△).

tumored thymus enzyme becomes more dissimilar immunologically from the standard thymic and liver proteins which, in turn, appear to be very similar (if not identical). Results similar to those depicted in Figure 8 were obtained for normal and leukemic AKR/J mice maintained as a separate stock for several years in this institute by sibling matings. In order to exclude the possibility that the changes in the radioimmunoassay competition displacement curves of cystathionase from the thymomas are due to a protease or to some other soluble catalytic modifying element produced in these tumors, a mixing experiment was performed. In this experiment, competition radioimmunoassays for cystathionase in supernatant fluids from a homogenate of a 10-month-old AKR mouse thymus, 10-month-old AKR mouse spontaneous thymoma, and a 1:1 mixture of the two extracts were carried out. The results demonstrated that the slope of the competition displacement curve of a mixture of normal and tumored thymus extracts has been determined equally by either extract (i.e., it falls on the predicted line for an equal mixture)— the tumor extract does not appear to affect the immunoreactivity of the enzyme in the normal thymus extract and vice versa.

Immunoreactivity of Cystathionase in Thymuses of Normal and Moloney Leukemia Virus-Infected Balb/c Mice. In contrast to the latent period of 7–8 months required for the development of AKR mouse spontaneous leukemia, a much shorter (approximately 2.5 months) latent period is required for the development of lymphatic leukemia in mice injected at birth with type C virus. In Figure 9 are shown the results of competition radioimmunoassays for cystathionase in the thymuses of two 9-week-old Balb/c mice—one healthy and one leukemic—the leukemic one having been injected intraperitoneally at less than 1 day of life with the Moloney strain of murine leukemia virus (thymus, spleen, and lymph nodes of the latter mouse were grossly enlarged). It appears that, compared with cystathionase in the normal thymus extract, the enzyme in the tumored thymus competes less efficiently in the immunoassay, similar to what was observed in AKR mouse spontaneous thymic lymphoma (Figure 8).

Discussion

The initial goal of the present work was to develop a radioimmunoassay for cystathionase, for such an assay would permit the detection of enzyme protein in the absence of catalytic activity as well as increase the sensitivity of detection of the enzyme (i.e., a major limitation of the best available catalytic assays for cystathionase is the inability to detect the enzyme in a cell-free homogenate of a single mouse thymus). Our rationale for attempting to establish such an assay was that an appropriately sensitive and specific assay could contribute to an understanding of the molecular basis for the cysteine auxotropy/cystathionase deficient phenotype of a variety of mouse hematopoietic tumor cells.

In the present study, the development and some of the biological applications of a competition radioimmunoassay for mouse liver cystathionase are described. Regarding specificity, it was essential to prove that the radiolabeled probe was representative of the enzyme in question. The Ouchterlony tests demonstrated that homogeneous rat liver cystathionase was fully cross-reactive with the mouse enzyme (Figure 3b) which, in turn, was antigenically homogeneous (Figures 3a and 3b). It is recognized that the formation of precipitin lines in double gel immunodiffusion is not an absolute criterion for immunologic homogeneity, for such an analysis only indicates the presence of precipitating antigen–antibody complexes and is, therefore, an estimate of the minimum numbers of components. Importantly, however, mouse liver cystathionase purified to homogeneity was used in the analysis. Virtually identical competition curves for rat and mouse liver enzyme were obtained with ^{125}I-labeled rat liver cystathionase as tracer (Figure 5), a finding which indicates that both enzymes are antigenically identical with this serum under these conditions. The quantitative studies shown in Figure 6 and in Table II support the contention that the radioimmunoassay is largely if not wholly specific for cystathionase. It is unlikely that there is significant contamination of our labeled tracer with other radioactive protein, because the assays for mouse cystathionase by catalytic activity and by immunoreactivity yielded fully parallel results. Finally, the parallel rat/mouse liver enzyme heat inactivation results also argue against the presence of a contaminating protein in the purified rat liver enzyme preparation, for such a cross-reactive, contaminating protein would also have to be present in the purified mouse liver enzyme preparation and would in addition have to exhibit a heat inactivation rate similar to that of the rat liver enzyme preparation. The assay has permitted quantitative measurements of enzyme concentration in various mouse organs, with liver appearing as the richest source tested thus far. This is in keeping with earlier results obtained by catalytic acitvity measurements in rats (Mudd et al., 1965).

At the risk of obtaining less than maximal sensitivity, we have used in our competition radioimmunoassays an antiserum dilution which bound 75% of the racer. When comparing competition displacement curves for the purified mouse and rat liver enzymes, a lower antiserum dilution would afford a better chance of detecting antibodies with higher affinities (steeper slopes) for antigenic determinants in one or the other of the two proteins. No antibody populations with differing affinities or titers for either protein were detected, however (Figure 5A).

The ability to detect 1 ng of enzyme protein under these conditions afforded a sensitivity in the order of 10^3-fold greater than that obtained with our enzymatic assay. This level of sensitivity allows detection of the enzyme in single mouse organs (e.g., thymus, spleen) where it could not be detected by enzymatic assays. The ability to assay for the protein in single cells is essential to an investigation of the biochemical mechanism leading to cysteine auxotrophy in mammalian leukemic cells.

An immunologic analysis of cystathionase in tumored vs. normal appearing murine thymus from animals with virus-associated "thymic" leukemia has revealed a striking difference in the immunoreactivity of the protein in these two tissue types. In a preliminary experiment analogous reduction in cystathionase immunoreactivity has been observed in an extract of exogenous, Moloney virus induced Balb/c mouse thymic tumor

(Figure 9). This implies a potential degree of generality to this phenomenon at least so far as type C virus associated disease is concerned. Of interest is the observation that with advancing age the antigenic reactivity of normal thymus enzyme does not change significantly. It remains isoimmune with the adult liver protein. This is the case in spite of the progressive age-related atrophy which is known to affect this particular organ. In contrast, cystathionase-related antigens in the extracts of the AKR tumored organs react with significantly lower affinity, and the suggestion from the available data is that the older the AKR animal and the more infiltrated the tumor, the less reactive is the available antigen. The result of the mixing experiment of tumored and normal AKR mouse thymuses strongly suggest that tumored thymus extract cannot convert normal thymic cystathionase into a less immunoreactive molecule. Similarly, the results indicate that normal thymic extract cannot modify immunoreactive protein(s) in tumored thymus so that they now react "normally."

The available data are compatible with either of two explanations for the thymic cystathionase activity depletion phenomenon with tumor development: (a) the presence of a catalytically inactive, weakly cross-reacting (modified ?) protein product of the cystathionase gene; or (b) progressive reduction in normal cystathionase synthesis with the residual nonspecific immunoreactivity due to background levels of one or more residual, cross-reactive antigen(s) which have no cystathionase activity and are not products of the cystathionase gene. These alternative possibilities are testable and are presently under investigation. Nevertheless, the present body of evidence strongly suggests that the concentration of antigenically "normal" (this work) and catalytically active (Iglehart et al., 1977) cystathionase protein in thymus-derived tumor cells is markedly reduced compared with that in normal, age-matched thymocytes.

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