

Inactive Enzyme Molecules in Aging Mice: Liver Aldolase

(senescence/fructose-1,6-diphosphate aldolase)

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ABSTRACT Evidence is presented that there is a considerable accumulation of inactive fructose-1,6-diphosphate aldolase (EC 4.1.2.7) in the liver of senescent mice. Liver aldolase was purified from 3-month-old mice and used to immunize rabbits. It was demonstrated with the monospecific antibody thus produced that the liver aldolase of young adult (3 month) and aged (31 month) mice are antigenically identical. With the antibody, inactive enzyme molecules (crossreacting material) in liver homogenate of old mice were detected. The liver aldolase of senescent mice had half as much active enzyme per mg of protein, as well as per antigenic unit, as did the liver aldolase of young adult mice. The accumulation of faulty enzyme molecules may be one of the causes of debilitation leading to senescence and death.

A possible cause of senescence and death may be the production and accumulation with age of altered, partially active, or catalytically inactive enzyme molecules. The increase of such enzyme molecules with age may place an ever increasing stress on an organism. The presence of faulty molecules among the enzymes of the protein-synthesizing machinery may result in a situation in which an organism continues to produce damaged proteins at an exponential rate and eventually has an "error catastrophe" (1), at which point it can no longer function under the burden of damaged enzymes. The result of such a catastrophe is death.

It has been our interest to test the validity of the above hypothesis. Initial studies in our laboratory have shown that in the nematode, *Turbatrix aceti*, the specific activity of the enzyme isocitrate lyase (EC 4.1.3.1) decreases with age and that concomitantly there is an increase in totally inactive enzyme molecules detected as antigenically crossreacting material (CRM) (2). Subsequently, Lewis and Holliday (3) described similar results for the enzyme glutamate dehydrogenase (EC 1.4.1.2) in aging hyphae of *Neurospora crassa*. In both the *Turbatrix* and *Neurospora* systems the antibody used was not prepared against a purified enzyme, thus leaving some doubts as to the complete identity of the enzyme in homogenates of old and young animals. It is, therefore, necessary to perform this kind of study with a completely purified enzyme and antibody system that can be accurately characterized.

In the present study, we examined age-related changes in enzyme structure and function in a mammalian system in order to verify our previous findings and to determine the universality of the phenomenon of accumulation of CRM with age. We have chosen to work with the fructose-1,6-diphosphate aldolase of mouse liver (EC 4.1.2.7). In order to

take advantage of the information one can obtain from sensitive and specific immunological techniques, monospecific antibody to purified liver aldolase of young adult mice was prepared and used throughout these studies.

MATERIALS AND METHODS

Animals. All experiments were performed with virgin female mice, of the low tumor strain C57Bl/6, obtained from the colony maintained at the Weizmann Institute of Science, Rehovoth, Israel. Mice of two age groups were used in the experiments reported here. Young adult mice were 2-3 months old and senescent mice were 31 months old when they were killed. Rabbits used in the production of antisera were purchased from a local breeder.

Crude Aldolase Preparation. The aldolase activity, both enzymatic and antigenic, of individual animals was assayed in a crude homogenate prepared by homogenization of single livers in 12 ml of cold 10 mM Tris·Cl buffer (pH 7.5) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.25 M mannitol. Samples were homogenized in the cold (4°) in a Sorvall Omnimixer for 1 min at maximum speed. The homogenate was centrifuged for 3 hr at 24,000 × g, and both the pellet and floating lipid were discarded. Homogenates were stored frozen at -20°, and aliquots were thawed as needed. Thawed enzyme was maintained at 0-4° at all times. All dilutions of liver homogenate were made in cold 50 mM Tris·Cl buffer (pH 7.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol (dilution buffer).

Mouse-Liver Aldolase Was Purified by the method of Gracy *et al.* (4). 49 C57Bl/6 female mice 3 months of age were killed by cervical dislocation, and their livers were removed into cold homogenization buffer. The livers were washed to remove all excess blood and homogenized for 60 sec in a Waring Blendor (34 g of liver in 140 ml). Details of the purification are presented in Table 1 and Fig. 1. The results are very similar to those obtained with rat and rabbit-liver aldolase (4, 5). The enzyme was shown to be pure by the following criteria. Rechromatography on a phosphocellulose column with substrate elution did not alter the specific activity of the purified enzyme, which eluted in a single sharp peak. Electrophoresis on a cellulose acetate membrane showed a single band of protein that corresponded to the single band of enzyme activity (Fig. 2). Sedimentation equilibrium ultracentrifugation by the method of Gracy *et al.* (4) showed a single symmetrical protein band (Fig. 3) with $s_{20}^0 = 7.2$.

Abbreviation: CRM, crossreacting material.

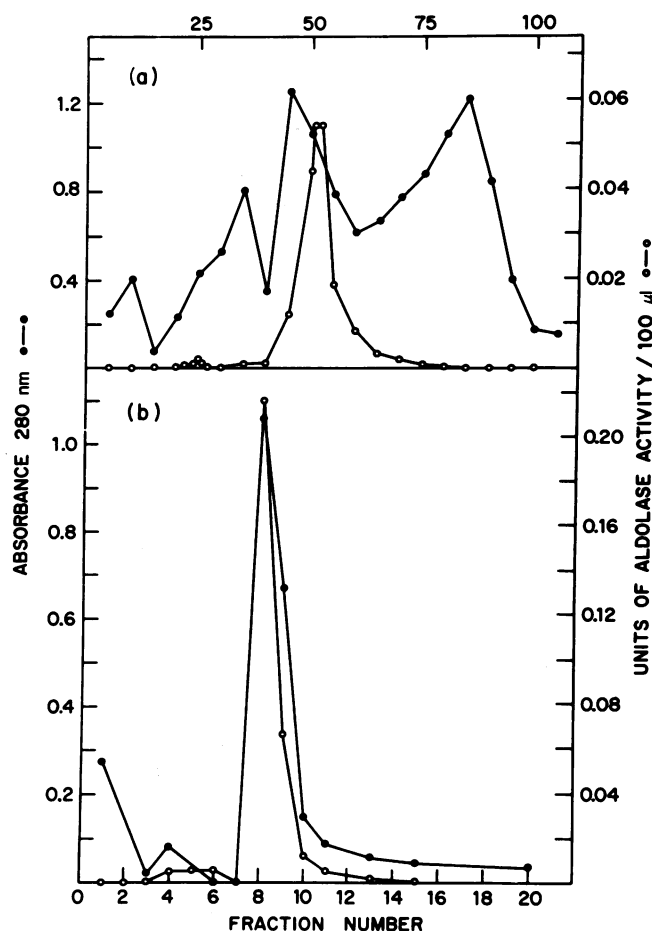


FIG. 1. Purification of mouse-liver aldolase of 3-month-old mice by the procedure of Gracy *et al.* (4). (a) Salt gradient elution of mouse-liver aldolase from phosphocellulose column. 9-ml Fractions were collected from a 53×3 cm column. Fractions 40–75 were pooled, concentrated by ammonium sulfate precipitation, and used for further purification of the enzyme. (b) Substrate (fructose-1,6-diphosphate) elution of mouse-liver aldolase from a phosphocellulose column. The concentrated eluate of the column described in Fig. 1a was placed on a 53×3 cm column. 11-ml Fractions were collected. Fractions 8–12 were pooled and concentrated by ammonium sulfate precipitation. This concentrate was used as purified enzyme.

Production of Antiserum to Purified Mouse-Liver Aldolase.

Three rabbits were bled for preimmunization normal serum. Each rabbit then received multiple injections in the foot pads and along the flanks of 500 μ g of purified mouse-liver aldolase from 3-month-old mice in complete Freund's adjuvant. 2 Weeks later, a boost of 500 μ g of pure enzyme in complete Freund's adjuvant was given along the flanks and the rump. A similar boost of 1 mg of pure enzyme in complete Freund's adjuvant was given 1 month later, and serum was then collected at weekly intervals for 3 weeks. The serum of each individual rabbit was pooled. In the experiments reported here the serum pool of one single animal was used throughout. The antiserum thus obtained was monospecific for mouse-liver aldolase (Fig. 4).

Aldolase Activity was Assayed as described by Gracy *et al.* (4), with the exception that 15 μ g of glycerolphosphate dehydrogenase-triosephosphate isomerase mixture (Boeh-

TABLE 1. Purification of liver aldolase from 3-month-old C57Bl/6 female mice

	Total protein (mg)	Total activity (units)	Specific activity (units/mg per min)	Purification (fold)	% Recovery
Homogenate	1500	75	0.05	—	—
Salt gradient Eluate of first phospho- cellulose column	478	58.5	0.123	2.5	78
Fructose- diphosphate eluate of sec- ond phospho- cellulose column	27.5	47.2	1.72	34	63

ringer Mannheim Corp.) was substituted for 10 μ g of each individual enzyme. Aldolase activity was measured by following the rate of oxidation of DPNH at 340 nm on a Unicam recording spectrophotometer at 25°. One unit of aldolase activity is defined as the amount of enzyme catalyzing the cleavage of 1 μ mol of fructose-1,6-diphosphate per min at 25°.

Protein Was Determined by the method of Lowry *et al.* (6).

RESULTS

7 Young adult mice, 2–2.5 months old, and 5 senescent mice, 31 months old, were compared. The 50% survival point of the mouse population from which the mice were obtained was 24 months. At 31 months of age, 10–20% of the mice were still alive. The specific activity of liver aldolase in old mice is significantly lower than that of young mice (Table 2). The active enzyme at these two ages does not appear to differ in its stability at 4° nor in its enzymatic activity as judged by K_m for fructose diphosphate and fructose-1-phosphate. Electrophoresis of whole liver homogenates from young and old mice on cellulose acetate membranes and subsequent staining for enzyme activity demonstrated identity between the electrophoretic mobility of the active enzyme present in the old and young mice.

The differences in specific activity of liver aldolase in homogenates of old and young mice is due to either an absolute difference in the amount of enzyme present or the presence of altered enzyme molecules that are inactive or partially active. In order to determine which of these possibilities is valid, we used antibody specific for mouse-liver aldolase to search for the presence of CRM. The monospecificity of the antibody used has been demonstrated by double diffusion in gel (Fig. 4). This antibody recognizes purified mouse-liver aldolase from young adult mice, the aldolase in liver homogenate of young adult mice, and the aldolase in liver homogenate of senescent mice all as a single antigen. The addition of antibody to liver homogenate caused an immediate drop in aldolase activity, as measured 5 min after mixing. This instantaneous inactivation was dependent upon the amount of antiserum added and did not increase during a 1-hr time interval at 4°. Subsequent incubation

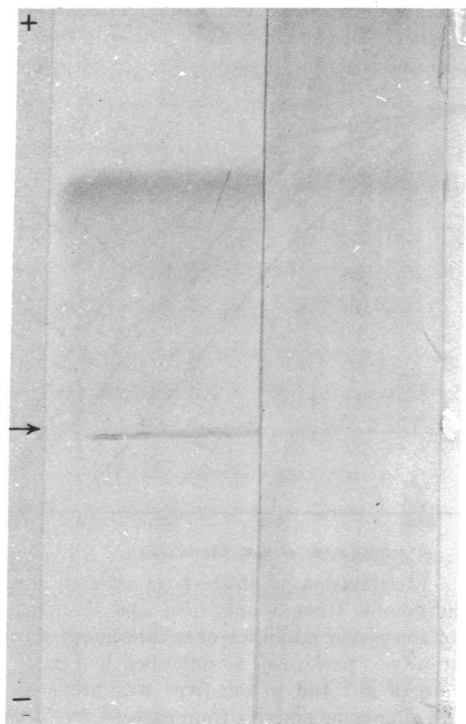


FIG. 2. Cellulose acetate electrophoresis of purified mouse-liver aldolase. Electrophoresis and staining for enzyme activity were performed according to Penhoet *et al.* (7). Protein was stained with 1% amidoblack in 7% acetic acid. 10 μ g of enzyme were used for the electrophoresis. After electrophoresis, the cellulose acetate strip was cut longitudinally. *Left*, protein staining; *right*, staining for enzyme activity. *Arrow* indicates origin.

at 4° overnight led to specific immunoprecipitation of aldolase. This precipitation was dependent upon the amount of antibody added. Equal amounts of antibody precipitated

TABLE 2. Comparison of body and liver weight of old and young mice and some properties of their liver aldolase*

	2- to 3-month-old mice	31-month-old mice
Body weight(g)	20.4(19-21.5)	30.1(27.7-32.1)
Liver weight (g)	1.14(1.00-1.40)	1.94(1.72-2.09)
Aldolase specific activity (units/mg of protein)	0.058(0.045-0.071)	0.029(0.025-0.038)
Stability of aldolase in homogenate†	94.3(85-98)	94.5(89-102)
K_m (fructose-diphosphate) ($\times 10^{-6}$)‡	4.9(4.2-5.5)	5.3(4.2-5.9)
K_m (fructose-1-phosphate) ($\times 10^{-4}$)§	5.0(2.5-7.7)	4.0(2.9-5.7)

* Results are given as averages, with range in parentheses.

† Percent of original activity remaining after incubation at 4° for 24 hr.

‡ K_m (fructose diphosphate) was determined between 0.003 and 0.1 mM substrate concentration.

§ K_m (fructose-1-phosphate) was determined between 0.03 and 1 mM substrate concentration.



FIG. 3. Sedimentation velocity ultracentrifugation of purified mouse-liver aldolase. The enzyme was centrifuged in 2.5° double-sector cells in an An-D rotor at 60,000 rpm at 20° in 10 mM Tris·HCl-1 mM EDTA-10 mM 2-mercaptoethanol-0.1 M NaCl (pH 7.5), at a protein concentration of 0.8 mg/ml (4). The photoelectric scanner recording is the derivative of the absorbance at 280 nm.

much less active enzyme from the homogenate of liver of senescent mice than from the homogenate of liver of young adult mice when the initial enzyme activity of both preparations was identical. It required from 1.7-2 times as much antibody to precipitate an equal amount of enzyme activity from homogenates of livers of old mice as from homogenates of livers of young mice (Fig. 5). The difference in instantaneous inactivation of the enzyme from the two age groups that appears in part of the curve (0.1 and 0.13 ml of antibody) may be of significance, although this result can be questioned. This difference may suggest that within the CRM some, if not all, of the molecules are of such a conformation as to maintain the antigenic nature of the region of the active site of the enzyme.

In order to eliminate the possibility that there may be some nonspecific inhibitor of precipitation in homogenate of old liver that might mimic the effect of CRM, we mixed homoge-

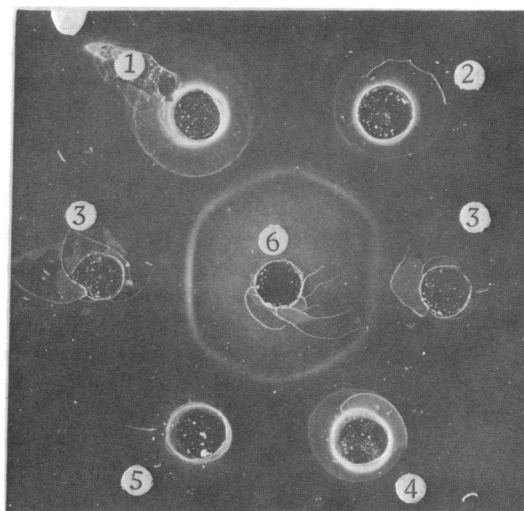


FIG. 4. Determination of monospecificity of anti-mouse-liver aldolase serum by double diffusion in agar gel. Immunological identity of pure mouse-liver aldolase with aldolase in whole homogenate from young adult and senescent mice is demonstrated. 1 and 5, liver homogenates of two young adult mice (2.5 months old); 2 and 4, liver homogenates of 2 senescent, 31-month-old mice; 3, pure mouse-liver aldolase; 6, anti-mouse-liver aldolase. The double diffusion in gel on microscope slides was performed as described by Campbell *et al.* (8).

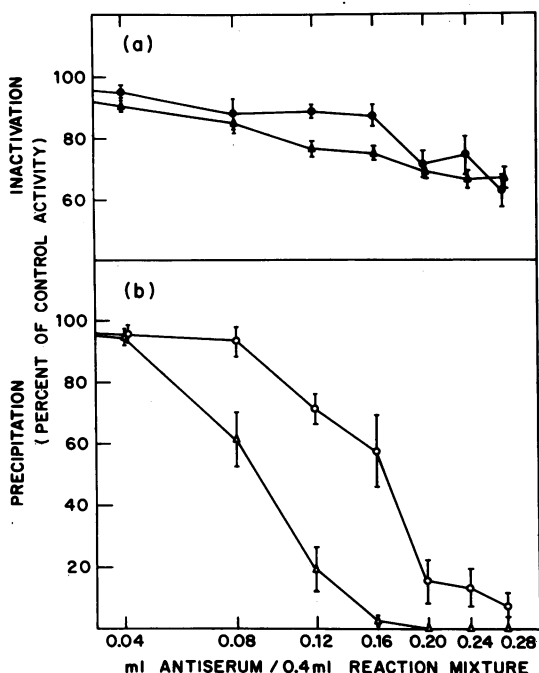


FIG. 5. Inactivation and precipitation by antibody of aldolase in liver homogenates of 2-month-old and 31-month-old mice. To liver homogenate containing 0.03 units of aldolase activity were added either antiserum or normal rabbit serum in the volumes indicated; the mixture was then brought to a final volume of 0.4 ml with dilution buffer. 0.1 ml of this mixture was assayed within 5 min of mixing to determine instantaneous inactivation (a). The remaining 0.3 ml was incubated at 4° overnight and centrifuged at $3000 \times g$ for 30 min. The precipitate was discarded, and 0.1 ml of the supernatant fluid was assayed for residual enzyme activity (b). Serum alone showed low aldolase activity, and this was subtracted from the readings. Normal rabbit serum and buffer controls behaved identically, and the results of these two were pooled to obtain control values. Δ , Inactivation: young mice; \bullet , inactivation: old mice; Δ , precipitation: young mice; \circ , precipitation: old mice. Vertical bars indicate standard error of the mean.

nates of old and young liver containing equal enzyme activity. The precipitin reaction was performed on the mixture. Fig. 6 demonstrates that the presence of old liver homogenate did not reduce the ability of antibody to precipitate aldolase, nor did the presence of young liver homogenate enhance the precipitation over what might be predicted for the mixture. The predicted degree of precipitation was based on the results of previous experiments depicted in Fig. 5, in which it was found that the enzyme of old animals contains twice as much antigen per unit of enzyme activity as does the enzyme of young animals.

DISCUSSION

In a previous paper (2), we reported the detection of increased amounts of inactive molecules of the enzyme isocitrate lyase in populations of aged nematodes. In this report we have confirmed this phenomenon in a purified mammalian system. We have also found the same increase in CRM for other enzymes of nematode and mammalian systems (Zeelon, Gershon, and Gershon; Gershon and Gershon, in press). These observations, along with those in *Neurospora* (3),

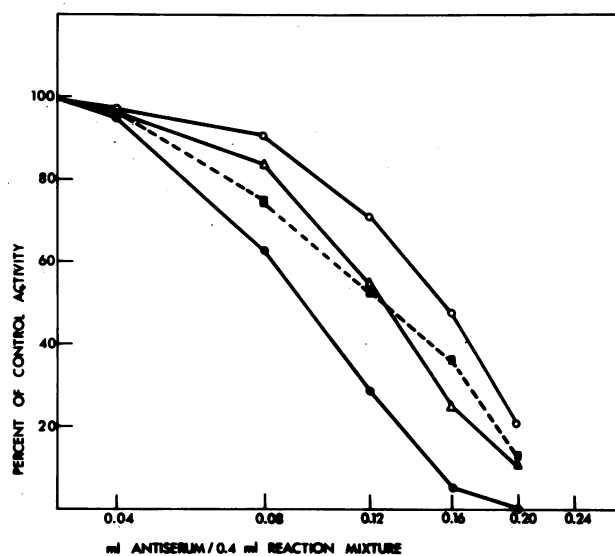


FIG. 6. Precipitation by antibody of aldolase in a mixture of liver homogenates from 2-month-old and 31-month-old mice: the lack of nonspecific inhibition or enhancement of precipitation. Precipitation was performed as described in Fig. 5. The mixed homogenate of old and young liver was prepared by mixing 0.015 units of enzyme activity from each to give a final activity of 0.03 units. \bullet — \bullet , Young homogenate; \circ — \circ , old homogenate; Δ — Δ , mixture of young and old homogenates; \blacksquare — \blacksquare , theoretical degree of precipitation based on the assumption that the old homogenate contains twice as much antigen per unit of enzyme activity as does the young homogenate.

indicate that the production of considerable amounts of CRM of enzymes is a wide-spread phenomenon in aging organisms.

The liver aldolase of senescent mice shows half the specific activity of that of young adult mice with a concomitant increase in CRM, detected with monospecific antibody prepared against pure mouse-liver aldolase from young adult mice. It is important to note that this antibody recognized the liver aldolase of young adult and senescent mice as being identical antigenically, as demonstrated by double diffusion in agar gel.

The drop in specific activity of aldolase in the livers of old mice does not appear to be an artifact of the *in vitro* preparative techniques, inasmuch as both the aldolase of young and old mice show identical high *in vitro* stability. This identical stability, as well as identical electrophoretic mobility of the active enzyme on cellulose acetate membranes and identical K_m for fructosediphosphate and fructose-1-phosphate, strongly suggest, but do not prove, that the active enzyme found in the liver homogenates of 31-month-old mice is identical with the active enzyme present in the liver of 3-month-old mice. This enzyme does not appear to be partially active, but rather completely intact, thus leaving the CRM detected in homogenates of old liver to be considered as inactive enzyme.

The mechanism of alteration of enzyme molecules leading to their inactivation and detection as CRM is not known. This modification may be either a chance event or it may be a programmed event related to age as a continuation of the differentiation process.

One can conceive of random errors in protein synthesis leading to alterations in the primary structure as a cause of

enzyme inactivation. This hypothesis has been suggested to be a possible mechanism by the use of base analogs (3, 9). The possibility that such a mechanism does actually operate in cells should be directly determined by chemical analysis of CRM. Modification of proteins after synthesis, by either programmed or chance mechanisms, can also be considered. Our results so far tend to indicate a programmed and complete inactivation of affected enzyme molecules. Modification of proteins after synthesis has been directly shown for lens α -crystallin (10) and for glucose-6-phosphate dehydrogenase of erythrocytes (11). This latter enzyme is inactivated to a large extent by undetermined processes long after protein synthesis has ceased in erythrocytes.

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