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EFFECT OF AGING ON ENOLASE FROM RAT MUSCLE, LIVER AND HEART

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Summary

Muscle enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) and liver enolase from young and old rats have been purified to homogeneity and several properties of the respective young/old pairs, including K_m, behavior on polyacrylamide gels, sensitivity to heat and specific activity have been compared. No difference has been detected. On the other hand, the heart isozyme shows an age-related increase in a heat-sensitive form of the enzyme. The results strongly support the idea that some enzymes become altered in aging animals but others do not.

Several enzymes have been shown to become altered in aged animals [1—7]. Available evidence strongly supports the idea that post-translational rather than sequence changes are responsible for the altered properties of the enzymes [8,9]. On the other hand, aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) from rat liver [10,11], creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) and aldolase from human muscle [12] and triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) [13] from *T. aceti* have been reported to be unaffected by age, though only triosephosphate isomerase was studied as a pure enyzme. A number of enzymes in blood tissues of old subjects have also been shown to be unaltered, based upon results of immunological titration [14]. A distinction should be made between the latter enzymes which are freshly synthesized and packaged in cells such as erythrocytes and leukocytes and

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those tissues in which enzymes turn over in the more usual sense of the term. The blood tissues may be 'young' even though they are synthesized in old subjects.

With so few enzymes studied, particularly in higher animals, few data are available which would help to predict which enzymes or types of enzyme would be expected to show age-related alterations. In order to provide a greater base of information, we have purified enolase to homogeneity from muscle and liver of young and old rats and examined the respective enzyme pairs for age-related differences. In addition, partially purified heart enolase has been examined. The liver enzyme, to our knowledge, has not previously been obtained in pure form.

No differences in properties were found between 'old' and 'young' muscle or 'old' and 'young' liver enolase, respectively. By contrast, the heart enzyme showed marked differences in heat-sensitivity between preparations from young and old animals.

The three enolases should not be considered to be the same protein derived from different tissues; they are quite distinct. Liver contains mostly enolase 1 ($\alpha\alpha$), muscle contains only enolase 3 ($\beta\beta$) and heart is reported to consist mostly of a third isozyme, enolase 2, presumably the heterodimer ($\alpha\beta$) along with liver and muscle types [15].

Methods

Purification of muscle enolase. Hind leg muscles from individual Sprague-Dawley and Fisher 344 rats were used for enzyme purifications. Young and old animals were 6—12 months and 28—32 months of age, respectively. In one case, 3—4-month-old animals were used. The animals were killed by guillotine and the tissues were dissected out and used immediately or stored at −80° C. No difference in the properties of the enzymes was observed after purification, even after prolonged storage (up to 2 years) of muscle tissue.

The procedure of Rider and Taylor [16] was altered to avoid the heating step, so that any heat-sensitive enzyme which might be present in 'old' preparations would not be lost. An $(NH_4)_2SO_4$ fractionation was used instead. The buffer systems were also changed. All procedures were carried out at 4° C.

Muscle tissue (20—30 g) was homogenized in an Omnimixer in 6 vols. of 10 mM Tris-maleic acid/5 mM $MgSO_4$, pH 7.0 (buffer A). The homogenate was centrifuged at 50 000 × *g* for 30 min and the supernatant liquid filtered through four layers of gauze. It was then brought to 55% saturation by addition of 0.326 g/ml $(NH_4)_2SO_4$. After 20 min the precipitate was removed by centrifugation at 27 000 × *g* for 10 min and the supernatant fluid was refiltered through gauze. An addition 0.161 g/ml $(NH_4)_2SO_4$ was added to bring the mixture to 80% saturation. After 30 min, the precipitate was collected by centrifugation. It was then taken up in a small amount of buffer A and dialyzed for 4 h against three changes of buffer of 1000 ml each.

The product was chromatographed on a column of CM Sephadex C-50 (2.5 × 100 cm) using buffer A. The enzyme was not retained and emerged at 160—220 ml. After concentration on a Amicon PM-10 membrane filter, the enzyme was dialyzed for 4 h against 10 mM Tris-HCl buffer/10 mM $MgSO_4$, pH

8.5. It was then loaded onto a pre-equilibrated column of DEAE-Sephadex A-50 (2.5 × 100 ml) and eluted with the same buffer. Fractions 130—160 (2 ml each) were pooled, concentrated as before and chromatographed on a column of Sephadex G-150 equilibrated with the Tris/$MgSO_4$ buffer. Pure enzyme eluted from the column at a volume of 250—310 ml.

Purification of rat liver enolase. Sprague-Dawley rats were used. Young rats were 10—12 months and old rats were 30 months of age. All buffers contained 1 mM β-mercaptoethanol. All columns measured 2.5 × 100 cm and fractions were 2 ml unless stated otherwise.

Livers (25—50 g) were homogenized in 4 vols. of buffer A using an Omni-mixer. The homogenate was treated as for the muscle enzyme except that the $(NH_4)_2SO_4$ fraction was collected at 45—67% saturation. The precipitate was dissolved in 10—15 ml of buffer A, dialyzed for 4 h against the same buffer and chromatographed on a column of CM-Sephadex as for muscle enolase. The enzyme was concentrated, dialyzed against Tris-HCl buffer (10 mM)/10 mM $MgSO_4$ (pH 7.6) and applied to a pre-equilibrated column of DEAE-Sephadex A-50. The column was first developed with 400 ml of this buffer. A small amount of enzyme activity was eluted. The main peak of enzyme activity was obtained using 400 ml of 0—0.2 M NaCl gradient. The product was concentrated using a PM-10 filter and then chromatographed on a column of Sephadex G-150 using the above buffer. After concentration (PM-10 filter), the product (5 ml) was dialyzed for 3 h against 10 mM Tris-HCl, pH 7.5, and applied to 2.3 ml of pre-equilibrated Affi-gel Blue (100—200 mesh) in a small column (0.5 × 12 cm). Fractions (1 ml) were collected in tubes pre-loaded with $MgSO_4$ so that the final concentration was 10 mM. The enzyme was not retained on the Affi-gel and appeared in the early fractions. Those fractions with the highest activity were pooled and contained pure enolase.

Purification of enolase from rat heart. Sprague-Dawley rats were used. Ages varied from 3 to 30—36 months as indicated. The purification procedure is a modification of that reported by Rider and Taylor [16]. Heart tissue was homogenized in 4 vols. of buffer A and carried through the stage of chromatography on CM-Sephadex as for liver enolase. All buffers contained 1 mM β-mercaptoethanol. The concentrate from the CM-Sephadex column was dialyzed for 4 h against 12.5 mM Tris-HCl/2.5 mM $MgSO_4$ buffer, pH 7.5. The dialysate was chromatographed on a column of QAE-Sephadex (2 × 17 cm) using in sequence, 100 ml (2 vols.) of the Tris-HCl buffer, 150 ml of a 0—0.1 M NaCl gradient and 50 ml of starting buffer containing 0.2 M NaCl. Three peaks with enolase activity emerged from the column, one with each elutant. The largest (middle) peak, corresponding to heart enolase, was subsequently chromatographed on Sephadex G-150 (2.5 × 100 cm) using 10 mM Tris-HCl/5 mM $MgSO_4$, pH 7.5.

Treatment of the heart enzyme on Affi-gel Blue does not increase the purity of the preparation. In contrast to the liver enzyme, heart enolase is slightly retained on the column.

Protein determination. Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as the standard.

Gel electrophoresis, SDS-electrophoresis and isoelectric focusing. Gel electrophoresis was carried out by the procedure of Kuchler [18] in 7.5% acrylamide

gels at 4 mA/gel in 0.05 M Tris-glycine buffer, pH 8.3. Because of the poor mobility of muscle and heart enolase, these enzymes were also electrophoresed by the procedure of Rodbard and Chrambach [19]. From 5 to 100 μg of protein were loaded in various runs. SDS gels were run according to the method of Weber and Osborn [20] on 10% gels. Isoelectric focusing was carried out in polyacrylamide gels according to the procedure of Wrigley [21] using ampholytes with a pH range of 6—8. Isoelectric focusing was also performed on sucrose gradients.

Enolase assay. The assay was performed using the method of Warburg and Christian [22] at 30° C. 1 unit of activity is defined as the amount of enzyme required to bring about a change in absorbance of 0.1 unit per min.

pH optimum. The assay was carried out as above but the pH was adjusted to range from pH 6.5 to 8.0 in the various experiments.

Determination of K_m. Activity was assayed at 30° C and pH 7.4 in the presence of increasing amounts of substrate (2-phosphoglyceric acid) in a total volume of 1 ml until a maximum rate was achieved. For Mg^{2+} determinations, the enzyme was first dialyzed for 48 h against 0.05 M imidazole/1 mM EDTA buffer (pH 7.4). Dialysis was then continued for another 48 h in the same buffer without EDTA. After this time, the dialysate showed no enolase activity unless Mg^{2+} was added. The K_m was determined as above after addition of increasing amounts of $MgSO_4$.

Heat-inactivation studies. Pure muscle and liver enolase were dialyzed overnight against 10 mM Tris-HCl/10 mM $MgSO_4$, pH 8.5 and 10 mM Tris-HCl/5 mM $MgSO_4$, pH 7.5, respectively. The enzyme, in 1.5—2 ml (250 μg/ml), was placed in a water bath at 61° C for muscle enolase and 56° C for liver enolase. Aliquots of 50—100 μl were removed at appropriate intervals and immediately chilled in an ice-bath for subsequent assay. The semi-purified heart enzyme (300 μg/ml) was treated similarly in the second of the above buffers at 59° C.

Incubation of muscle enolase with heart preparations. Pure 'young' muscle enolase (1600 U) was added to 7.5 mg $(NH_4)_2SO_4$ fraction from 'old' hearts to check for proteolytic action. Incubations were carried out at the preparative temperature (4° C) but only for 24 h to exaggerate any effects. Enzyme activity and heat-sensitivity studies were then carried out as described above. The relatively crude heart preparation contained too little heart enolase activity (90 U) to affect the temperature pattern of the added muscle enzyme.

Results

The yield of muscle enolase varied between 16 and 30% in numerous purifications from both young and old animals, with around 20% being most common. No particular step seemed responsible for this variation in yield. A similar overall yield (21%) was reported by Rider and Taylor [16] for their procedure. Neither the strain of rat used (Sprague-Dawley vs. Fisher 344) nor the age of the animals was responsible for differences in yield.

'Young' and 'old' muscle and liver enolase were pure as judged by single protein bands appearing after electrophoresis on acrylamide gels at all concentrations tested and with and without SDS. Fig. 1 shows the results obtained

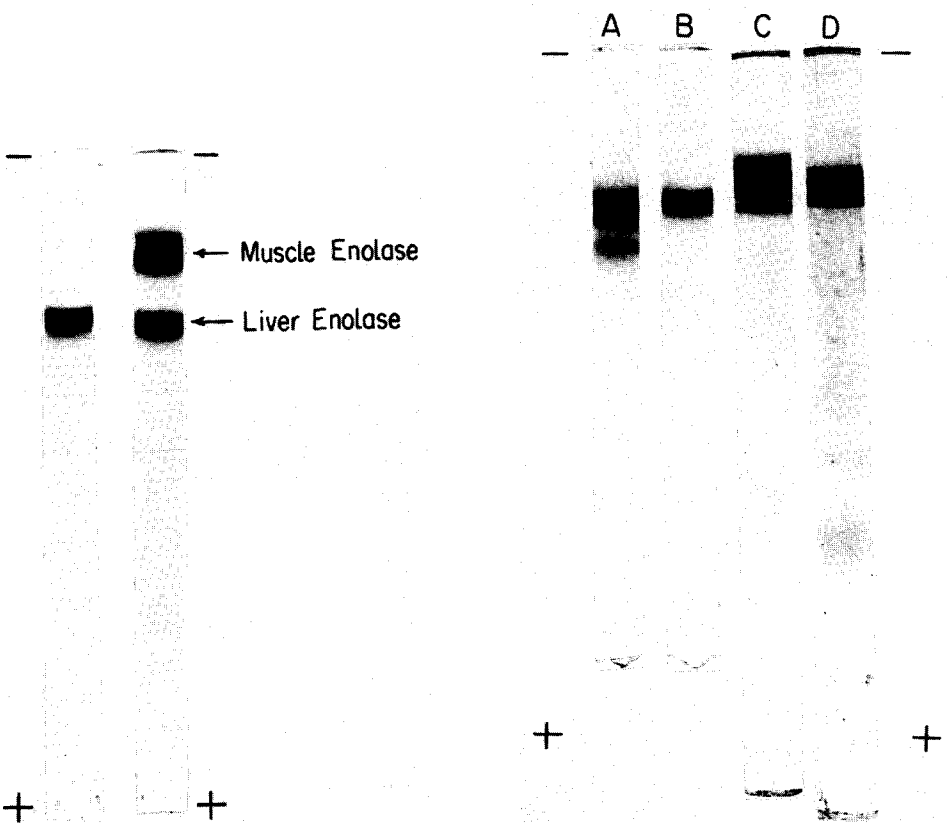

Fig. 1. Polyacrylamide gel electrophoresis of 'young' muscle and liver enolase. These gels were heavily loaded with enzyme so that any impurities would be apparent. Muscle enolase from old rats and mixed preparations from young and old rats were identical with the muscle enolase gel shown above.

Fig. 2. Gel electrophoresis of 'young' rat liver enolase. A, B, gel electrophoresis of enzyme preparation before and after treatment, respectively, with Affi-gel Blue. C,D, same preparations on SDS gels. No differences were observed with 'old' preparations.

with 'young' preparations. In general, isoelectric focusing appears to be an unsuitable procedure for testing the purity of rat enolases. Focusing of muscle enolase in gels at pH 6—8 yielded several protein bands, though only one showed enzyme activity. When run in a sucrose gradient, the enzyme sometimes showed a small second (p*I* 6.5—6.9) and even a third component (p*I* 7.6—7.8) besides the main enzyme fraction at p*I* 7.2. The pure liver enzyme, after isoelectric focusing on gels, showed two bands of equal activity, determined by the method of Sharma and Rothstein [23]. The above results are propably due to the well established occurrence of artifacts caused by the ampholytes [24]. In fact, enolase from yeast has been proved to produce such artifacts after isoelectric focusing [25].

The specific activity of pure rat muscle enolase was 1150—1200 units/mg. No difference was observed in enolase isolated from young, mature or old rats, or from Sprague-Dawley vs. Fisher 344 rats.

The heat-sensitivity of muscle enolase is linear and shows no age-related

TABLE I

PURIFICATION OF RAT LIVER ENOLASE

Treatment	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fold purification	Yield (%)
Crude homogenate	4500	14 472	3.2	1	100
$(NH_4)_2SO_4$	1856	13 627	7.3	2.3	94
CM Sephadex	468	7 673	16.4	5.1	53
DEAE-Sephadex	13.8	4 212	306	96	29
Sephadex G-150	4.8	2 840	591	185	20
Affi-gel	3.1	2 756	893	279	19

change. Similarly, there are no age-related differences in spectral properties, though such changes occur in enolase from *T. aceti* [3,8]. The K_m was not significantly different for 'young' and 'old' muscle enolase ($10.8 \cdot 10^{-5}$ and $9.8 \cdot 10^{-5}$ M, respectively) nor was there a difference in the Mg^{2+} requirement ($1.5 \cdot 10^{-4}$ and $1.75 \cdot 10^{-4}$ M respectively).

Rider and Taylor [16] estimated that their rat liver enolase was 80% pure. Though we have modified their procedure somewhat, using different buffers, the purification proceeds similarly. Table I outlines a typical purification procedure. Rechromatography of the semi-purified preparations on DEAE-Sephadex or Sephadex G-150 was not useful in increasing purity. However, use of Affi-gel Blue was completely effective in this respect (Fig. 2).

Specific activities were 850—893 units/mg for both 'young' and 'old' liver enolase in all cases. No differences were observed in heat-lability patterns, which yielded identical straight lines. No difference was detectable after isoelectric focusing in sucrose gradients. The K_m was essentially the same, being respectively, $6 \cdot 10^{-5}$ M and $3 \cdot 10^{-5}$ M. In brief, there is no apparent difference in the properties of liver enolase obtained from young and old rats.

Comparison of liver and muscle enolase

The muscle enzyme has a higher specific activity (1200 units/mg vs. 893

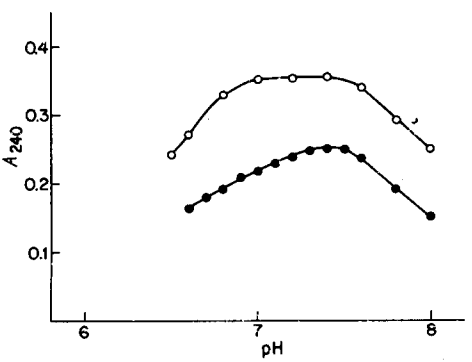

Fig. 3. Effect of pH on the activity of muscle and liver enolase. ○———○, liver enolase; ●———●, muscle enolase.

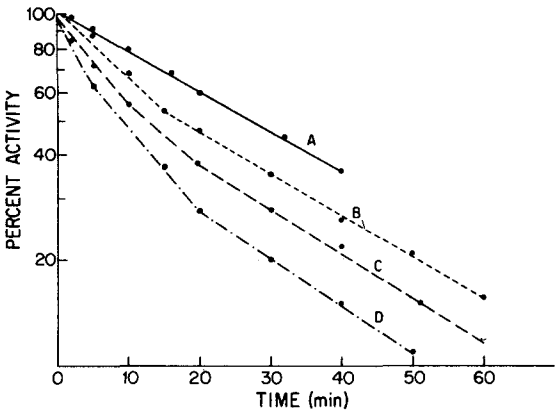

Fig. 4. Heat-lability of partially purified rat heart enolase. The ages of animals were: A, (•———•), 3 months; B, (•- - - - -•) 12 months; C, (•— — —•) 24 months; D, (•· — · — ·•) 30—36 months. Temperature, 59°C.

units/mg) than the liver enzyme. The ratio of activities is similar to that obtained by Rider and Taylor [16] using a different assay system. The liver enzyme is less stable on storage. The isoelectric points determined by isoelectric focusing in sucrose gradients are 7.2 for the major band formed by pure muscle enolase and 5.6 for the liver enzyme ($(NH_4)_2SO_4$ fraction). The latter enzyme under our experimental conditions, does not bind to Affi-gel but does bind to DEAE-Sephadex. The muscle enzyme behaves in the opposite manner. As reported by Rider and Taylor [16], the molecular weights appear to be identical on SDS gels. The effect of pH on activity is shown in Fig. 3. The K_m for Mg^{2+} was $4 \cdot 10^{-4}$ M, for the liver enzyme, but removal of the metal apparently causes damage as only 20% of the enzyme activity was recovered. No such problem occurred with the muscle enzyme. Muscle enolase shows no detectable isozyme after chromatography on DEAE-Sephadex columns, whereas in each of several purifications of 'young' liver enzyme, an isozyme appeared in the void volume. It amounted to 3—10% of the total activity recovered. No age-related difference was observed in this respect.

Heart enolase

Though the enzyme was not obtained in pure form, the QAE-Sephadex step assured removal of the muscle and liver type isozymes. The partly purified enzyme still showed four protein bands after gel electrophoresis. The specific activity achieved was 670 units/mg, representing a 220-fold purification. In young animals, the isozyme distribution in crude heart tissue showed about 20% muscle type, 75% heart type and 5% liver type from the QAE-Sephadex column. There were only minor age-related changes in this pattern.

Heat-sensitivity experiments showed substantial differences between the semi-purified enolase from young vs. old hearts (Fig. 4). The enzyme from 3-month-old animals yielded a straight line. With each increase in age, hearts showed an increasing amount of heat-sensitive enzyme. In each case, the more heat-stable component parallels exactly the 'young' pattern and presumably represents unchanged enzyme.

The differences in heat-sensitivity are not a result of proteolytic action, as purification of the enzyme in the presence of the proteolytic enzyme inhibitor, phenylmethylsulfonyl fluoride, had no effect on the heat-sensitivity patterns. Moreover, pure 'young' muscle enolase loses no activity after incubation for 24 h with the 'old' heart $(NH_4)_2SO_4$ preparation and the heat-sensitivity curve of the muscle enzyme remains unchanged after this treatment.

Discussion

Previously reported evidence that certain enzymes in aged mammalian tissues are unaltered seems satisfactory but some of it is based upon work with crude homogenates and contains a number of anomalies. For example, aldolase B from old rat liver showed a small heat-labile component and human muscle aldolase showed a variety of heat-lability patterns, not in accordance with the age of the subject. Experiments with aldolase from old rat liver based on catalytic activity per nmol of sequence seems to provide clear cut evidence for lack of change. Recently aldolase from old rat liver was shown to be unaltered if fresh tissue preparations were used [26]. The earlier alteration reported by Gershon and Gershon [6] was ascribed to the authors' use of stored homogenates which resulted in the proteolytic removal of a C-terminal tyrosine residue in the 'old' preparations. Our findings that pure muscle enolase and liver enolase are not altered in old rats confirm and make unassailable the conclusion that in higher animals, altered enzymes are not necessarily a concomitant of aging. Taken together with the changes reported for old rat liver superoxide dismutase [5], rat muscle phosphoglycerate kinase (Sharma, Prasanna and Rothstein, unpublished data), heart and muscle aldolase [7] and rat heart enolase, it becomes clear that enzymes in rodents may or may not become altered with age. This situation parallels that found in *T. aceti*, in which pure triosephosphate isomerase, in contrast to isocitrate lyase, phosphoglycerate kinase and enolase, was found to be unaltered.

What process can satisfactorily explain the presence of both altered and unaltered enzymes in aging organisms? Errors in the protein synthesizing system, (e.g. transcription or translation) should affect all proteins and this is therefore an unlikely if not untenable explanation for the mechanism of formation of altered enzymes. Chemical changes such as deamidation also seem to be an unlikely solution to the problem as no change of charge occurs which is detectable by isoelectric focusing [5,8,27] nor are oxidation of SH groups [8] or sulfone formation involved in altered enolase (Sharma and Rothstein, unpublished results). An explanation based on the proposed slowing of protein turnover in old organisms has been put forth by Rothstein and co-workers in a number of publications [8,9,28—30]. If protein turnover slows, enzymes will have an increased 'dwell' time in the cells and could, depending upon their structure, form relatively stable intermediates (altered enzymes) as a result of a subtle denaturation. Some enzymes may possess no such stable intermediate forms and may in part denature to structures no longer recognizable as being related to the parent enzyme. Such enzymes would be found to be unaltered in old organisms but would be present in reduced amounts. Triosephosphate isomerase in *T. aceti* [13] and creatine kinase and aldolase in human muscle

[12] may follow such a pattern. Other enzymes may remain unaltered because of their intrinsic stability.

As to whether protein synthesis indeed slows with age, there is abundant evidence that it does so in *T. aceti*. A substantial age-related slowing of protein degradation has recently been reported by Prasanna and Lane [31] and Sharma et al. [32] have shown that synthesis and degradation of both soluble proteins and individual protein (enolase) slow markedly with age. The case for slowed protein turnover in higher animals is less clear. There are several reports [3—35] that cell-free systems from old animals are less capable of incorporating labeled amino acid into protein, and nutritional studies are suggestive of a slower protein turnover in aged humans. However, no definitive study has been performed which proves the case. Thus, though it is clear that some proteins are altered during aging and others are not, the reasons for this situation remain speculative.

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