Inactive Enzyme Molecules in Aging Organisms. Nematode Fructose-1,6-diphosphate Aldolase†

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ABSTRACT: The use of antiserum prepared against crude homogenates of young adult *Turbatrix aceti*, a free living nematode, enabled the detection of antigenically cross-reacting material (CRM) to the enzyme fructose-1,6-diphosphate aldolase. It was found that with age there is an accumulation of CRM which has either a reduction or total loss of catalytic activity. It was shown by electrophoresis in cellulose acetate, thermal inactivation, determination of K_m for fructose 1,6-diphosphate, and immunological techniques, that these findings could not be attributed to age-dependent changes in isozymic forms of the enzyme. The mechanisms by which altered enzymes may be produced are discussed. It is proposed that the production and accumulation of inactive enzymes and faulty proteins may be of detrimental effect to the organism.

Although aging is a well-recognized phenomenon, the processes which cause it are still largely obscure. Orgel (1963) has postulated the appearance of errors in protein molecules as a cause of aging and suggested that the protein synthesizing machinery introduces incorrect amino acids into nascent proteins. The initial frequency of such errors is assumed to be extremely low but it increases exponentially due to the progressive accumulation of faults in those enzymes which participate in protein synthesis. As a result of the increase in the number of errors with age, the specific activities of enzymes derived from old animals may be expected to be lower than those derived from young animals. Accumulation of such altered molecules might be a burden on metabolism since more molecules would have to be synthesized in order to maintain necessary levels of activity. It is hypothesized that once a critical level of faulty proteins is reached, death ensues.

Harrison and Holliday (1967) have shown that feeding of *Drosophila* with amino acid analogs for a short period during the pre-imaginal period reduces the life span of the adult flies. Although this reduction in life span was attributed to an increase in the frequency of errors in the proteins of analog-treated flies no substantiating evidence was offered. Gershon and Gershon (1970) found that the reduction with age of the specific activity of isocitrate lyase in crude preparations of the free living nematode, *Turbatrix aceti*, was accompanied by an accumulation of inactive enzyme molecules.

The existence of inactive or partially active faulty enzyme molecules was detected by immunological methods in the form of cross-reacting material (CRM).

The free-living nematode *Turbatrix aceti* is suitable for aging studies (Gershon, 1970) and has been chosen for the following reasons: its life span is short, with a mean of 35–40 days (Zeelon and Gershon, unpublished results); it has a fixed number of cells and there is no cell replacement except in the reproductive system (Pai, 1928); synchronized populations can be raised axenically. Previous studies have shown a decline with age in the specific activities of all of the nonlysosomal enzymes studied (Erlanger and Gershon, 1970; Gershon and Gershon, 1970). The aim of this present study was to find out whether the accumulation of CRM with age is of universal nature and not peculiar to isocitrate lyase. The fructose-1,6-diphosphate aldolase of *T. aceti* was chosen as a second representative enzyme. For better understanding of the nature of changes in aldolase with increasing age, parameters such as electrophoretic mobility, K_m for fructose 1,6-diphosphate, and the rate of thermal denaturation of the enzyme from nematodes of various ages were compared with the degree of CRM detected.

Materials and Methods

Animals. Axenic stock cultures of *T. aceti* were maintained at 30° as previously described (Gershon, 1970; Zeelon and Gershon, 1973). Stock cultures were initiated by seeding 10^6 animals in 100 ml of medium (Rothstein and Cook, 1966). After 2 weeks at 30°, the populations numbered over 10^7 animals/100 ml.

Age-Synchronized Populations of T. aceti. Age synchronization of *T. aceti* was performed as previously described (Gershon, 1970). Worms (0–3-day old) were collected by filtration through a 5 × 6 cm column of glass beads (Superbrite 3M, 0.2 mm in diameter) (Zeelon and Gershon[1]). The nematodes were collected by a 10-min 4000*g* centrifugation at 4° and washed in 100 ml of sterile synthetic nematode medium (Hansen, 1966), supplemented with 3% acetic acid (pH adjusted to 3.4 with concentrated HCl). The nematodes were resuspended to 50,000 worms/ml and maintained in the same medium supplemented with 500 μg of hydroxyurea/ml. This concentration of hydroxyurea has been shown to be an efficient inhibitor of DNA synthesis in *T. aceti* (Gershon, 1970). Such inhibition of DNA synthesis effectively prevents reproduction without influencing worm growth or life span (Gershon, 1970). The age-synchronized cultures thus produced were grown at 30° and harvested for biochemical studies after various intervals of growth.

Cleaning of the Nematodes. Nematodes from age-synchronized cultures were harvested by centrifugation in the cold at 3000*g* for 10 min. The nematodes were separated from insoluble constituents of the medium by adding a cold 35% w/w sucrose solution to the pellet and exposing it to a 5-min centrifugation at 1500*g* in a swinging bucket. In this procedure, the live nematodes float on the surface while dead animals and insoluble medium residues settle in a pellet. The washed nematodes were recentrifuged in 35% sucrose followed by five rinses of 15 vol of water each. The washed

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nematodes were suspended in 50 mM Tris-Cl buffer (pH 7.4) containing 1 mM EDTA and 10 mM β-mercaptoethanol (TEM buffer).

Preparation of Nematode Homogenate (Crude Aldolase Preparation). Aldolase activity, both enzymatic and antigenic, was assayed in crude homogenates of *T. aceti*. Cleaned synchronized populations of nematodes suspended in cold TEM buffer were homogenized in an ice-cold 5-ml French pressure cell (Aminco) under 2000 kg of applied load. The homogenate was centrifuged at 20,000*g* for 60 min at 4°. The pellet which contained less than 5% of the total aldolase activity was discarded. The supernatant thus obtained is referred to as crude enzyme preparation. All the experiments were performed on freshly prepared homogenates.

Enzyme Assay. The assay for aldolase activity was performed as described by Gracy *et al.* (1970). One milliliter of reaction mixture contained 0.8 ml of 50 mM triethanolamine buffer (pH 7.4), 10 mM EDTA, 0.1 mM NADH,[1] 0.1 mM fructose 1,6-diphosphate, and 10 μg/ml of a mixture of triosephosphate isomerase and glycerolphosphate dehydrogenase (Boehringer Mannheim Corp.), 0.1 ml of 0.1 M sodium azide, and 0.1 ml of crude enzyme. The azide was added in order to reduce the level of non-$FruP_2$-dependent NADH oxidation. Aldolase activity was determined by following the oxidation of NADH at 340 nm with a Unicam Sp 800 spectrophotometer at 25°. For each determination of aldolase activity, a control without $FruP_2$ was run and its value was subtracted from the activity found with $FruP_2$. Control values ranged from 10 to 15% of those with $FruP_2$. One unit of aldolase is defined as the amount of enzyme required to cleave 1 μmol of $FruP_2$/min at 25°. Protein concentration was determined according to Lowry *et al.* (1951). DNA concentration was determined by the method of Kissane and Robins (1958).

Determination of the Half-Life of Aldolase. Cycloheximide (500 μg/ml) added to synchronized nematode populations was found to cause 95% inhibition of protein synthesis, as determined by studies of the incorporation of [^{35}S]methionine into acid-insoluble material with correction for free [^{35}S]methionine pool size. This inhibition was verified at all ages studied. Maximal inhibition was achieved within 30 min of exposure. For the determination of the half-life of aldolase, the activity of the enzyme per milligram of protein was determined at different times after the blocking of protein synthesis. The logarithm of the specific activity was plotted as a function of the time of exposure to the inhibitor, and the half-life was calculated from the slope. In several experiments the activity of aldolase at different times after the inhibition of protein synthesis was expressed per microgram of DNA. The ratio of the material which reacted as protein (sum of protein + free amino acids) to DNA was not altered over the duration of the inhibition experiment.

Electrophoresis on Cellulose Acetate Membrane. Nematodes (1.5×10^6) were suspended in 1 ml of homogenization solution prepared as described by Lebherz and Rutter (1969) and homogenized as described above. The concentration of protein in the supernatant, after 60 min of centrifugation at 20,000*g*, was at least 10 mg/ml. Homogenate (2 μl) was applied to cellulose acetate strips and electrophoresis was carried out in 0.082 M Tris-Cl buffer (pH 8.6) containing 10 mM β-mercaptoethanol and 1 mM EDTA. This buffer was used instead of barbitone buffer (pH 8.6) which was found to decrease nematode aldolase activity and did not support its electrophoretic mobility.

Localization of aldolase activity was performed as described by Penhoet *et al.* (1966). Glass microscope slides were coated with 0.8 ml of 0.5% Noble agar in 0.01 M $NaAsO_2$ buffer (pH 7.5), containing final concentrations of 0.01 M $FruP_2$, 0.001 M NAD^+, 0.12 mg/ml of glyceraldehyde-3-phosphate dehydrogenase (Boehringer Mannheim Corp.), 0.024 mg/ml of phenazine methosulfate, and 0.4 mg/ml of nitro blue tetrazolium chloride. The slides were stored over wet filter paper at 4° until used. After the electrophoresis was completed aldolase activity was localized by placing the cellulose acetate strips on the microscope slides. The reaction was allowed to progress and the color was developed at room temperature.

Rabbit Anti-Nematode Serum. The serum was prepared by immunizing a rabbit by repeated intradermal and intramuscular injections of crude enzyme preparation from 5-day old nematodes in emulsion with an equal volume of Freund's complete adjuvant. Before immunization, the rabbit was bled for normal serum (Gershon and Gershon, 1970).

Inhibition of Aldolase Activity by Antiserum. Various amounts of antiserum were added to 14.5 milliunits (mU) of aldolase and the volume was brought to 0.3 ml with TEM buffer. The mixture was kept at 4° for 10 min and 0.1 ml was withdrawn and assayed for aldolase activity as described above. The degree of inhibition was calculated by subtracting the residual from the original activity.

Precipitation of Aldolase. To 14.5 mU of aldolase activity various amounts of antiserum and TEM buffer were added to a final volume of 0.3 ml. After 20 hr of incubation at 4°, the mixture was centrifuged at 3000*g* for 20 min. The supernatant (0.1 ml) was assayed for aldolase activity. For each concentration of antiserum, a control containing normal rabbit serum was also assayed.

Results

Crude enzyme preparations from synchronized populations of various ages were assayed for aldolase activity as described under Materials and Methods. There is a considerable reduction in the specific activity of aldolase in crude preparations which commences at the age of 18 days and continues thereafter (Figure 1). It has been shown in previous studies (Gershon, D., unpublished results) that the amount of protein per nematode does not change considerably after the age of 10 days and remains at a plateau of 12 mg of protein per 10^6 nematodes throughout the adult life. The possibility that the results might be due to increased proteolytic activity in the homogenates of senescent *T. aceti* was ruled out in two ways. Firstly, the *in vitro* stability for 24 hr at 4° was the same for homogenates of both old and young animals. Secondly, experiments were performed in which homogenization and all further treatments were carried out in the presence of 10 mg/ml of bovine serum albumin (BSA). The presence of this high concentration of BSA in no way altered the amount of enzyme activity detected (Zeelon, 1972). The conclusion, therefore, is that old nematodes have about one-third the aldolase activity of young nematodes.

The reduction in specific activity of aldolase might be attributed to an increase in the rate of degradation accompanied by either no change or even a reduction in the rate of synthesis of the enzyme with age. In order to find out whether the rate of degradation of the enzyme changes with age, the

[1] Abbreviations used are: NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; $FruP_2$, fructose 1,6-diphosphate; CRM, cross-reacting material.

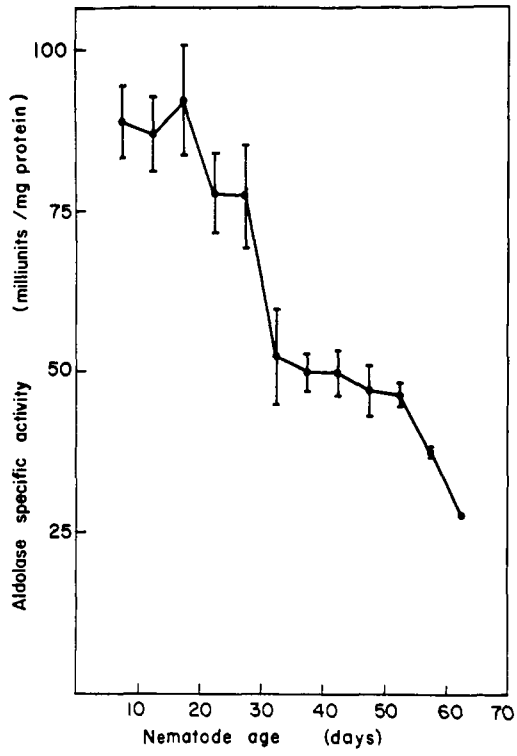

FIGURE 1: Specific activity of aldolase in nematodes of various ages. Synchronous populations of nematodes of various ages were homogenized and crude preparations were assayed for aldolase activity as described under Materials and Methods. Each point represents the average of four individual determinations. The vertical lines denote the standard error of the mean.

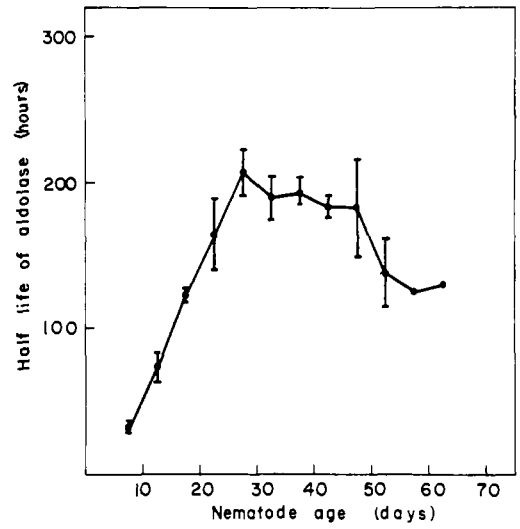

FIGURE 2: Half-life of aldolase in nematodes of various ages. Cycloheximide (500 μg/ml) was added to synchronized nematode populations of various ages containing 70,000 worms/ml. At various time intervals aliquots were removed, crude enzyme preparations prepared, and the specific activity of aldolase determined. Enzyme half-life was determined as described under Materials and Methods. Each point represents an average of three individual experiments. The vertical lines denote the standard error of the mean.

half-life of the enzyme at various ages was determined. Figure 2 shows that by using cycloheximide at concentrations which give over 90% inhibition of protein synthesis we have found an age-dependent stabilization of aldolase activity. At the age of 7 days the half-life of aldolase was 40-hr. It increased progressively until a half-life of 200 hr was attained at 28 days and remained stable until the age of 48 days. Thereafter, the half-life of aldolase becomes shorter and reaches a value of 120 hr at the age of 60 days.

Inasmuch as one cannot attribute the fall in specific activity of aldolase to an increased rate of degradation in senescent nematodes, three other possibilities for the reduction in aldolase specific activity with age can be suggested. (a) Although we found stabilization of the enzyme with age, it might still be possible that the rate of its synthesis decreased drastically, thus causing a reduction in the number of enzyme molecules present. (b) An isozymic form of aldolase with low specific activity may appear in aging nematodes. Such a situation has been described for other enzymes, such as the lactic dehydrogenase of rat liver (Kanungo and Singh, 1969). (c) Altered enzyme molecules with either reduced activity or molecules entirely lacking in activity may appear with age.

The use of antiserum has enabled us to distinguish among the three above-mentioned possibilities. In Figure 3 it can be seen that about twice as much enzyme activity is inhibited in homogenates of young as compared to old nematodes. The amount of enzyme inhibited per fixed volume of antiserum decreased with age. Fifteen microliters of antiserum inhibited 30% less activity from 30-day old nematodes as compared to the inhibition showed in preparations of 10-day old

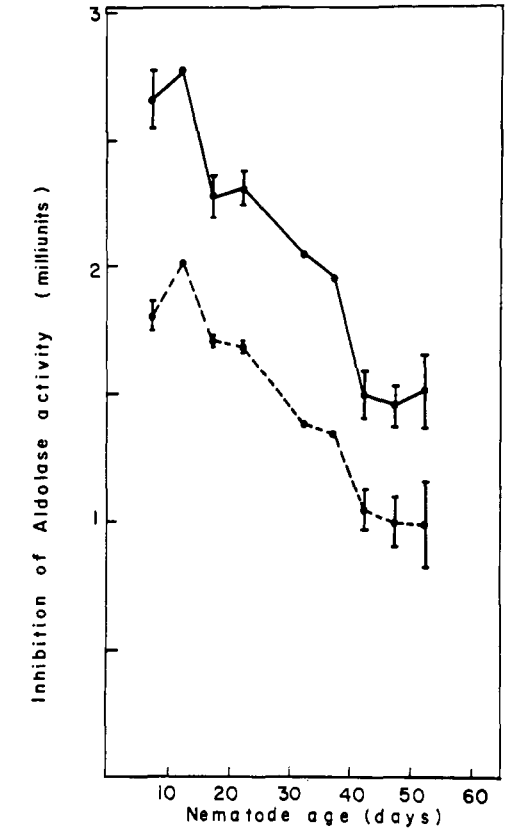

FIGURE 3: Inhibition by antiserum of aldolase from nematodes of various ages. Crude preparations of enzyme from nematodes of various ages were prepared and inhibition with two antiserum concentrations was performed as described under Materials and Methods. Each point with vertical bars represents an average of at least two experiments. Vertical bars denote the standard error of the mean. Points which do not have vertical bars represent single experiments: (- - - -) 10 μl of antiserum/0.1 ml of homogenate-antibody mixture; (—) 15 μl of antiserum/0.1 ml of homogenate-antibody mixture.

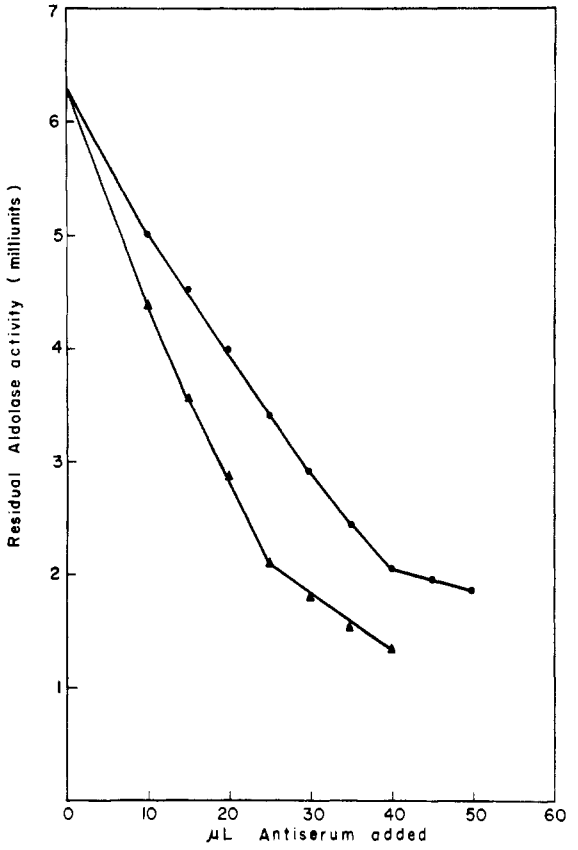

FIGURE 4: Comparison of the inhibition by antiserum of aldolase from 6- and 51-day old nematodes. Crude enzyme preparations of 6- and 51-day old nematodes were prepared as described under Materials and Methods. Inhibition with various amounts of antiserum was performed as described under Mateials and Methods: (▲) aldolase of 6-day old *T. aceti;* (●) aldolase of 51-day old *T. aceti.*

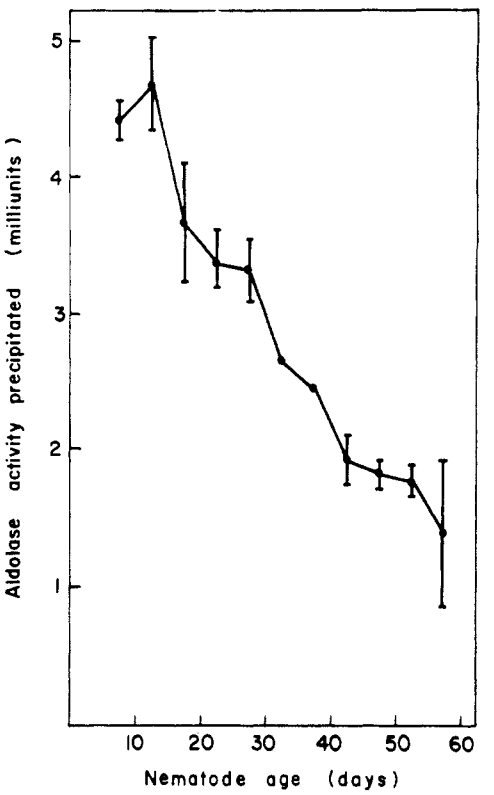

FIGURE 5: Precipitation by a fixed amount of antibody of aldolase from nematodes of various ages. Crude preparations of aldolase from nematodes of various ages were prepared as described under Materials and Methods. Antiserum (60 μl) was added to 0.24 ml of homogenate containing 14.5 mμ of aldolase activity. After 20 hr of incubation at 4°, the preparations were centrifuged at 3000*g* for 30 min at 4°. Each supernatant (0.1 ml) was assayed for residual enzyme activity. The amount of enzyme precipitated was determined by subtracting residual activity from original control activity. Each point with vertical bar represents an average of at least three individual experiments. Vertical bars denote the standard error of the mean. The two points without bars represent single experiments.

animals. The degree of inhibition was further decreased with age and in preparations of 40-day old animals reached only 60% of that of 10-day old nematodes. The mechanism of inhibition of enzyme activity by an antibody is presumed to be the result of steric hindrance caused by antibodies which react with antigenic sites near or at the active site (Cinader, 1967). The need for more antiserum for inhibition of equal aldolase activity in preparations from old as compared to those from young nematodes suggests that for the same enzyme activity old nematode preparations may contain more enzyme molecules than do young preparations. Support of this interpretation comes from the experiment described in Figure 4, which was performed to determine if indeed the enzyme molecules of old and young nematodes are antigenically indentical. Upon the addition of increasing amounts of antiserum, the maximal inhibition of aldolase activity from old nematodes was equal to that achieved with preparations from young nematodes. The amounts of antiserum which were required to achieve equal inhibitions of enzyme activity were 1.8-fold greater for preparations of old nematodes as for young ones.

Confirmation of these inhibition experiments was obtained in studies of the precipitation of aldolase activity with antiserum. Using 20 μl of antiserum and enzyme preparations of equal activity from animals of various ages, the enzyme activity precipitated from homogenates of old worms was less than a third of the enzyme activity precipitated from young nematodes (Figure 5).

No precise determination concerning the relative amounts of enzyme molecules in the various ages could be made from these results, because while the experiment was performed under conditions of optimal proportion of antibody to enzyme for preparations of young nematodes, this optimal proportion was not necessarily achieved in old preparations. This became obvious since it was determined that old preparations contain more antigen per unit of enzyme activity than do young preparations. It thus became necessary to investigate whether total aldolase activity of old nematodes could be precipitated by the antiserum which was prepared against homogenates of young nematodes and, if so, how much antiserum was required for complete precipitation. Total precipitation of aldolase was achieved in homogenates of old and young nematodes, with the precipitation of the former requiring 1.8 times as much antiserum as the precipitation of the same activity in homogenates from young nematodes (Figure 6). This result parallels that which was obtained for antibody-mediated inhibition of enzyme activity.

The above results may be interpreted in one of two ways. It may be suggested that the aldolase of young and old nematodes is a single, antigenically identical molecular species

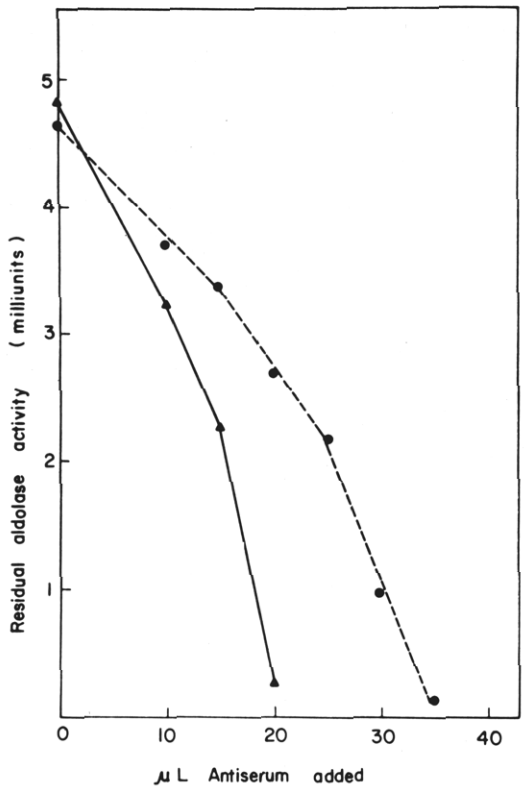

FIGURE 6: Precipitation of aldolase from young and old nematodes. Homogenates were prepared as described under Materials and Methods. Precipitation with various amounts of antiserum was performed as described in Figure 5: (▲) 6-day old nematodes; (●) 51-day old nematodes.

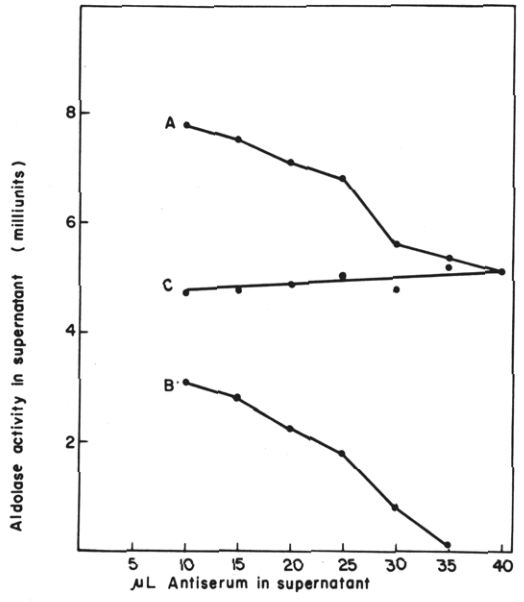

FIGURE 7: Determination of residual antiserum activity after precipitation of enzyme from old animals. Homogenates were prepared as described under Materials and Methods. Precipitation of aldolase from 51-day old nematodes was performed in duplicate as described in Figure 5. The supernatant (0.082 ml) of one tube was assayed for residual aldolase activity and the supernatant (0.246 ml) of the other tube was added to 0.054 ml of homogenate of 6-day old nematodes containing 14.5 mU of enzyme activity. The procedure of precipitation was as described in Figure 5. The supernatant (0.1 ml) after the second precipitation was assayed for aldolase activity. (A) Aldolase activity in the supernatant after the precipitation of aldolase from 6-day old nematodes with the supernatant from the precipitation of aldolase from 51-day old nematodes. (B) Residual aldolase activity in the supernatant after precipitation of enzyme from 51-day old nematodes (in 0.082 ml). (C) A calculated line which is the result of A minus B. It shows the residual activity attributable to aldolase of 6-day old nematodes after subtraction of the aldolase activity of 51-day old nematodes which remained unprecipitated.

which contains inactive or partially active molecules in senescent populations. It may equally be suggested that several isozymes of aldolase exist in young nematodes and that their relative proportions change with age. Thus one might suggest that the preponderant isozyme of young animals might be of high specific enzyme activity and that of old animals of low specific activity. The antibodies produced against homogenates of young nematodes would be primarily against the predominant isozyme of young worms, but would also contain smaller amounts of antibody to the other presumptive aldolase isozymes which are in low titer in young animals and increase with age. The antiserum would then be competent to precipitate all isozymes of aldolase but would be required in different amounts for young and old preparations. Thus, if there were alterations in the relative amounts of various aldolase isozymes with age one should expect to observe the following. Antiserum prepared against aldolase of young nematodes would contain but a low titer of antibody to the predominant isozyme(s) of senescent animals. Increased volumes of antiserum would be required to inhibit or precipitate the increasing amounts of these isozymic forms in homogenates of worms of increasing age. At the same time the high titer antibody to the predominant isozyme(s) of young animals should be found in excess and would therefore remain in the supernatant fluid after precipitation. This can be attacked experimentally by determining whether free antibody capable of precipitating enzyme from young worms remains free in the supernatant after precipitation of enzyme from senescent worms. Such an experiment was indeed performed (Figure 7). Various amounts of antiserum were used to precipitate a fixed amount of aldolase from a crude homogenate of senescent nematodes. The supernatant fluids of these precipitations were assayed for residual antibody activity by determining their ability to precipitate aldolase from a crude homogenate of young adult *T. aceti*. When adjustment was made for the amount of enzyme activity of the senescent *T. aceti* which remained in the supernatants, it became clear that all the antibody was efficiently active in precipitating the

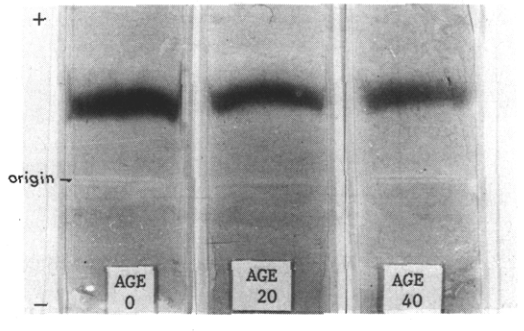

FIGURE 8: Electrophoresis of aldolase from nematodes of various ages. Homogenates for electrophoresis were prepared from nematodes of the following ages: 0, 20, and 40 days. Electrophoresis on cellulose acetate strips was carried out for 65 min at 250 V and 1 mA, and the position of the enzyme was determined by staining for aldolase activity as described under Materials and Methods.

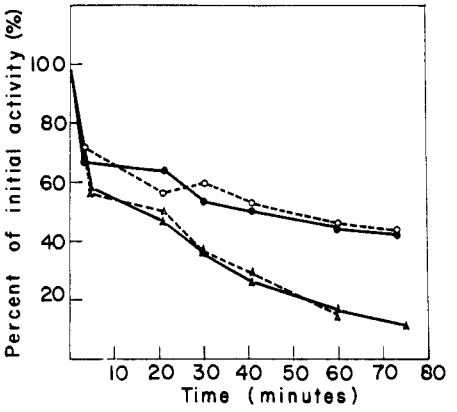

FIGURE 9: Thermal denaturation of aldolase from 5- and 45-day old nematodes at 45 and 50°. Homogenates of nematodes of various ages were prepared as described under Materials and Methods, and diluted to contain an activity of 4.84 mU in a volume of 0.1 ml. The homogenates were incubated at 45 and 50°. Samples were taken at various times and assayed for residual activity: (▲—▲) 5-day old nematodes, denaturation temp 50°; (△- - -△) 45-day old nematodes, denaturation temp 50°; (●—●) 5-day old nematodes, denaturation temp 45°; (○- - - -○) 45-day old nematodes, denaturation temp 45°.

aldolase of senescent worms over the entire range of antigen excess; none of the antibody remained in the supernatant to precipitate aldolase of young worms (Figure 7). These results indicate that the same enzyme is found in old and young nematodes, and that if isozymes do exist their proportions do not change with age.

Another proof of the identity of aldolase derived from young or old nematodes was provided by electrophoresis of crude enzyme preparations on cellulose acetate membranes. In Figure 8 it can be seen that aldolases from *T. aceti* of ages 0, 20, and 40 days do not differ in their electrophoretic mobility.

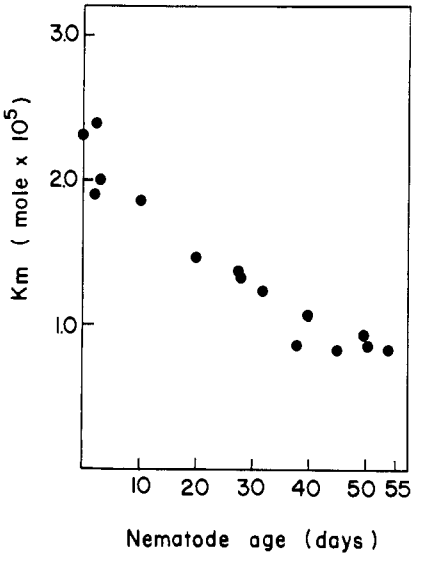

FIGURE 10: K_m of aldolase from nematodes of various ages. Homogenates were prepraed as described under Materials and Methods. Crude homogenate (0.1 ml) containing 4.84 mU of enzyme activity was added to 0.9 ml of reaction mixture containing fructose 1,6-diphosphate at concentrations from 0.1 to 0.005 mM. The initial velocities were determined. $1/v$ was plotted as a function of $1/S$, and K_m was calculated from the slope.

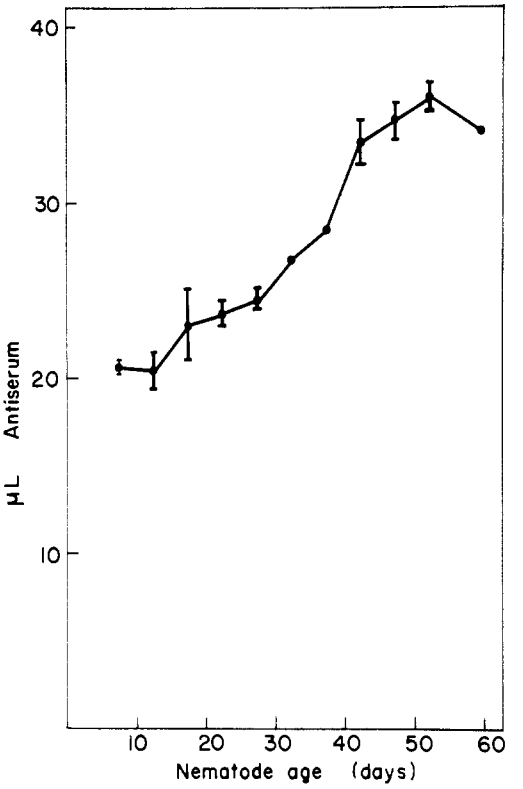

FIGURE 11: The amounts of antiserum required for the precipitation of 4.84 mU of aldolase activity from nematodes of various ages. Homogenates of nematodes of various ages were prepared and precipitation with various amounts of antiserum was performed as described in Figure 5. The residual activities were determined and extrapolation was made for the amount of antiserum required for total precipitation of the activity. The amount of the antiserum which is required for the precipitation of 4.84 mU of enzyme was calculated. Each point with vertical bars represents at least three experiments. Vertical bars denote the standard error of the mean. Points without bars represent single experiments.

It is clear that if there were some differences in their primary structures they were small and did not change the charge of the enzyme to such an extent as to enable electrophoretic separation. Thus, one of the classic means of demonstrating isozymes failed to reveal them in this aldolase system.

Differences in the primary structure of an enzyme might change its thermal stability, and so isozymes that could not be detected by former procedures might be detected if they differ in this property. In Figure 9 it can be seen that aldolase from 45-day old nematodes did not differ from aldolase of 5-day old nematodes in its thermal stability at two denaturation temperatures. Therefore, any differences between aldolases from young and old nematodes did not affect the thermal stability of the enzyme. Slight differences in aldolases from young and old nematodes were, however, found by the determination of the K_m of the enzymes. Figure 10 shows a small gradual decrease of K_m with age. Aldolase from 3-day old nematodes has a K_m value of 2×10^{-5} M and aldolase of 45–50-day old nematodes has a K_m of 0.9×10^{-5} M. Although this difference in K_m is only twofold, it was reproducible and consistent. Nevertheless, taken by itself, such a small difference cannot support speculation about the nature of changes in the enzyme with age.

It has, therefore, been reconfirmed that in senescent *T. aceti* there appears to be an accumulation of enzyme cross-reacting material (CRM) which is either partially or totally devoid of

enzyme activity and cannot be attributed to the appearance of new isozymes. In order to find the ratio of CRM to enzyme activity at various ages the amounts of antiserum which were required for total precipitation of a fixed enzyme activity were determined. In Figure 11 it can be seen that the amount of antiserum required for total precipitation of this fixed amount increases with age to a peak in senescent worms which is 1.8 times that of young adults.

Discussion

The specific activity of aldolase in the nematode *T. aceti* declines as a function of increasing age. The specific activity of the aldolase found in crude homogenates of 50-day old nematodes is about 50% of that of 7-day old worms. Such a drop in specific enzyme activity has been described by us previously for several nematode enzymes (Erlanger and Gershon, 1970; Gershon and Gershon, 1970).

This drop in specific activity of aldolase in crude preparations does not reflect a reduction in the number of enzyme molecules per animal, but rather the presence of enzyme molecules which are altered and either partially or totally devoid of enzyme activity. Immunological tests show that 1.8-fold more enzyme molecules are present per given enzyme activity in preparations of 50-day old animals than in preparations of 7-day old nematodes. We have ruled out the possibility that the change found in aldolase of old animals was caused by the appearance of new isozymes during the development of the nematode. Such a change, occurring in early development, has been described in other species by Lebherz and Rutter (1969) and by Hershkovitz *et al.* (1967). The electrophoretic mobility of the active enzyme as determined on cellulose acetate membranes is unchanged and no isozymes could be detected in any of the age groups. The attainment of equal maximal inhibition, albeit with increasing amounts of antibody, in aldolase preparations from various ages strongly indicates that the aldolase subunits in young and in old nematodes are identical or extremely similar. In support of this conclusion is the work of Chen *et al.* (1970) who showed that antialdolase A serum could not fully inhibit aldolase which was composed of A and C subunits. As the proportion of the C subunits in the enzyme increased less inhibition was obtained. Precipitation of aldolase of senescent nematodes by antibody under conditions of varying degrees of antigen excess as well as in the equivalence zone of maximal precipitation led to the total removal of free antibody from the reaction mixture. Thus, no residual anti-aldolase antibody remained to precipitate enzyme from young adult nematodes. Thus, it has been demonstrated that no additional isozymic forms of aldolase appear in senescent *T. aceti*. If there are isozymic forms of the enzyme which we have not been able to detect, they certainly appear not to change in their relative proportions with age. We conclude that the active enzyme molecules in old and young nematodes are similar, and there is no change regarding the subunit composition of the enzyme. The only change which has been observed is the K_m which is 2.0×10^{-5} M in young nematodes and which changes gradually with age and reaches a value of 0.9×10^{-5} M in 50-day old nematodes.

One can propose two possible mechanisms to explain the age-related reduction in specific enzyme activity. The first one assumes postsynthetic modification of enzyme molecules. These modifications may be similar to those found by Lai *et al.* (1970) in rabbit muscle aldolase in which the deamidation of asparagine leads to the presence of α and β subunits. Flatmarck and Slatten (1968) have also described a postsynthetic alteration in cytochrome *c*. Although these modifications have not been shown to alter the specific activity of the enzyme, this may not be true for all postsynthetic alterations, as for example, the inactivation by dephosphorylation of glycogen synthetase. If indeed such modifications do occur, their detection will be possible only if the rate of modification is greater than the rate of degradation, thus leading to an accumulation of modified enzyme molecules. The observed extension of the half-life of aldolase with increasing nematode age might facilitate such detection. The second mechanism which may be proposed as being responsible for the production of defective enzyme molecules in aging organisms is that suggested by Orgel (1963, 1970) in which errors in the machinery of protein synthesis are envisaged. Such errors will lead to changes in the primary structure of newly synthesized enzymes resulting in both partially and completely inactive enzyme molecules.

The accumulation of cross-reacting material with age has been found with several enzymes in various phylogenetically diverse species, such as the present findings with aldolase and isocitrate lyase (Gershon and Gershon, 1970) of *T. aceti*, liver and muscle aldolases of mice (Gershon and Gershon, 1973a,b), and glutamate dehydrogenase in *Neurospora crassa* (Holliday, 1969). It, therefore, seems to be a general phenomenon related to aging. It is not known what the direct effect of the accumulation of CRM is on aging cells but it is possible that the cell may suffer from insufficient enzyme activity in some crucial metabolic pathways. Also, the production of faulty structural proteins which are components of membranes may have a detrimental effect on the physiological functions of the cell.

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Model Reactions Which Establish a Facile Reduction of Pyridoxal Phosphate and Analogs by 1,4-Dihydropyridines†

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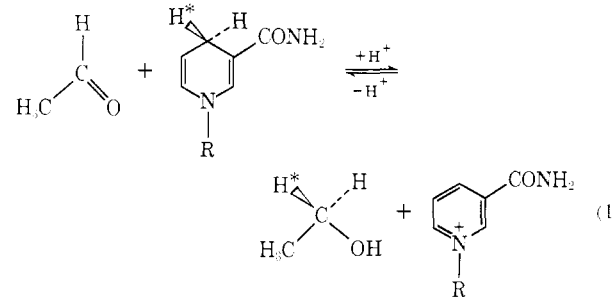
ABSTRACT: A kinetic study of the reaction of pyridine-4-carboxaldehydes with 1,4-dihydropyridines is reported. 3-Hydroxypyridine-4-carboxaldehyde (PyrCHO), but not pyridine-4-carboxaldehyde, is reduced to the corresponding alcohol by N^1-(*n*-propyl)-1,4-dihydronicotinamide (NPrNH) in refluxing methanol. Under the same conditions, PyrCHO and pyridoxal are reduced by 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridine (Hantzsch ester, HE). Pyridoxal phosphate (pyridoxal-P) and pyridoxal are readily reducible by HE and NPrNH in *ca.* 50% aqueous methanol at 30°. The apparent second-order rate constants for the reaction of HE with pyridoxal are comparable at 30° in aqueous methanol and in boiling neat methanol. Inclusion of 5×10^{-3} M EDTA or 1.2×10^{-3} M hydroquinone in buffered aqueous methanol reaction mixtures of NPrNH and pyridoxal-P did not influence the rate, and nmr product analysis of the reaction of HE and pyridoxal in both refluxing CH_3OD and 48% CH_3OD–D_2O (30°) established solvent deuterons not to be incorporated into product pyridoxine. These results establish the reduction to be a non-free-radical mediated direct hydrogen transfer from dihydropyridine to aldehyde which does not require trace metals as catalysts. In separate experiments, metal ion catalysis of the reduction of pyridoxal-P and PyrCHO by HE was established. The order of metal ion catalysis ($Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+} > 0$) was found to be that previously established for complexation to pyridine and salicylaldehyde (Brewer, D, G., and Wong, P. T. T. (1966), *Can. J. Chem. 44*, 1407; Mellor, D. P. and Maley, L. (1947), *Nature (London) 159*, 370; Martell, A. E., and Calvin, M. (1952), "Chemistry of the Metal Chelate Compounds," New York, N. Y., Prentice-Hall, p 546). From the pH dependence of the apparent second-order rate constants for the reaction of pyridoxal-P with HE and NPrNH, the individual second-order rate constants for reduction of the various ionic species of pyridoxal-P were calculated. The rates of dihydropyridine reduction were found to parallel the rates of imine formation (Auld, D. S., and Bruice, T. C. (1967a), *J. Amer. Chem. Soc. 89*, 2083) and transamination (Auld, D. S., and Bruice, T. C. (1967b), *J. Amer. Chem. Soc. 89*, 2090) for like ionic species (Table VI).

The nicotinamide nucleotides, nicotinamide adenine dinulceotide (NAD^+) and its 2′-phosphoric acid derivative ($NADP^+$) with their reduced forms (NADH, NADPH), are coenzymes in a very large number of enzymatic oxidations and reductions (Bruice and Benkovic, 1966). Alcohol dehydrogenases of both yeast and liver, for example, contain Zn^{II} and NADH at the active sites and catalyze the stereospecific transfer of a hydride ion (or its equivalent) to aldehyde substrates (for a review see Popják, 1970). In order to better understand the mechanisms of catalysis of "hydride" transfer from dihydronicotinamides to aldehydes, searches have been made, to no avail, for aldehydes which are reducible in water at ambient temperatures by dihydronicotinamides (Bruice and Benkovic, 1966). Though enzymatic reduction of pyridoxal-P[1] by pyridine nucleotide has been established (Morino and Sakamoto, 1960; Holzer and Schneider, 1961) the



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[1] Abbreviations employed are: nicotinamide adenine dinucleotide and its reduced form, NAD^+ and NADH; 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridines (Hantzsch ester), HE; 1-propyl-3-carbamidopyridinium ion and *N*-propyl-1,4-dihydronicotinamide, $NPrN^+$ and NPrNH; 3-hydroxypyridine-4-carboxaldehyde, PyrCHO; pyridoxal phosphate, pyridoxal-P; 3-hydroxypyridine-4-methanol, $PyrCH_2OH$.