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Age-dependent Properties of Acid & Alkaline DNases in Chick Brain

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Acid and alkaline DNase activities in partially purified preparations from young and old chick brain were measured. The specific activity of acid DNase from old brain was lower by about 50% than that of enzyme from young brain, whereas alkaline DNase exhibited only marginal difference in activity of the two preparations. Study of various properties, viz. heat-stability and effect of exogenous compounds like Mg^{2+}, Hg^{2+}, Zn^{2+}, PHMB, on these enzymes revealed that while acid DNase in old brain is more susceptible to heat and heavy metal ion inhibition, alkaline DNase is devoid of any age-dependent variation in its properties.

Age-related alterations in the activities of certain enzymes in various animals have been reported by a number of investigators[1-3]. Earlier studies from this laboratory have also shown that the activity of both acid and alkaline DNases in chicken brain homogenates decrease with age[4-6]. In the case of acid DNase, apart from the decreased specific activity, the total activity also decreased beyond the 10th day of post-hatching. However, the total activity of alkaline DNase remained at a constant level beyond the 10th day of hatching, although the specific activity decreased by about 50%. The physiological significance of such reduced activities of these enzymes with age is not known. Several theories have been proposed to offer a biochemical basis for the process of aging[7-11]. As per these theories, the reduced activity of any given enzyme could be due to any one or a combination of the following factors: (i) introduction of several errors in the amino acid incorporation during the synthesis of enzyme protein, (ii) an age-specific repression of the genes responsible for the eventual synthesis of the enzyme, and (iii) an age-dependent change in the conformation of the protein which may have resulted in the altered activity.

The present investigation, therefore, is an attempt to gain further insight about how the behaviour of DNases from young and old chick brain differs as a result of a variety of perturbations imposed upon them *in vitro*. Several properties, viz. heat-stability and effect of exogenous compounds on both acid and alkaline DNases, were studied with the partially purified enzyme preparations from young and old brain. Our results in general show that acid DNase activity is decreased with age, possibly due to the age-specific alterations suffered by the protein responsible for this activity. On the other hand, alkaline DNase from old brain exhibits comparable specific activity and properties as that from young brain.

Materials and Methods

Either fertilized eggs or chicks of specified age (white leghorn) were obtained from a local poultry farm. Sixteen days incubated eggs were chosen as the source for young brain enzyme, whereas the old brain enzyme was prepared from male chicks of age more than one year.

Highly polymerised calf thymus DNA, *p*-hydroxymercuribenzoate and 2-mercaptoethanol were purchased from Sigma Chemical Company, USA. All the reagents used were of analytical grade.

The cerebral hemispheres of chick brain were removed and a 10% homogenate was prepared in cold glass distilled water using Potter Elvehjem type homogenizer with a Teflon Pestle. Brain extracts were obtained by centrifuging the homogenates at 30,000 *g* for 60 min at 0-4°C. Solid ammonium sulphate was added to the supernatant to give saturation as calculated from the formula of Noda and Kuby[12]. Before assaying the enzyme activities, the pellet was dialysed against water at 0-4°C until the ammonium ions were completely removed.

The DNases were assayed in both crude and purified fractions according to the method of McDonald[13] by measuring the acid-soluble deoxyribose at the end of the incubation. The activity was also followed by the increase in UV absorption at 260 nm of the acid-soluble fraction. Other details regarding the assay of DNases have already been described[4]. Protein was estimated, depending upon the concentration of the proteins in the sample, either by the Biuret method[14] or as per Lowry *et al.*[15].

Kinetics of heat inactivation were studied with crude extract as well as with the partially purified enzyme. In the case of the partially purified enzyme, the solution was diluted with water to a protein concentration 2-3 mg/ml. The enzyme preparation was maintained at the desired temperature. At varying time intervals, a small

aliquot was withdrawn and assay of the enzyme activity was carried out as usual.

In order to study the effect of various exogenous compounds on enzyme activity, the required concentrations were maintained in the standard assay mixture and then the enzyme was assayed as usual. Solutions of various compounds, except those of EDTA and PHMB, were made in glass-distilled water. In the case of EDTA, care was taken to see that the *p*H of stock solutions is 7.0. Stock solution of PHMB was prepared in 0.1*N* NaOH.

Results

In view of the presence of an inhibitor for acid DNase in chick brain[16], the possibility of the presence of some inhibitor(s) for DNases in higher amounts in old brain was checked by mixing the enzymes from young and old chick brains in various proportions. Table 1 shows the results obtained in such experiments. It can be seen that in either case no significant reduction in the activity could be noticed when enzyme from old brain was mixed with that from young one. At every level of mixing, the observed activity was in close agreement with the expected activity.

In general, acid DNase activity was found to be less susceptible to heat as compared to alkaline DNase. For example, alkaline DNase activity suffers loss when the preparation is incubated at 50°C, while a significant loss of activity in the case of acid DNase could be noticed only at a preincubation temperature of 60°C. After these preliminary studies with crude brain extracts, it was thought that the measurement of each of the DNase activity is bound to be influenced, at least to some extent, by the other DNase activity. It was therefore considered appropriate to purify these activities at least to the extent of obtaining them free from each other. The data on the partial purification and separation of acid and alkaline DNase activities from young and old brain presented in Table 2 show precipitation of acid DNase and alkaline DNase activities at 60-85 and 30-50% ammonium sulphate saturation respectively. It is also to be noted that the cross contamination of the DNase preparations at either age is negligible (Table 2). In terms of the absolute fold purification, approx. 3-and 1.5-fold was achieved in the case of acid and alkaline DNase respectively. Further, while the specific activity of acid DNase in partially purified preparation showed a 50% reduction due to old age, the decrease in the specific activity of alkaline DNase in old brain was only about 30% and even this reduction was statistically significant only at 5% level (Table 3).

The effect of preincubation of the partially purified preparations at 65°C on the stability of acid DNase is

Table 1—Acid and Alkaline DNase Activities Observed in Mixed Homogenates from Young and Old Chick Brain

[Extracts from young (14 days of embryonic life) and old (1 year) chick brain were prepared and DNases were assayed as described in Materials and Methods. Specific activity is expressed as μg of acid-soluble deoxyribose P liberated/mg protein/2hr. The results are averages of three different incubations]

Enzyme protein (mg)		Specific activity*	
Young +	Old	Acid DNase	Alkaline DNase
4	0	12.45	15.9
0	4	1.40	10.2
1	3	4.70 (4.16)	10.0 (11.6)
2	2	6.44 (6.93)	12.2 (13.0)
3	1	9.45 (9.70)	13.0 (14.5)

*Numerals given in parentheses are the expected values

Table 2—Separation of Acid and Alkaline DNases from Young (16 Days of Incubation) and Old (1½ Years) Chick Brain

[The yield, at a given saturation of ammonium sulphate, is expressed as percentage. The total activity in original brain extract is taken as 100%. Method of assay is described under Materials and Methods. The results given here are from a typical experiment. Similar results were obtained in other experiments also]

Fraction	Acid DNase			Alkaline DNase		
	Total activity units	Yield (%)	Sp. activity	Total activity units	Yield (%)	Sp. activity
			Young chick brain			
Brain extract	13723	100	13.73	6572	100	14.5
0-30%	66	0.48	1.93	157	2.4	0.88
30-50%	199	1.45	1.30	2655	40.4	18.8
50-60%	44	0.32	1.95	88	1.34	0.40
60-85%	6203	45.2	44.14	138	2.1	0.54
			Old chick brain			
Brain extract	1524	100	3.9	3183	100	7.4
0-30%	66	4.3	1.2	29	0.9	1.3
30-50%	49	3.2	1.2	1019	32	11.0
50-60%	0	0	0	67	2.1	0.9
60-85%	450	29.5	21.0	0	0	

Table 3—Specific Activities of Acid and Alkaline DNases in Young and Old Chick Brain

[The specific activities are expressed as described in Table 1. Each value represents the average ±SD of four or more experiments. Whenever a value was found to be significantly different from the corresponding value at young age, the level of significance is shown in parentheses]

Fraction	Acid DNase		Alkaline DNase	
	Young	Old	Young	Old
Crude extract	15.5±3.7	4.3±1.0 ($P<0.001$)	9.9±3.3	6.6±0.9 ($P<0.05$)
Partially purified fraction	40.5±7.3	19.5±5.1 ($P<0.001$)	13.7±3.6	9.9±1.4 ($P<0.05$)

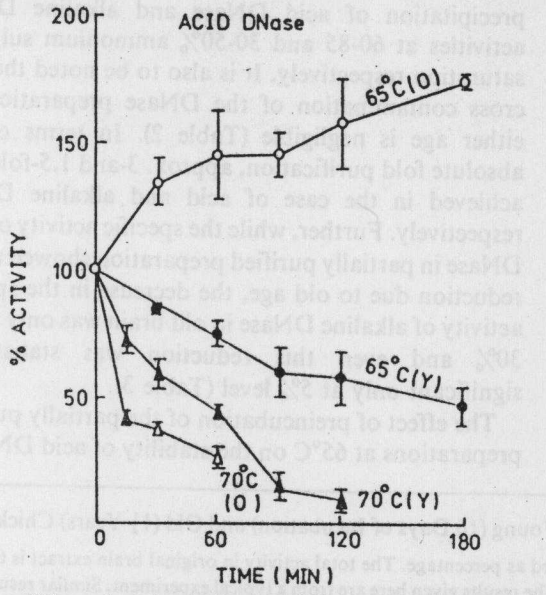

Fig. 1—Effect of preincubation at different temperatures for varying periods on the partially purified acid DNase preparations from young and old chick brain [for other details see text]

shown in Fig. 1. While the enzyme from young brain suffered loss of activity with time in a multiphasic manner, the enzyme from old brain experienced increase in activity with time again in a multiphasic manner. However, when the preincubation temperature was raised to 70°C, it resulted in rapid loss of activity irrespective of the age, the rate of loss being more in the case of old enzyme.

Preincubation of partially purified alkaline DNase, either from young or old chick brain, at 45°C for varying periods resulted in considerable loss of activity (Fig. 2); however, the rate of loss of activity was similar in both the cases. When the preincubation temperature was raised to 60°C, there was only 44 and 40% activity after 15 min in young and old enzyme preparations respectively. Further, the activity was completely lost within 3 min when the temperature was raised to 70°C. In another experiment, the enzymes were preincubated

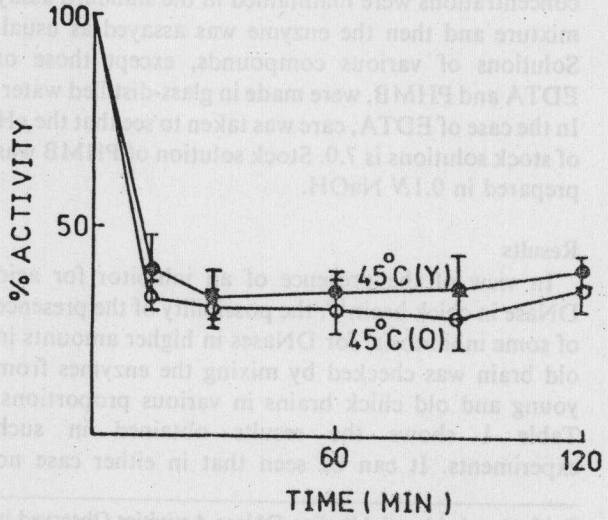

Fig. 2—Effect of preincubation at 45°C for varying periods on the partially purified alkaline DNase preparations from young and old chick brain [for other details see text]

for a fixed time of 15 min but at varying temperatures (45, 50 and 60°C). The results showed the same trend as described above (data not shown).

The effect of various exogenous compounds on acid DNase obtained from young and old chick brain is shown in Table 4. The acid DNase from both young and old chick brain showed activation in presence of Mg^{2+}. However, the enzyme from young brain exhibited maximum activation in presence of 5 m*M* Mg^{2+}, whereas that from old brain showed maximum activity only at 10 m*M*. Concentrations beyond these levels resulted in decreased activity. The enzyme activity was inhibited by either Hg^{2+} or Zn^{2+} and the inhibition was more in the case of enzymes obtained from old brain. PHMB exerted inhibition only at 1 m*M* level and the inhibition was more in the case of old enzyme. Further, urea at 2*M* concentration activated the enzyme of old brain but not of young one, while at 6*M* level urea was inhibitory to enzymes of both ages, once again the inhibition being slightly but significantly more in the case of old enzyme. 2-Mercaptoethanol, EDTA and NH_4^+ were found to be devoid of any effect on acid DNase activity (data not shown). The point, however, to be noted is that whenever either activation or inhibition is seen this effect is significantly more in the old brain preparation.

The results of the study on the effect of Mg^{2+} and EDTA on the activity of alkaline DNase presented in Table 4 show that the enzyme from old brain requires much lesser amount of Mg^{2+} (0.1 m*M*), for its maximum activity than the enzyme from young brain (1 m*M*). This result is further substantiated from the observed effect of EDTA. Enzyme from old brain was more sensitive to EDTA as compared to the enzyme from young brain.

Table 4—Effect of Some Exogenous Compounds on Partially Purified Acid and Alkaline DNase Activities in Young and Old Chick Brain

[Activities are expressed as percentages ± SD from six experiments where control value is taken as 100%]

Addition (m*M*)		Chick brain	
		Young	Old
		Acid DNase activity	
Control		100	100
Mg^{2+} :			
	1.0	125±12	119± 3
	5.0	140±14	127± 5
	10.0	118±17	152±14
	30.0	67± 4	49± 9
Hg^{2+} :			
	0.05	92± 9	67±12*
	0.10	38±10	21± 5*
Zn^{2+} :			
	0.05	103± 9	103± 4
	0.10	96± 5	61± 9*
	5.00	30± 5	21± 5*
PHMB :			
	0.10	104± 6	96± 8
	1.00	63± 7	47±10*
UREA :			
	2M	93± 7	131± 6*
	6M	76± 5	61± 2*
		Alkaline DNase activity	
Mg^{2+} :			
	0.1	143±11	195±15†
	1.0	232±26	135±10
	5.0	64± 3	64± 7
	10.0	65± 4	58± 6
EDTA :			
	0.01	96± 6	82± 5*
	0.10	34± 3	17± 5*
	0.20	30± 3	21± 5*

*This value is significantly different from the corresponding value of young enzyme, $P<0.025$.

†This value is significantly different from the corresponding value of young enzyme, $P<0.001$.

Further, the effect of various exogenous compounds like Hg^{2+}, Zn^{2+}, 2-mercaptoethanol, PHMB, urea and NH_4^+ on alkaline DNase from young and old brains was also tested. Hg^{2+} (0.01 m*M*), Zn^{2+} (0.01 m*M*), PHMB (0.1 m*M*) and urea (6*M*) inhibited the activity significantly (>30%) whereas 2-mercaptoethanol (10 m*M*), urea (2*M*) and ammonium ion (20 m*M*) showed activation (>40%) irrespective of the age (data not shown).

Discussion

Reduction in the activity of any enzyme in an aging tissue could be due to various reasons. One such possibility is the presence of excessive amounts of inhibitor(s) for the enzyme in old tissue. That animal tissues have natural inhibitors for both acid and alkaline DNases has been well documented[17-20]. Recently Bhattacharya *et al.*[16] have demonstrated the presence of an inhibitor for acid DNase in chick brain and clonal lines of neuroblastoma cells. The inhibitor was proteinous in nature and trypsin-sensitive. Curiously, the highest levels of this inhibitor were found in embryonic chick brain at which time acid DNase also exhibited high activity. The results of our experiments in which homogenates from old brains were mixed with those from younger ones revealed no extra amount of inhibitor in the old homogenates (Table 1). At any level of mixing the observed activity was same as expected. Of course it is still possible that the inhibitor, if present in higher amounts in old brain, is tightly bound to the enzyme and therefore has no chance to exert its influence on the younger enzyme. In any case our results do indicate that there is no extra lossely-bound inhibitor of either acid or alkaline DNase in old chick brain.

Seeking a biochemical explanation for the lowered activities of certain enzymes in aging tissues, Orgel[7] proposed that this might be due to the errors introduced in primary structure during the biosynthesis of the enzymic protein. Since the 'errors' would result in introduction of wrong amino acids at various points of the primary structure of the protein, this defective enzyme protein could be expected to behave somewhat differently as compared to the normal enzyme. While the earlier work with aldolase in crude mouse liver[1] and mouse muscle homogenates[21] indicates the presence of 'altered' enzyme in old tissues, data accumulated subsequently from different laboratories on various enzymes both in mammalian species and free living nematodes do not support unequivocally the 'error' theory. On the other hand, Rothstein[11] believes that the changed activity of various enzymes in old tissues could be due to conformational change in the protein and not necessarily due to a change in the primary structure. Indeed subsequent work from the same laboratory on purified enolase from young and old *Turbatrix aceti*, a free living nematode[3], and on thymidine kinase of mouse cerebellum by Caron and Unsworth[22] does not lend support to the concept that observed change in the specific activity of these enzymes is due to the change in the primary structure. On the other hand, they believe that the observed differences might be due to conformational change and synthesis of a different molecular form of the enzyme.

Be as it may, our present data with partially purified acid DNase from young and old brain do give the scope to suspect that the acid DNase from old brain is

'different' from that of young brain. Thus, preincubation of acid DNase from old brain at 65°C resulted in activation, while the same treatment to the young enzyme resulted in gradual loss of activity with time (Fig. 1). Further, acid DNase from old brain is much more sensitive to both Hg^{2+} and Zn^{2+} (Table 4). Whenever an effect was seen on the acid DNase activity due to an exogenous factor, this effect was found to be, to a greater extent, in aging preparations (Table 4). Also the old enzyme needed higher amounts of Mg^{2+} to attain maximum activity (Table 4). From these considerations, it can probably be assumed that the acid DNase elaborated in old brain cells is 'altered' or 'different' from the one in young tissue. It is however not possible from the present data to conclude whether the reduced specific activity of this enzyme in aging tissue is due to a change in the primary structure itself or due to post-translational conformational change.

In contrast, the results concerning the altered properties of alkaline DNase in old age are equivocal. Thus, to begin with, the reduction in the specific activity of this enzyme in partially purified preparations due to old age is only about 30%. Even this difference was found to be statistically significant only at 5% level (Table 3). It is therefore probably justified in assuming that this decrease is only marginal. Also, heat inactivation studies (Fig. 2) revealed that both young and old enzymes suffer loss of activity due to preincubation at 45°C in a similar fashion. Further, the old alkaline DNase seems to be more responsive to Mg^{2+} activation. Complementary to this property is the observation that alkaline DNase from old brain is slightly more sensitive to EDTA (Table 4). The perturbations caused by several other effectors on alkaline DNase activity are similar irrespective of age (see results section). We are therefore inclined to believe that the alkaline DNase present in aging chick brain may not be different from that of the young age. Further work with homogeneous preparations of these enzymes should provide more definitive answers.

Acknowledgement

We wish to place on record the kind encouragement we received from Prof. Gurubakhsh Singh, the then Vice-Chancellor of this University. KPS and NIS are Pool-Officer and junior research fellow respectively of the Council of Scientific & Industrial Research, New Delhi.

References

1 Gershon H & Gershon D (1972) *Proc natn Acad Sci, USA*, 70, 909-913.
2 Reiss V & Gershon D (1976) *Eur J Biochem*, 63, 617-623.
3 Sharma H K & Rothstein M (1978) *Biochemistry*, 17, 2869-2876.
4 Shrivastaw K P & Subba Rao K (1975) *J Neurochem*, 25, 861-865.
5 Subba Rao K & Shrivastaw K P (1976) *J Neurochem*, 27, 1205-1210.
6 Subba Rao K & Shrivastaw K P (1979) *J Biosci*, 1, 69-74.
7 Orgel L E (1963) *Proc natn Acad Sci, USA*, 49, 517-521.
8 Kanungo M S (1970) *Biochem Rev*, 41, 13-23.
9 Kanungo M S (1975) *J Theor Biol*, 53, 253-261.
10 Rothstein M (1975) *Mech Ageing Develop*, 4, 325-338.
11 Rothstein M (1977) *Mech Ageing Develop*, 6, 241-257.
12 Noda L & Kuby S A (1957) *J biol Chem*, 226, 541-549.
13 McDonald M R (1955) *Meth Enzym*, 2, 437-447.
14 Gornall A G, Bardawill C J & David W W (1949) *J biol Chem*, 177, 751-766.
15 Lowry O H, Rosebrough N J, Farr A L & Randall R J (1951) *J biol Chem*, 193, 265-275.
16 Bhattacharya P, Moskal J R & Basu S (1977) *Proc natn Acad Sci, USA*, 74, 842-845.
17 Laskowski Sr M (1971) in *The Enzymes*, Vol.4 (Boyer P D, ed.), pp.289-311, Academic Press, New York.
18 Bernardi G (1971) in *The Enzymes*, Vol. 4 (Boyer P D, ed.), pp. 271-287, Academic Press, New York.
19 Lesca P (1976) *J biol Chem*, 251, 116-123.
20 Keenan T, Freudenstein C & Franke W W (1977) *Cytobiologie*, 14(2), 259-278.
21 Gershon H & Gershon D (1973) *Mech Ageing Develop*, 2, 33-41.
22 Caron P C & Unsworth B R (1978) *Mech Ageing Develop*, 8, 181-195.

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5 Subba Rao K & Shrivastaw K P (1976) *J Neurochem*, 27, 1205-1210.
6 Subba Rao K & Shrivastaw K P (1979) *J Biosci*, 1, 69-74.
7 Orgel L E (1963) *Proc natn Acad Sci, USA*, 49, 517-521.
8 Kanungo M S (1970) *Biochem Rev*, 41, 13-23.
9 Kanungo M S (1975) *J Theor Biol*, 53, 253-261.
10 Rothstein M (1975) *Mech Ageing Develop*, 4, 325-338.
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