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DROSOPHILA DEOXYRIBONUCLEASES. I.

VARIATION OF DEOXYRIBONUCLEASES IN *DROSOPHILA MELANOGASTER*

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SUMMARY

A technique for detecting deoxyribonucleases after their fractionation by disc electrophoresis has been used to study these enzymes in *Drosophila melanogaster*. A complex spectrum of enzymatic activities has been found which undergoes striking variation during development. An analysis of the substrate and divalent cation requirements of these activities indicates that this organism produces at least seven distinct enzymes throughout the course of development. The stage-specific appearance of certain activities is discussed in regard to their possible function.

INTRODUCTION

Until quite recently the emphasis of most work involving deoxyribonucleases has been on their use as tools for investigating DNA structure. *Escherichia coli*, which has been most extensively studied for this purpose, has been shown to produce a minimum of six distinct enzymes[1,2]. At the present time at least six different enzyme types have also been characterized in mammalian tissues[3,4]. As it became apparent that most organisms produce a wide spectrum of deoxyribonucleases, the question of their distribution and function became more important. The existence of such a large variety of enzymes suggests that some of them have cellular functions other than the most obvious one of digestion[5].

With this question in mind, an investigation of the deoxyribonucleases of Drosphila has been undertaken. Drosophila was chosen because of its potential for genetic studies as well as the fact that the stages of its tissue development are often strictly divided between the processes of growth, differentiation, and histolysis. The development of methods for culturing large numbers of well synchronized Drosophila[6] has made it possible to study these processes individually with conventional biochemical procedures. In addition, the giant chromosomes of this organism display tissue and stage-specific differentiation which provide the possibility of relating protein fluctuations to observable gene activities[7].

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In order to provide the necessary background for studying these problems, a survey has been made of the major deoxyribonuclease activities present in this organism at various times during development. This phase of the work has been facilitated by a method for detecting deoxyribonucleases in acrylamide after their separation by disc electrophoresis. The procedure, which has been described elsewhere[8], is similar to standard disc electrophoresis with the exception that high molecular weight DNA is trapped in the gel matrix during polymerization of the gel. After separation of the proteins by electrophoresis, the gels are incubated at conditions appropriate for deoxyribonuclease activity. During the incubation, the trapped DNA is degraded at those positions occupied by deoxyribonucleases. After the degradation products and proteins have been removed by a second electrophoresis step, the remaining DNA is stained. The original positions of the deoxyribonucleases are identified as discontinuities in the substrate background. By utilizing the speed and resolution of the standard disc electrophoresis procedure, this method permits a more rapid survey of deoxyribonuclease activities than has previously been possible.

A preliminary report of this survey appeared with the description of the method[8].

MATERIALS AND METHODS

The Oregon R wild-type strain of *Drosophila melanogaster* was cultured and isolated as described by MITCHELL and co-workers[6,9]. Larval ages were calculated from the middle of a 2-h egg collection, pupal ages from the time of puparium formation, and fly ages from the day of emergence. Homogenization was performed at 0° in a cone glass grinder with standard electrophoresis buffer at a concentration of 75 mg *D. melanogaster* per ml. Homogenates were prepared within the hour preceding electrophoresis and were centrifuged at 75 000 × *g* for 15 min before use.

Electrophoresis was performed in 7.5% acrylamide gels containing 0.75 mg native DNA per ml gel or 0.67 mg denatured DNA per ml gel. Electrophoresis was terminated 0.5 h after the bromphenol blue marker had run off the end of the gel. Incubation was performed at 37° for 35 min. The primary incubation buffers were 0.085 M acetate–0.005 M $MgCl_2$ and 0.06 M sodium citrate–0.005 M EDTA. Densitometry tracings were made from all gels after their ends had been broken off at the original position of the bovine serum albumin marker. No nucleases were observed to migrate faster than this marker protein.

RESULTS

Developmental study

Representative densitometry tracings obtained from two stages of *D. melanogaster* development are presented in Figs. 1 and 2. From such patterns it is possible to distinguish between enzymatic activities not only on the basis of their electrophoretic mobility but also on the basis of their requirements for specific substrates and incubation conditions. Such distinctions are useful for an analysis of the patterns because of the great variety and fluctuation of enzymatic activity that have been found during development.

An analysis of the deoxyribonucleases, found at seven developmental stages, has been made using two types of measurements taken from such patterns. Firstly,

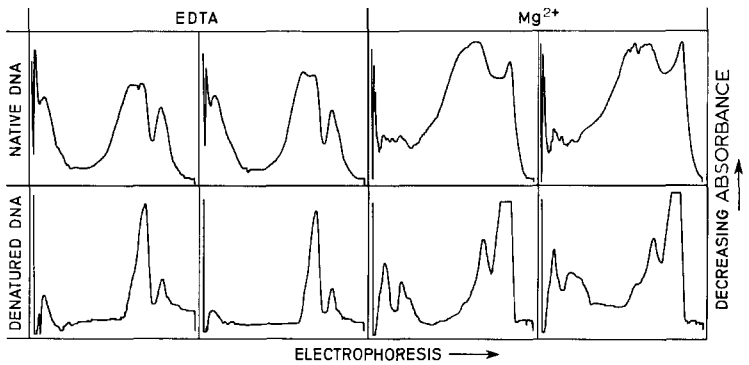

Fig. 1. Gel deoxyribonuclease patterns. All patterns were obtained from the same homogenate of 87-h larvae. Densitometry tracings obtained from duplicate gels are presented for each set of incubation conditions. Incubation was at pH 4 in the presence of EDTA or Mg^{2+} as described in the text. The relative lengths of the patterns have been adjusted photographically to facilitate intercomparisons.

the electrophoretic migration of the activities has been measured relative to the migration of a marker protein (bovine serum albumin) in order to permit direct comparisons between different patterns. These data are presented in Tables I and II. The relative migration (R_m) measurements represent the ratio of the distance traveled by the activity to the distance traveled by the bovine serum albumin marker. R_m values, obtained from different developmental stages, have been averaged when they are close enough to suggest continuity of a single activity through several stages. The R_m values of activities which appear within the range R_m 0.1–0.4 were not averaged because of their failure to satisfy any clear pattern. As a further analysis of the patterns, the heights of the peaks above background have been measured to provide a rough quantitative estimate of enzymatic activity. These data are plotted in Fig. 3 against the average R_m values, calculated in Tables I and II.

In order to eliminate the possibility of variation sometimes found between different substrate samples, these experiments were carried out with preparations of DNA that had been checked against a standard preparation. All results represent the

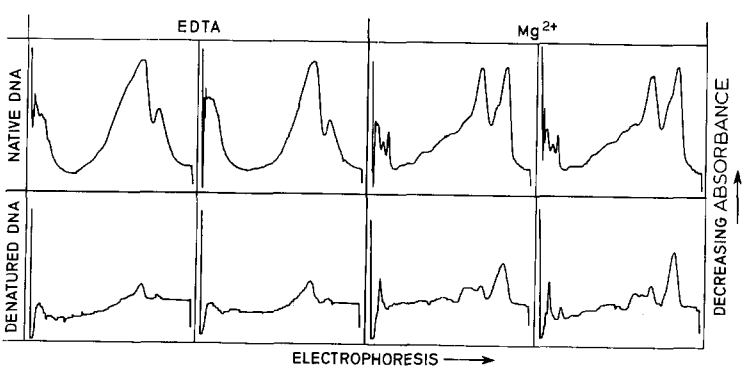

Fig. 2. Gel deoxyribonuclease patterns obtained from 15-days-old flies. Assay conditions are the same as those described for Fig. 1.

TABLE I

RELATIVE ELECTROPHORETIC MIGRATION OF DEOXYRIBONUCLEASES FROM *D. melanogaster*

Incubation at pH 4.0 in the presence of Mg^{2+}. Values in brackets were obtained from a shoulder of a larger peak, and the slope of the curve at the shoulder did not change sign.

Animal age		*Migration relative to bovine serum albumin*											
Native DNA													
61-h	Larvae	0.85	0.77	0.70	0.61				0.22	0.14		(0.07)	0.0
87-h	Larvae	0.86		0.65	0.58				0.18	0.13	0.09	0.08	0.0
115-h	Larvae	0.84	(0.76)	0.68	(0.58)		(0.39)					0.08	0.0
6-h	Pupae	0.83		0.69	0.57				0.29			(0.09)	
67-h	Pupae	0.85	(0.74)	0.69	(0.64)				0.25			0.09	
1-day	Flies	0.87	(0.78)	0.70	0.59	(0.49)	0.42					0.09	0.0
15-day	Flies	0.85	(0.79)	0.69	(0.56)	(0.45)			(0.33)		0.10	0.07	0.0
Average		0.85	0.77	0.69	0.59	0.47	0.41					0.08	0.0
Denatured DNA													
61-h	Larvae	0.83		0.70	0.62		0.40	0.26	0.22	0.13		0.07	
87-h	Larvae	0.83		0.69	0.58				0.23	0.18		0.07	
115-h	Larvae	0.79		0.68	(0.57)		(0.45)					0.08	
6-h	Pupae	0.80	0.74	0.70			0.45						
67-h	Pupae			0.70			0.39					0.05	
1-day	Flies	(0.82)		0.69	(0.57)								
15-day	Flies	0.84		0.69	0.60		0.45					0.06	
Average		0.82		0.69	0.59		0.42					0.07	

TABLE II

RELATIVE ELECTROPHORETIC MIGRATION OF DEOXYRIBONUCLEASES FROM *D. melanogaster*

Incubation at pH 4.0 in the presence of EDTA. Brackets have the same significance as in Table I.

Animal age		*Migration relative to bovine serum allumin*								
Native DNA										
61-h	Larvae						(0.13)	(0.06)	0.0	
87-h	Larvae	0.79		0.68	0.62			0.07	0.0	
115-h	Larvae	0.78		0.69	(0.64)	(0.52)		(0.05)	0.0	
6-h	Pupae	0.80		0.70	(0.62)			(0.06)	0.0	
67-h	Pupae			0.70				0.09	0.0	
1-day	Flies			0.70					0.0	
15-day	Flies	0.79		0.69				(0.07)	0.0	
Average		0.79		0.69	0.63			0.07	0.0	
Denatured DNA										
61-h	Larvae							0.06		
87-h	Larvae	0.79		0.69				0.06		
115-h	Larvae	0.78		0.68						
6-h	Pupae	0.80	0.75	0.70						
67-h	Pupae			0.70						
1-day	Flies			0.69						
15-day	Flies	0.80		0.69				0.06		
Average		0.79		0.69				0.06		

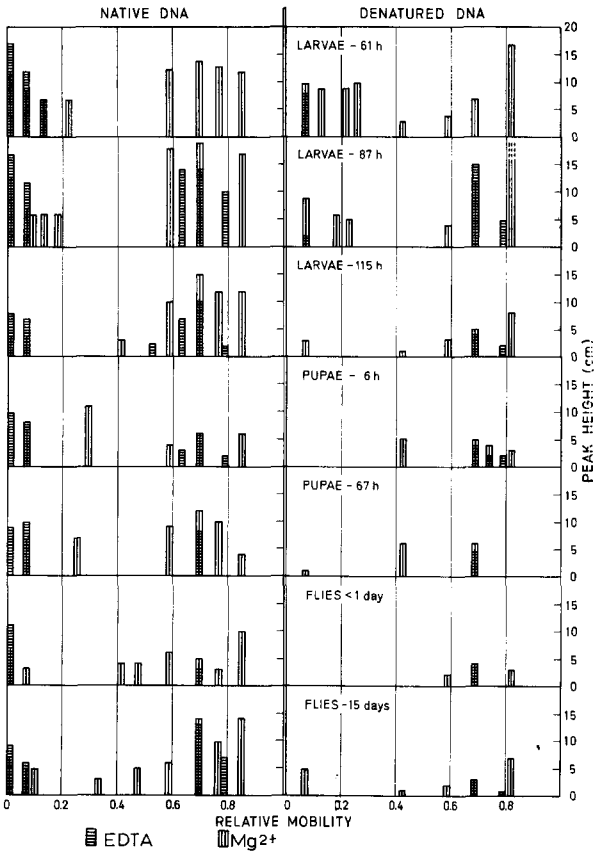

Fig. 3. Deoxyribonuclease patterns obtained from various developmental stages of *Drosophila melanogaster*. These data were compiled from densitometry tracings of stained gels. The height of each peak above background is plotted against its migration relative to bovine serum albumin. All gels were incubated at pH 4.0 in the presence of either EDTA or Mg^{2+}. Further details are given in the text.

average of values obtained from two identically treated gels. Because of the close spacing of some peaks, the absence of an activity at a particular stage of development cannot be considered significant when other large peaks are also present in this region.

Error estimation

Those tracings in Figs. 1 and 2, which were obtained from duplicate gels, demonstrate the reproducibility of the method. In a sample of 54 pairs of duplicate gels the average peak height difference found between corresponding peaks was 0.9 cm. The differences were generally independent of the peak heights themselves, and the maximum differences observed were 2.5 cm for native and 3.6 cm for denatured DNA. Because of the wide range of specific activities present in a homogenate, it is not possible to include every activity in a single gel within the linear range of the assay. Nevertheless, the peak-height data do provide a useful basis for crude comparisons of relative enzyme activity. It should also be noted that the highest peaks provide

only a minimum estimate of enzymatic activity, because most of the DNA has been degraded at these positions.

These and unpublished experiments have consistently demonstrated a very erratic behavior of the small peaks appearing in the range R_m 0.1–0.4. In a comparison of 20 duplicate experiments covering five developmental stages all corresponding R_m values agreed within R_m 0.04 with the exception of those falling in the 0.1–0.4 range. The values within this range were therefore not averaged in Tables I and II.

Supplementary controls

It has previously been shown that purified pancreatic deoxyribonuclease I does not degrade the DNA in the gel during electrophoresis, or during any of the other stages in the procedure which are performed at 0° (see ref. 8). As the densitometry tracings in Fig. 4 demonstrate, however, this is not always the case for *D. melanogaster*

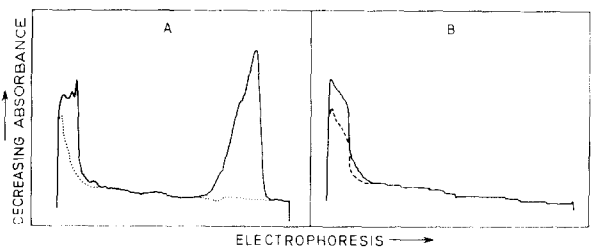

Fig. 4. Densitometry tracings representing enzymatic activity occurring at 0°. Two-thirds of the standard quantity of homogenate from 1-day-old flies was subjected to electrophoresis in gels containing native DNA. The standard nuclease detection procedure was followed except that the 37° incubation was replaced by an extended preincubation soaking of 60 min at 0° in pH 4.0 buffer. The preincubation buffers for Gels A and B (heavy lines) contained $MgCl_2$ and EDTA, respectively. — — —, densitometry tracings of a gel for which the preincubation soaking was omitted entirely; · · ·, of a gel which had received the complete treatment but contained no enzyme.

nucleases. The even background in these patterns suggests that none of the nucleases have been active during electrophoresis. Some enzymes are very active, however, at 0° during the presoaking steps of the procedure which precede the incubation and elution steps.

The data in Table III show that the R_m values of the individual activities do not change when the native DNA concentration is reduced by one-half. Thus, any interaction between these nucleases and the supporting matrix does not represent an enzyme–substrate interaction because it is equally expressed between the bovine serum albumin reference and the matrix. On the other hand, if Mg^{2+} is incorporated into the original gel together with native DNA, strong interactions probably occur during electrophoresis. In this situation none of the nuclease activity progresses past R_m 0.57, and most activity is expressed as a smear near the origin. The conclusion is, therefore, that most of the *D. melanogaster* nucleases do not interact specifically with DNA in the gel during electrophoresis, although such interaction can be induced by the presence of Mg^{2+}.

TABLE III

EFFECT OF DNA CONCENTRATION ON THE RELATIVE MIGRATION OF SOME *D. melanogaster* DEOXYRIBONUCLEASES

Two-thirds of the standard quantity of a homogenate from 1-week-old flies was assayed for deoxyribonuclease by the gel procedure. All gels contained native DNA and were incubated at pH 7.0 in 0.1 M Tris·HCl–0.005 M $MgCl_2$.

DNA concn. (mg/ml)	*Migration relative to bovine serum albumin*			
0.67	0.94	0.77	0.59	0.11
0.50	0.93	0.78	0.60	0.09
0.33	0.93	0.78	0.60	0.09
*Native DNA plus 0.001 M $MgCl_2$**				
0.67			0.57	smear —>

* Gel polymerized from a solution containing $MgCl_2$.

Organ distribution

A preliminary investigation has been made to determine how the observed nuclease activities are distributed among the larval organs of this insect. In each assay the number of organs tested corresponded to the equivalent number used in the standard whole animal extract. At this concentration the intestine and haemolymph displayed the most significant amount of activity, and the fat body produced one small peak. Neither the malpighian tubules nor the salivary glands contributed any activity. The activities, detected in intestine and haemolymph, are organ specific, indicating that separation of some of the activities can be achieved by dissection. The relation of these patterns to those obtained from the whole animal is not strictly additive and is, therefore, complicated by several factors including the probable presence of inhibitors. The problem of organ specific nucleases will be considered in detail elsewhere[10].

DISCUSSION

Minimum estimate of D. melanogaster deoxyribonucleases

Fig. 3 and Tables I and II reveal that a large number of peaks of enzymatic activity have been detected. This does not imply, however, that each peak was produced by a separate protein. On the contrary, there exists a strong possibility that several peaks represent isozymes, complexes, or aggregates of a more limited number of proteins. An estimate of the minimum number of enzymes may be obtained by first assuming that all activities represent aggregates of a single protein. An attempt is then made to single out those activities which disprove this assumption by possessing significantly different properties from the other activities. Each contradiction indicates that an additional enzyme is present.

Within the mobility range R_m 0.4–1.0, it is likely that the activities requiring Mg^{2+} are distinct from those requiring EDTA. This conclusion is suggested by the high concentration of the Mg^{2+}-activated components and the complete absence of the EDTA-activated components in 61-h larvae. Although these two types of activity

are found together at other stages of development, they are not in young larvae and therefore cannot be due to the same protein. At least three EDTA-activated components migrate to this area; one of which (R_m 0.63) is different from the other two by virtue of its inactivity in denatured DNA. The data, therefore, suggest the presence of at least two classes of EDTA-activated enzymes in this mobility range.

The data further show that of the six Mg^{2+}-activated components which appear within this range, the peak at R_m 0.77 is different from the others because of a strong preference for the native substrate. Thus, a minimum of two classes of Mg^{2+}-activated enzymes also appears in the range 0.4–1.0.

Observations taken from analyses of other organisms have shown that the activities appearing at the origin are usually characterized by a strong interaction with the substrate (ref. 8; J. B. BOYD, unpublished observations). *Drosophila melanogaster* pupae produce at least one such enzyme which is active only in EDTA. Since other developmental stages of the organism possess activity which appears at the origin in both Mg^{2+} and EDTA, at least two enzymes of this type are present over the total course of development. An extremely broad and predominent peak appears in pupae at R_m 0.25–0.29. Like the peak at 0.77, this one shows no activity in denatured DNA. These two activities are probably not aggregates of one another, however, because they are present at different times during development. Therefore, within the range 0.00–0.30, at least three activities have been detected which appear to be different from one another and from the others that have been singled out.

It is, therefore, concluded that of the large number of observed activites, at least seven probably represent distinct enzymatic types. The above analysis includes a number of assumptions and is attempted only to provide a crude estimate. This is very likely a conservative estimate, however, because minor activities have been observed to fuse into a single peak due to the limits of resolution of the method. It is anticipated that the application of additional criteria of separation will raise this estimate. Although a single incubation pH was used in the studies reported here, the data include most activities that have been detected with incubation buffers of higher pH. Several lines of evidence indicate that this effect is due to the fact that the ions of the incubation buffer rapidly enter the gel, but the pH within the gel itself changes much more slowly from what it was during electrophoresis. Thus the data probably include all enzymes that are active in the pH range 9–4. The majority of these activities are probably endonucleases, because the sensitivity of the method for endonucleases is much higher than that for exonucleases (J. B. BOYD, unpublished observations).

One of the assumptions implied in the above analysis is that the enzymes have the same mobility in gels containing native and denatured DNA. A given enzyme can be identified in both substrates by means of a single R_m value, when it has been shown that specific nuclease–substrate interactions have not occurred during electrophoresis. Experiments with both deoxyribonuclease I (ref. 8) and *D. melanogaster* nucleases (Table III), which were performed to test the possibility of such an interaction, have been negative. All information so far available suggests that if an enzyme interacts with the DNA during electrophoresis, it does so very strongly and remains at the origin. This evidence permits the tentative conclusion that the activities at R_m 0.62 (EDTA) and 0.77 (Mg^{2+}) display a strong preference for native DNA.

The possibility that some of the observed activities are derived from contami-

nating yeast can be excluded by several lines of evidence. 1. When the entire assay was performed on yeast instead of flies, no deoxyribonuclease activity was detected. This result indicates that the conditions of homogenization probably do not disrupt the yeast. 2. As will be reported elsewhere[10], there is no difference between the nuclease patterns of intestine obtained from larvae fed cellulose and normal food. 3. The nuclease patterns of the two fly stages studied are very similar, but the 1-day-old animals had not been allowed to eat. 4. The larval patterns are similar although the intestine of the last larval stage is almost entirely devoid of food. 5. Pupae contain little, if any, yeast.

Several attempts have been made to implicate aggregation in the production of these patterns. Omission of the large-pore gel and variation of the enzyme concentrations do not significantly alter the pattern. It is, therefore, unlikely that the concentration or "stacking" process of disc electrophoresis is responsible for any aggregation. This effect cannot be excluded for the erratic activities which appear in the range R_m 0.1–0.4. Attempts to elute and rerun the various activities have met with varying degrees of success. Much of this difficulty is probably due to the technical problem of separating so many activities by slicing the gel. The approach has been further pursued in a system in which the problems of separation have been reduced[10].

Patterns of deoxyribonucleases in development

One of the major advantages of using an insect with halometabolous development for such studies is that the processes of growth and differentiation are largely separated in the various developmental stages. The general pattern of the total deoxyribonuclease activity presented in Fig. 3 contains some striking features which correspond to particular stages of development. The most dramatic trend in this respect is the decrease in total activity during late larval life which is followed by a sudden drop at puparium formation. MAHAMMED, GONCALVES AND TROSKO[11], who have studied total deoxyribonuclease activity at pH 7 in *D. melanogaster*, have also found much lower activity in pupae than in larvae. They concluded that this drop in activity is correlated with the termination of the process of histolysis of the larval tissues. Our data, which cover the onset of metamorphosis more closely, are not consistent with this interpretation. These data show that the sharp decline in activity occurs within the 6 h following puparium formation. Since extensive histolysis of the larval tissues is going on at this time, the termination of histolysis and the drop in the total activity are not coincident. In fact, the total deoxyribonuclease activity is probably best correlated with the requirement of the animal for digestive enzymes. The existence of a large spectrum of deoxyribonuclease activities that has been suggested by the data presented here make it clear that further fractionation will be necessary before the functions of the individual activities can be established.

The appearance of certain activities at specific developmental stages does suggest functions for some of the enzymes. As an example, the paucity of EDTA-activated enzymes in 61-h larvae has been noted previously and may reflect the strong emphasis in these animals on cell growth instead of cell division. Another abrupt break in the deoxyribonuclease pattern is the disappearance from pupae of the Mg^{2+}-activated activity which is ordinarily present at the origin of gels containing native DNA. Since pupae lack a functional digestive system, this activity might

correspond to the predominant enzyme that has been found in the digestive system of another insect[12,13]. Also characteristic of specific developmental stages are the activities at R_m 0.62 (EDTA) in older larvae and R_m 0.25–0.29 (Mg^{2+}) in pupae. The latter may be involved in histolysis of the larval tissues.

These studies have shown that a large variety of deoxyribonucleases in *D. melanogaster* undergo striking and specific variation during the course of development. Since the use of whole animal homogenates necessarily limits the detail to which such studies can be carried, further studies of the individual activities and their tissue distribution are needed to reveal the metabolic significance of these enzymes.

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