THE CHROMATOGRAPHY OF NUCLEIC ACID PREPARATIONS ON DEAE-CELLULOSE PAPER

III. STUDY OF DEPOLYMERIZATION AND DENATURATION PROCESSES

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We have previously described a method of chromatography of nucleic acids on DEAE-cellulose paper[1–6]. In this method, paper strips or centrifuged paper pulp are used. From these results, it appears that the fractions which are important for the characterization of the DNA preparations are those eluted with alkaline eluants[7]. For this reason, we have attempted to increase the resolution of the fractionation procedure by searching for new eluants capable of resolving the molecules eluted in the alkaline pH range. In all the experiments reported here, centrifuged DEAE-cellulose paper pulp (CPP) has been used.

The hydrolysis processes due to deoxyribonuclease I* have been studied by using these techniques to follow the kinetics of the enzyme action at different enzyme concentrations. We also have followed the denaturation by heat and the kinetics of denaturation at different temperatures.

MATERIALS

DEAE-cellulose paper

DEAE-Whatman paper (ion-exchange cellulose—diethylaminoethyl cellulose DE 81) was used for the nucleic acid fractionation. The paper pulp was prepared as described previously[5].

Deoxyribonucleic acid

We used non-labelled DNA's extracted from calf thymus according to the method of KAY *et al.*[8] or extracted from bacteria by MARMUR's[9] method.

METHODS

DNA determination

The nucleotide concentration of the different DNA fractions was estimated from the U.V. spectrum recorded with a Beckman DK1 recording spectrophotometer,

* Deoxyribonuclease (bovine pancreas) = DNAase = deoxyribonucleate oligonucleotido-hydrolase (E.C. 3. 1. 4. 5).

taking the difference $E_{260m\mu}-E_{300m\mu}$ as a measure of this concentration (cells of 3 cm light pathway were frequently used).

Centrifugal chromatography

The procedure used is similar to that previously described[5,6].

Eluants

The study leading to the choice of eluants will be described in the results. However, it may be interesting to summarize here the eluants which were selected:

(1) Phosphate buffer 0.01 *M*,
(2) Phosphate buffer 0.01 *M* + 0.14 *M* NaCl,
(3) Phosphate buffer 0.01 *M* + 0.50 *M* NaCl,
(4) Phosphate buffer 0.01 *M* + 1.00 *M* NaCl,
(5) 0.05 *M* NH_3 + 1.00 *M* NaCl,
(6) 0.05 *M* NH_3 + 2.00 *M* NaCl,
(7) 0.2 *M* NH_3 + 2.00 *M* NaCl,
(8) 0.4 *M* NH_3 + 2.00 *M* NaCl,
(9) 1.0 *M* NH_3 + 2.00 *M* NaCl,
(10) 0.1 *M* NaOH,
(11) 0.5 *M* NaOH,
(12) 1.0 *M* NaOH.

The eluants are derived from those described in the BENDICH procedure[10].

RESULTS

Kinetics of DNAase action

Fig. 1 indicates the elution diagram obtained by chromatographing by the CPP method a calf thymus DNA, either in the native form or denatured by heating for 10 min at 100° and rapid cooling at 0°. Each eluant indicated on the abscissae was used in portions of 2 ml. The optical density found in the different portions used is indicated on the ordinate. The arrows show the eluants which were selected from the results obtained and which were able to separate discrete fractions, cleanly separable one from the other. Some characteristics of the graph should now be pointed out.

First of all, it can be seen that after the elutions with 0.2 *M* NH_3 and 0.4 *M* NH_3 used in conjunction with 2 *M* NaCl, a single wash was made with 0.05 *M* NH_3 alone. This step was to remove the residual NaCl. The amount of DNA fraction removed by this washing is very small and this is also the case when 0.4 or 1 *M* NH_3 is used without NaCl. It can be seen that NaCl is necessary to obtain an eluable fraction even when the pH is high. This shows that neither the NH_3 concentration nor the ionic strength are alone determinant. In fact, the elution appears to depend on both pH and ionic strength.

Figs. 2–5 show results obtained by subjecting a similar amount of DNA (namely 540 μg calf thymus DNA) to the action of various amounts of DNAase I (0.010 μg, 0.025 μg, 0.100 μg, 0.400 μg or 1.500 μg). The conditions of the CPP fractionation were the same in all the cases. DNA was incubated with the DNAase in the presence of 5.10^{-3} *M* $MgCl_2$ in a 0.01 *M* phosphate buffer (pH 7.8) at 37°. At 0, 20, 40 and 60

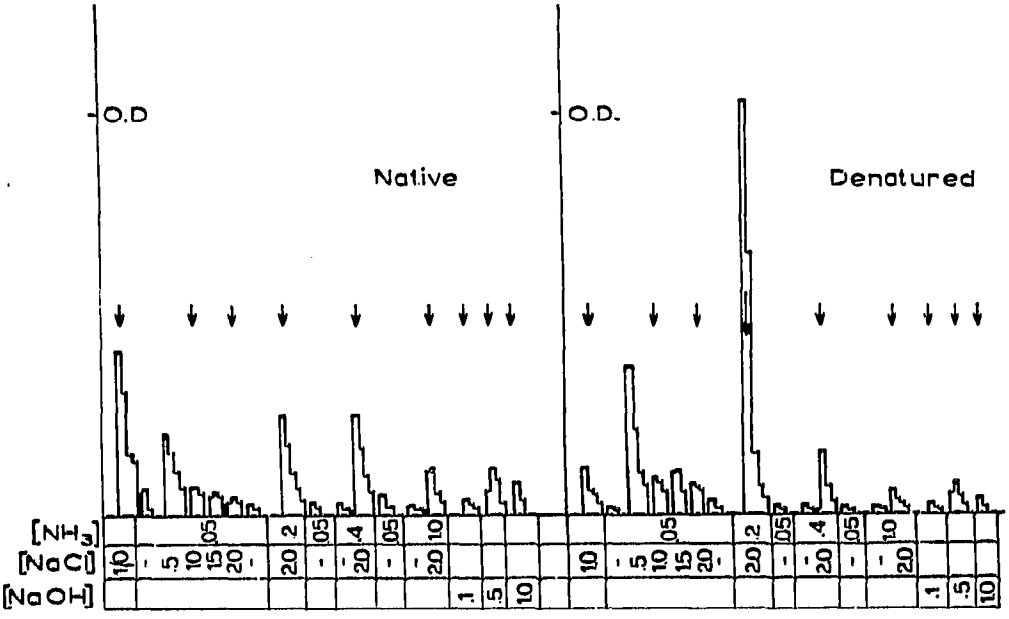

Fig. 1. DEAE-cellulose paper pulp (CPP) chromatography of a native or denatured calf thymus DNA.

min, an aliquot was withdrawn and added to a cold mixture of sodium dodecylsulfate and EDTA (final concentration: 2.5 %; 0.15 *M*).

After homogenization by shaking, the mixture was put on the CPP column and immediately centrifuged. It can be seen that the DNAase activity affects the different fractions eluted. The results indicate that, at low enzyme concentrations, only the more highly polymerized fractions are hydrolyzed by DNAase. For instance, with the lowest concentration of DNAase, fraction 8 (Fig. 2) passes through a maximum at 20 min whilst fraction 7 increases steadily from zero time. With a higher concentration (Fig. 3), fraction 8 decreases from zero time and fraction 7 goes through a maximum. With a higher DNAase concentration, fraction 7 increases from zero time, but fractions 5 and 4 pass through a maximum, etc. This shows the relationship existing between the different fractions eluted on CPP.

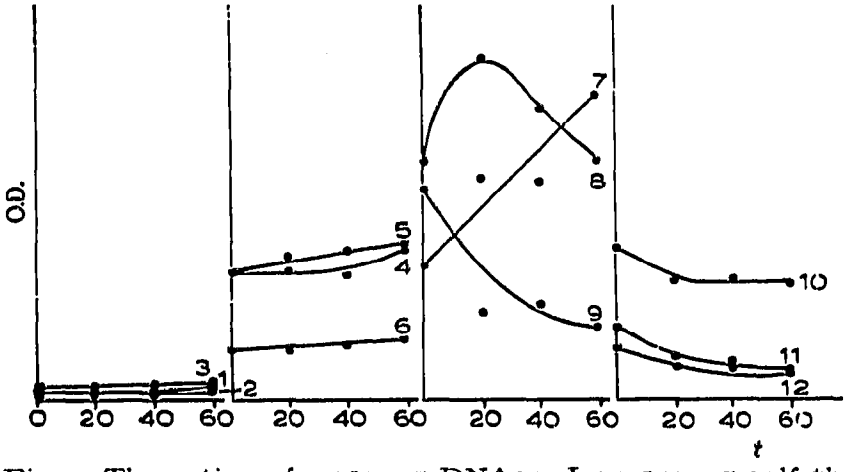

Fig. 2. The action of 0.010 μg DNAase I on 540 μg calf thymus DNA. Conditions: 2.5 g CPP; incubation temperature: 37°; 15 ml of the mixture were centrifuged at 3000 r.p.m. for 6 min at 0°; optical density 260 mμ—300 mμ. For numbers 1–12, see text.

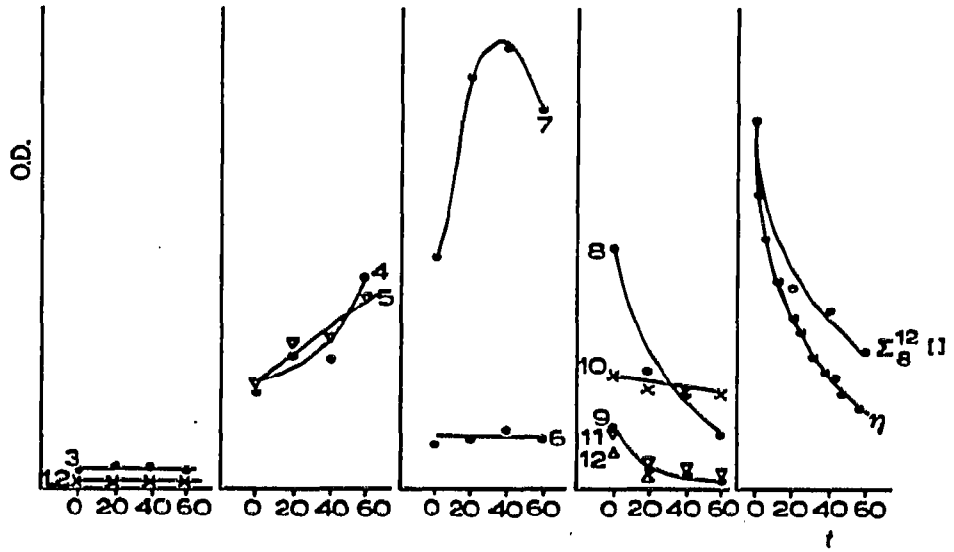

Fig. 3. The action of 0.025 μg DNAase I on 540 μg calf thymus DNA. For conditions, see legend to Fig. 2.

Results obtained by measuring the changes of viscosity in an Oswald micro-viscosimeter are also shown in Figs. 3 and 4. It can be seen that the decrease of viscosity parallels the decrease of concentration found in fractions 8 to 12, thus indicating that those fractions are mainly responsible for the viscosity of the DNA preparation. It can also be seen that between 0.4 and 1.5 μg DNAase, not much difference can be observed: no acid soluble fractions (1 and 2) are formed during the course of the experiment. With longer times, it is of course possible to obtain significant amounts of fractions 1 and 2 (ref. 6).

Kinetics of heat denaturation

Fig. 6 shows the melting curve of the calf thymus DNA used. This melting

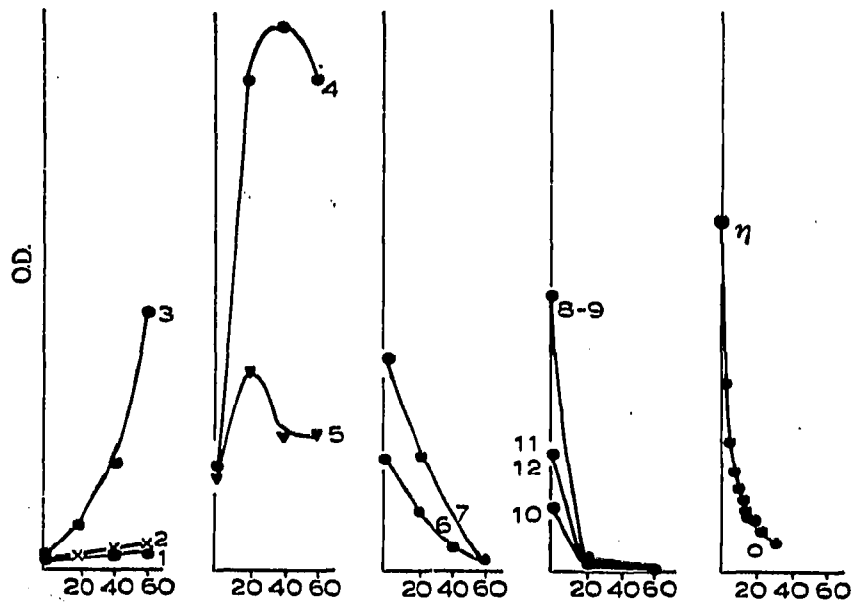

Fig. 4. The action of 0.100 μg DNAase I on 540 μg calf thymus DNA. For conditions, see legend to Fig. 2.

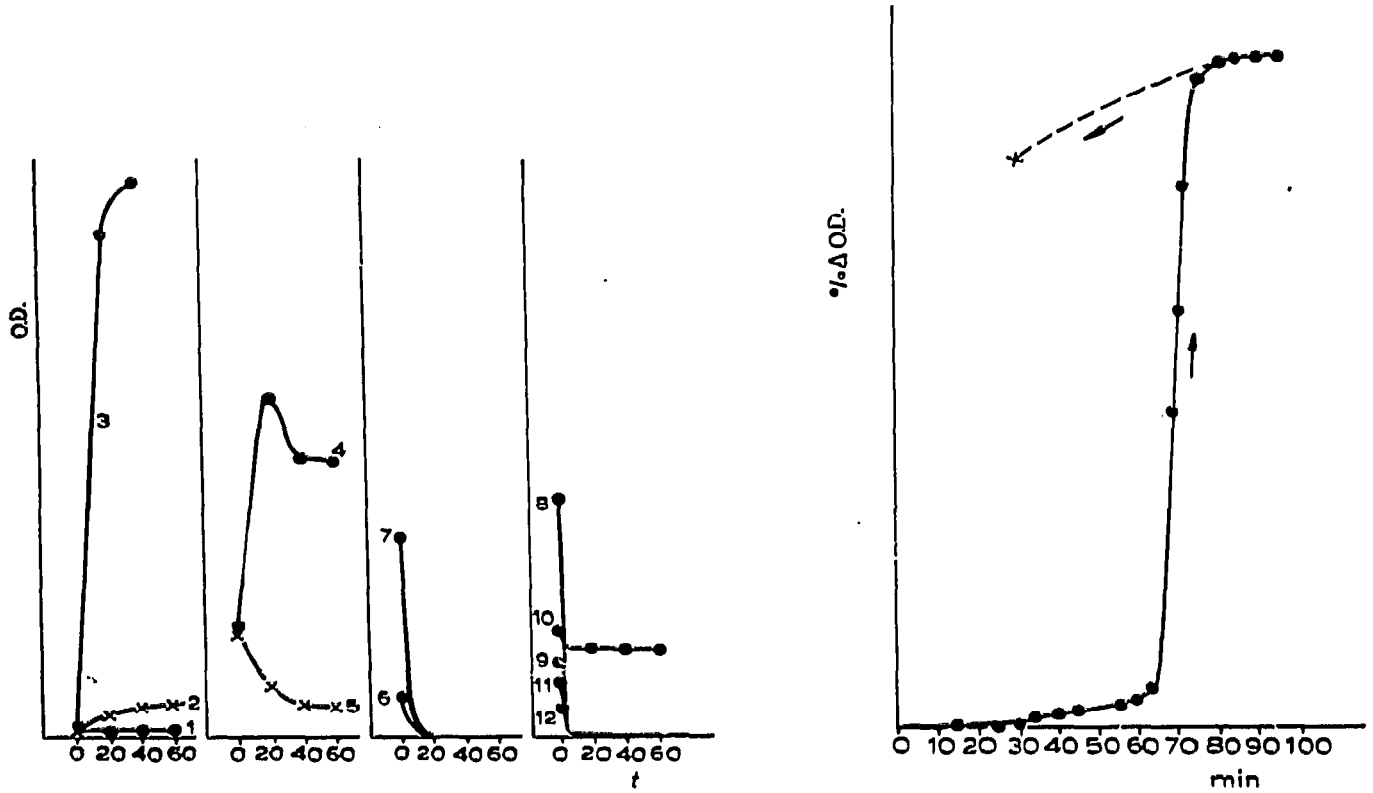

Fig. 5. The action of 0.400 μg or 1.500 μg DNAase I on 540 μg calf thymus. For conditions, see legend to Fig. 2.

Fig. 6. Melting curve of the calf thymus DNA used.

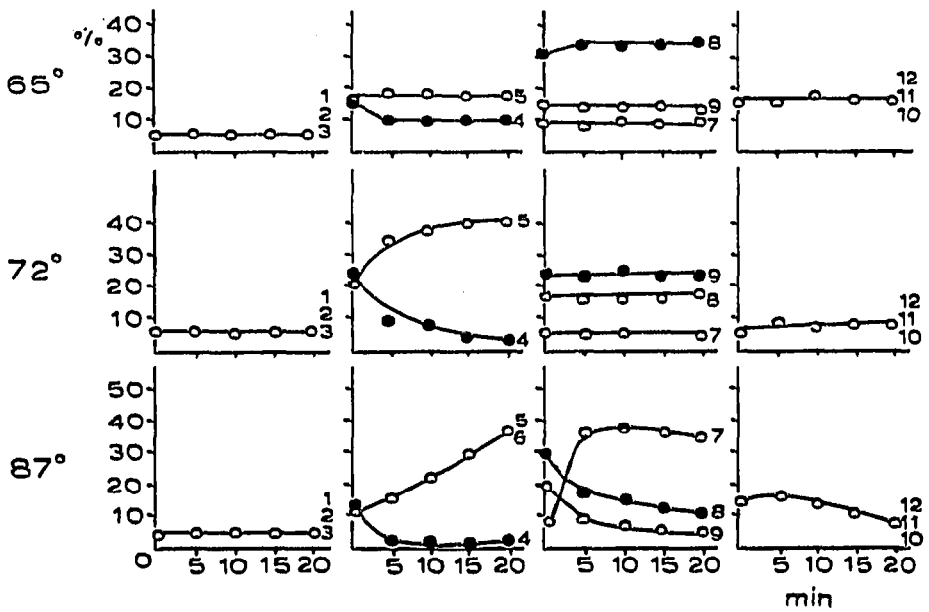

Fig. 7. DEAE-cellulose paper pulp chromatography of calf thymus DNA heated at 65°, 72° and 87° and rapidly cooled.

curve (and all the results shown here) was obtained under the experimental conditions described by MARMUR AND DOTY[11]: 20 μg DNA/ml in standard saline citrate solution (0.15 *M* NaCl plus 0.015 *M* Na-citrate).

The kinetics of melting was studied at 65, 72, 87 and 100°; the results are shown in Figs. 7 and 8. It can be seen that heating at a given temperature produces a slow modification in the relative importance of the different fractions. At temperatures lower than the melting point, nothing seems to happen. At higher temperatures, the general pattern of the phenomenon is the same as in the previous case, namely: decrease of the concentration of the highly polymerized fractions (fractions 8 to 12) accompanied by an increase in fractions 5 and 7. At 100°, after 10 min, almost all the DNA fractions are transformed into fraction 7, but the molecules present in this fraction, when heated at 100°, are gradually turned into molecules eluable with fraction 5. From Figs. 7 and 8, it can be seen that the amount of fraction 4 decreases

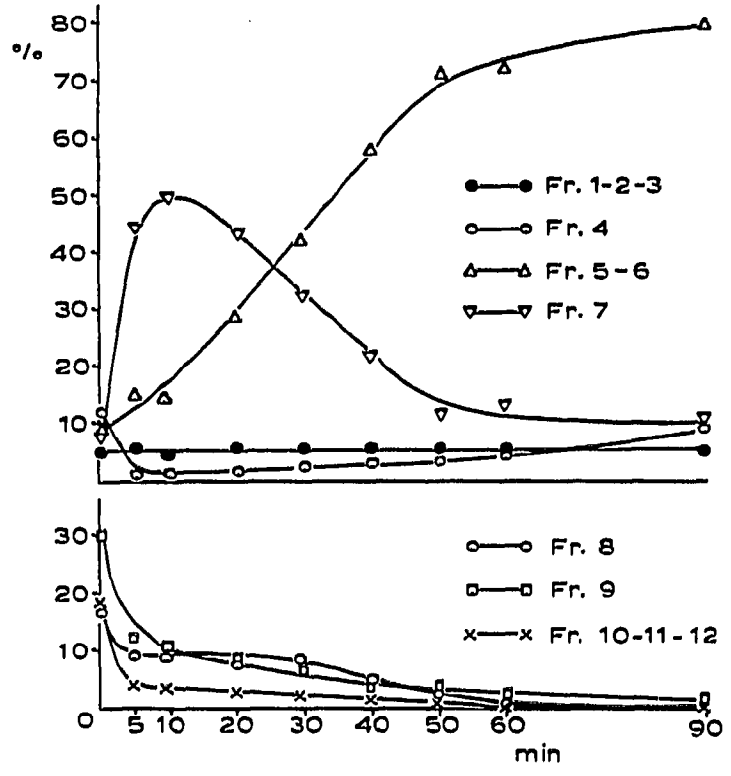

Fig. 8. DEAE-cellulose paper pulp chromatography of calf thymus DNA heated at 100° and rapidly cooled.

with time, being converted into fraction 5. This has been confirmed with an isolated fraction 4 eluted on CPP, dialyzed and heated at 100° for 10 min. Results are shown in Fig. 9.

From the results described here and from others we have obtained with other DNA preparations containing important acid soluble fractions, it can be seen that: fractions 1, 2 and 3 are not modified by heating at different temperatures; fraction 4 is the only one which undergoes a change and is eluted with higher ionic strength eluants, all the other fractions are shifted to an eluant of lower ionic strength or of lower pH. As the melting curves are generally obtained with slow gradual temperature

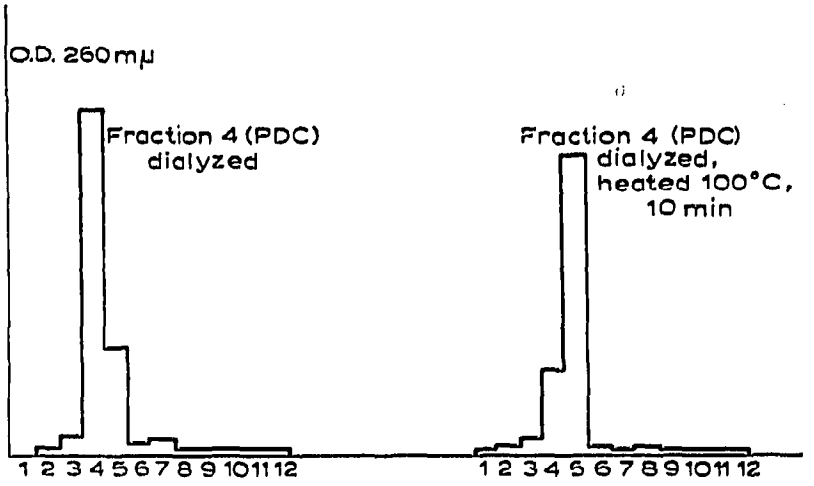

Fig. 9. Rechromatography on CPP of fraction 4 native or heat denatured.

increase, Fig. 10 summarizes the results obtained by heating for a period of 10 min at the different temperatures. In this figure the melting curve of the total DNA preparation is also indicated by a dotted line, together with the modifications observed in fractions 1, 3, 4, 5, 7, 8 and 12, showing that the melting itself is accompanied by a decrease of the importance of fraction 4 and fractions 8 to 12 and by an increase of the importance of fractions 5 to 7.

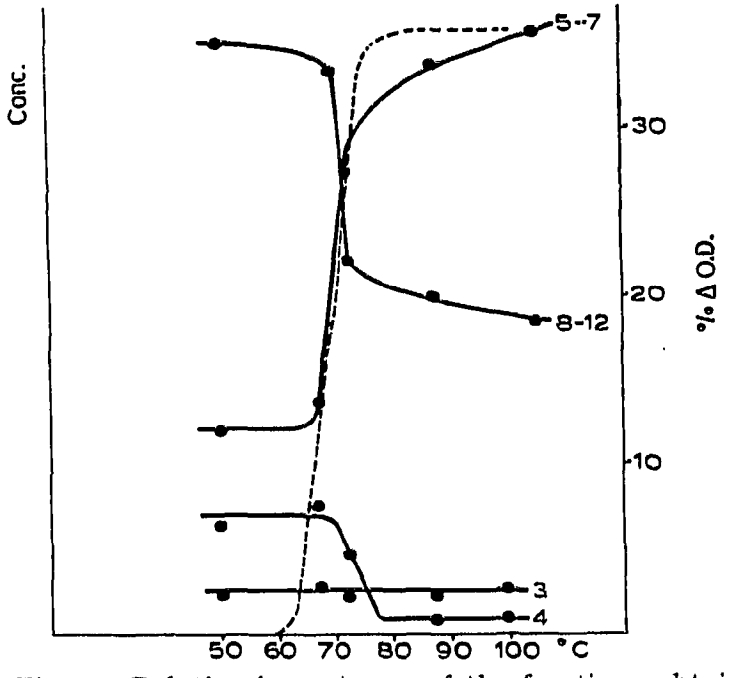

Fig. 10. Relative importance of the fractions obtained by heating for a period of 10 min at the different temperatures indicated.

DISCUSSION

The results shown here enable one to propose an extended scheme of elution on centrifuged DEAE-cellulose paper pulp. Using the eluants described here dis-

continuously, it is possible to obtain a large number of discrete fractions from calf thymus DNA preparations. It is important to point out that with bacterial DNA's a very much lower number of fractions is obtained. For instance, with *E. coli* DNA, prepared by a slightly modified MARMUR technique, one obtains a preparation containing 82 % of fraction 9. Therefore, the heterogeneity described by the figures published here only reflects the characteristic behaviour of calf thymus DNA and the implications of these diagrams obtained should not be generalized.

Results obtained with DNAase show that a relationship exists between the different fractions: each fraction can be attacked by DNAase and the hydrolysis products can be eluted with eluants of lower ionic strengths or lower pH's. Therefore, the different fractions contain molecules of various groups of molecular weights and results obtained with an analytical ultracentrifuge[7] are in agreement with the data shown here. It can also be seen that heat denaturation produces a modification in the relative importance of the different fractions, thus showing another type of relationship between these fractions. It has to be concluded that the CPP method does not permit one to separate native from denatured DNA (as fractions 7 or fractions 5, for example, can very well contain native or denatured molecules).

Another point of importance is that native fraction 4, when heated, shifts to fraction 5. As it is very difficult to believe that heating would produce polymerization of this fraction alone, we think that the position of the different macromolecules, in our diagrams, is due not only to the state of polymerization, but also is probably due to what we should call the "steric volume" of the molecules. For instance, fraction 4, when denatured by heating, is eluted with eluant 5 (which in the case of native DNA contains molecules of higher complexity than fraction 4). In general, the fact that a given fraction can contain either native or denatured "DNA" molecules indicates that the retention of a given fraction by the column is accounted for by its molecular weight as well as its shape, both of these two factors being of importance.

ACKNOWLEDGEMENTS

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SUMMARY

Our present results indicate that deoxyribonucleic acid (DNA) preparations of calf thymus origin contain different macromolecular species which can be separated by centrifugal DEAE-cellulose paper pulp (CPP) chromatography. These fractions can be attacked by enzyme action or by heating, and the present paper indicates the relationship between the different macromolecular species. Each fraction appears vulnerable to deoxyribonuclease I, the more highly polymerized ones being attacked first. The hydrolysis products are, in turn, hydrolyzed by the enzyme to smaller molecules.

During the melting phenomenon by heat denaturation, the different fractions of calf thymus DNA are modified in such a way that they are eluted with eluants 5 and 7. At a given temperature (when equal or superior to the melting point), the relative

importance of the different fractions varies with time. Fraction 5 appears as an end state of the melting process.

These results show that the CPP chromatography method can be applied to the study of changes occurring in a population of DNA molecules submitted to the action of various physical or biological agents.

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