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The fractionation of calf thymus DNA by multiple formation of sedimentable complexes with homologous lysine-rich histone KAP. Derivative melting curves and ultracentrifugation in CsCl concentration gradients

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ABSTRACT

The process of fractionation of total calf thymus DNA using a step precipitation of DNA by means of increasing concentrations of the homologous histone KAP was investigated. In addition to the known fractions three so far undescribed ones/in thymus/, characterized by buoyant densities in CsCl equal 1.692, 1.706 and 1.728 g/ccm, were identified. Considerable amounts of preparations seriously enriched in individual satellite fractions were obtained. The ability of GC-rich satellite DNAs to form more soluble complexes with histone KAP is suggested as reason for the observed fractionation.

INTRODUCTION

Heterogeneity of calf thymus DNA molecules in respect of their base composition, an already well established fact, had been shown by means of density gradient centrifugation[1-4] and analysis of derivative melting curves[5]. Schildkraut et al.[1] stated that calf thymus DNA is composed of the main fraction, showing buoyant density in CsCl equal to 1.699 g/ccm, and of a satellite fraction whose buoyant density equals 1.713 g/ccm. Polli et al.[2] found an additional satellite fraction of buoyant density equal to 1.719 g/ccm. In some other works the values: 1.698 g/ccm for the main fraction[3] and both 1.714 g/ccm and 1.721 g/ccm for satellite fractions[4] were given. In some other calf tissues satellite DNA fractions of buoyant density equal to 1.706 g/ccm in liver[6] and both 1.706 g/ccm and 1.709 g/ccm in kidney[7] were observed, besides the above mentioned ones.

The isolation of small amounts of satellite DNA fractions was so far performed by means of expensive and time consuming procedures based on repeated ultracentrifugation in Cs_2SO_4 or CsCl concentration gradient[8-11]. The results obtained by Sponar et al.[4] as well as experiments performed in our laboratory[12]

led us to a supposition that the phenomenon of precipitation of DNA fractions relatively richer in AT by complexing them with histone KAP may appear useful for the isolation of larger quantities of satellite DNAs. Therefore, we decided to investigate the effect of the step precipitation, by stepwise rising histone concentration, of those DNA fractions which remain in the supernatants.

The obtained fractions were characterized by means of their derivative melting curves whose selectivity was shown by Ansevin and Brown[13] not to be worse than that of CsCl density gradient centrifugation. Data obtained from the melting curves were compared with those from ultracentrifugation in CsCl.

MATERIALS AND METHODS

Calf thymus DNA was obtained by an initial deproteinisation as described by Zbarski et al.[14] followed by two subsequent deproteinisations acc. to Kay et al.[15]. It contained 0.6 % protein, as estimated by the method of Lowry et al.[16]. Its average molecular weight, amounting to ca. 29.10^6 daltons, was estimated on the basis of viscosity measurements[17].

Histone KAP from calf thymus was obtained by the method of Johns[18]. Its homogeneity was checked by means of electrophoresis on polyacrylamide gel performed acc. to Ilyin et al.[19].

DNA-histone complexes were obtained by mixing histone KAP and DNA at the weight ratio 0.3 : 1 or, in some series, 0.4 : 1, in 1.5 M NaCl containing 0.02 M phosphate buffer, pH 6.8, at DNA concentration ca. 1 mg/ccm. 50 ccm of such solution were dialysed at 4°C, subsequently for : 3 hours against 800 ccm of 0.5 M NaCl, 3 hours against 800 ccm of 0.3 M NaCl and 12 hours against 800 ccm of 0.15 M NaCl. All solutions contained 0.02 M phosphate buffer, pH 6.8.

The final volume of the dialyzed solution was measured and the precipitated DNA-histone complex was removed by centrifugation in a Servall SS-1 rotor for 30 minutes at 25000 g at 4-8°C. DNA concentration in the supernatant was estimated by UV spectrophotometry, assuming that one absorbance unit at 260 nm in 1 cm cells corresponds to the DNA concentration of 47 micrograms/ccm.

The precipitate /P I/ was dissolved in 1.5 M NaCl + 0.02 M

phosphate buffer, pH 6.8, and protein was removed on hydroxyapatite[12]. The supernatant /S I/ was divided into two parts. The minor part was used for the analysis of DNA and histone concentration and for recording melting curves before and after deproteinization. The rest of the supernatant was used for the next step of fractional precipitation : after adding NaCl up to 1.5 M concentration and an aliquot of histone KAP equal to 0.4 the weight of DNA present in this part of S I, the amount of DNA being known from the analysis of the minor part of S I, the solution was dialyzed in three steps and centrifuged in the same way as described above. The obtained precipitate /P II/ and supernatant /S II/ were processed in the same way as P I and S I and the whole procedure was repeated once again leading to the isolation of P III, P IV, S III and S IV /see diagram No 1/.

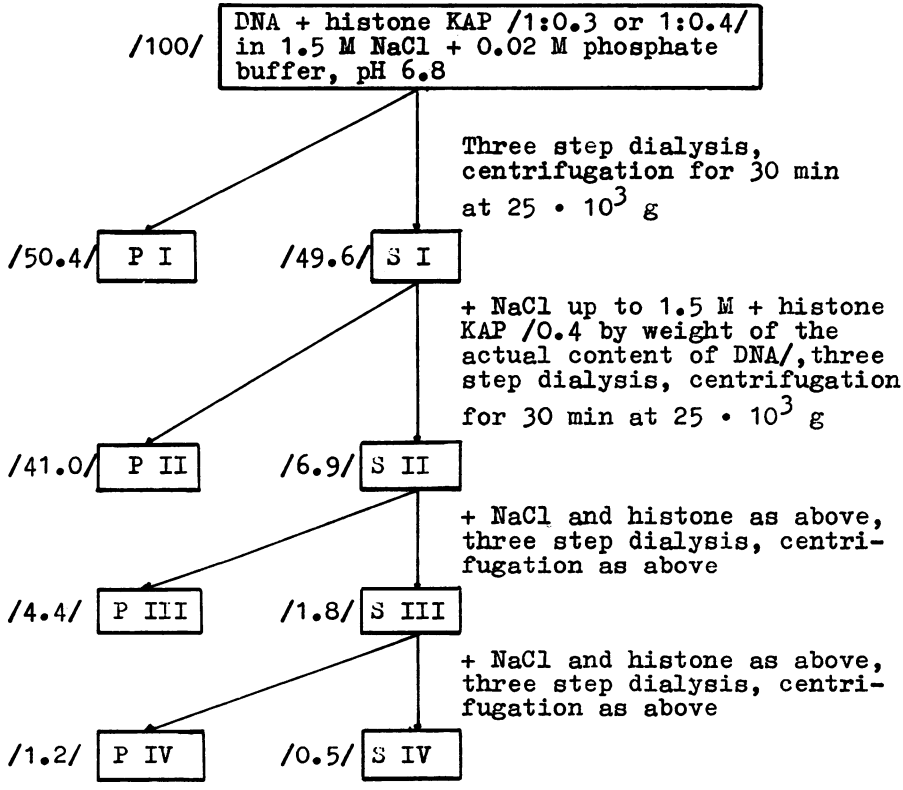
Preparative centrifugation in CsCl density gradient was performed in the 8 x 25 ccm titanium angle rotor of the MSE Super speed 65 ultracentrifuge acc. to Brunk and Leick[11]. Two solutions were prepared by dissolving : /A/ 6.68 g CsCl, analytical grade, in 4 ccm of the 0.04 M Tris-HCl buffer, pH 8.2, and /B/ 3.80 g CsCl and 0.2 mg DNA in 4 ccm of the above buffer. 4 ccm of the solution B were transferred into the 25 ccm centrifuge tube and underlayered from a pipette equipped on top with a syringe needle by 4 ccm of the solution A. The tubes were filled up with paraffin oil and centrifuged for 40 hours at 25°C and 33400 r.p.m. After centrifugation the content was removed by means of a MSE tube piercer and divided into 100 fractions. For that purpose the tube piercer was slightly modified by making an additional aperture near the hole in which the piercing needle is fitting. Such a modification prevents formation of air pressure causing small air bubbles to move across the investigated density gradient layers at the very moment of making the hole in the bottom of the tube. Each fraction was diluted by adding 1 ccm of the Tris-HCl buffer and A_{260} was measured.

Analytical density gradient centrifugation was performed in the same rotor equipped in adaptors for 2.5 ccm tubes. 0.8 ccm samples of the same two solutions, as described in the above

DIAGRAM OF THE FRACTIONATION PROCEDURES

P - precipitates, S - supernatants

The figures in parentheses represent the yield of DNA in the precipitates and supernatants expressed as percentage of total initial DNA.



section, and applied in the same manner, were centrifuged at 25°C and 36400 r.p.m. for 40 hours and after centrifugation each sample was divided into 53 equal fractions. The actual density of the subsequent fractions was estimated at 25°C on a Zeiss plunge refractometer /C. Zeiss, Jena/ equipped with interchangeable thermostated prisms of Abbe type, permitting precision of readings in the range of 1.10^{-5}. Densities were calculated from the equation $\rho = n. 10.8601-13.4974 \overset{20}{.}$ The obtained density gradient appeared linear within the limits of 1.745-1.675 g/ccm. The difference between the two subsequent fractions amounted to 0.0023 g/ccm. Samples for density measurements were taken from all fractions between the peaks and from only one fraction within the peak. Densities of the remaining fractions were obtained by interpolation. All those fractions were diluted by adding 0.5 ccm of 0.04 M Tris-HCl buffer, pH 8.2, and A_{260} was measured in microcells of a Unicam SP 500 spectrophotometer.

Melting curves were estimated on a Unicam SP 500 spectrophotometer. The temperature was measured by means of a thermistore mounted in the teflon stopper of the blank cell, several milimeters above the light path, and built into a bridge system. Derivative melting curves were calculated by dividing the subsequent $\Delta h = \Delta \left(\frac{A^{t}_{260}}{A^{25}_{260}} \right)$ values by the corresponding Δt, where A^{t}_{260} is the absorbance of the measured sample at 260 nm and t°C, A^{25}_{260} is the same at 25°C, Δ is the increase. The results were calculated in a normalized form by means of the equation : $100 \cdot (\Delta h / \Delta t) / \sum_{i} \frac{\Delta h_i}{\Delta t_i}$.

Such a normalization permits rapid evaluation of the percentage of DNA which melts within the given interval of temperature. Each normalized value was ascribed to the mean temperature for the given interval. 0.7 and 1.4°C steps were applied by manual ultratermostate contact thermometer regulation. A^{t}_{260} was measured after the stabilization of the temperature at the new level was stated.

Comparison of the results of the derivative melting curves and of density gradient centrifugation in CsCl was made on the basis of the equation $\delta T = \delta \rho \cdot 418$, where δT is the difference between the melting temperatures or between the temperatures corresponding to the maxima on the derivative melting curves for two DNA fractions differing in their AT/GC ratio, $\delta \rho$ is the difference between buoyant densities in CsCl gradient of the same two fractions. This equation was obtained from a combination of two equations :

$$GC = 100 \times \frac{\rho - 1.660}{0.098}$$ [1] and $$GC = 2.44\ (T_m - 53.9)$$ [21]

where the base composition of DNA is expressed in mole % of GC pairs.

RESULTS AND DISCUSSION

Direct analysis of total DNA. Our data obtained from both CsCl density gradient centrifugation /fig. 1/ and from the evaluation of derivative melting curves /fig. 2/ of total calf thymus DNA show again the existance of the already discovered fractions: the main /1.699 g/ccm/ and the two satellite /1.713 and 1.719 g/ccm/ ones. Besides those, however, some additional fractions can be easily distinguished: three fractions on the melting curve, whose melting temperatures correspond to buoyant densities equal to 1.692, 1.706 and 1.728 g/ccm [22], and four fractions on the density gradient centrifugation curves, with buoyant densities 1.687, 1.692, 1.706 and 1.728 g/ccm, respectively. Specific designations have been introduced for the particular fractions in order to make it easier for the reader to analyze and to classify the composition of the material obtained from various fractionation steps. They are summarized in Table no 1 together with some analytical data.

Fractionation of total DNA by means of histone KAP /see diagram no 1/. The first precipitate, P I, obtained by addition of histone KAP up to the ratio histone KAP/DNA = 0.3, is composed mainly of the AT rich fractions /fig. 1, 3B/. Contamination with G1, G2 and G3 fractions is also visible /Table No 1/. In the first supernatant /S I/, instead, a remarkable accumulation of G1, G2, G3 and G4 fractions is observed /fig. 2, 3A/ with G1 and G2 fractions participating in the largest

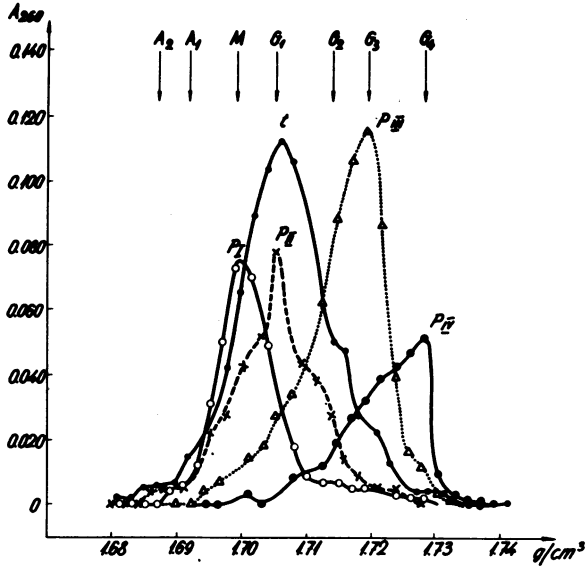


Fig. 1 Analytical centrifugation in CsCl density gradient of total calf thymus DNA and of DNA from the precipitates obtained by means of complex formation with histone KAP. Details of the procedure - see "Materials and Methods" and [11].
——— total DNA, —o— P I, --x-- P II, ...Δ... P III, -.-.- P IV

quantities.

The addition of histone KAP to S I up to the ratio histone/DNA=0.4 caused the formation of P II, composed mainly of the M, G1 and G2 fractions slightly contamined with G3 /fig. 1, 3B/. The resulting supernatant, S II, was composed mainly of G3, some G4 and only of traces of the less GC-rich fractions /fig. 2, 3A/.

By adding surplus histone KAP /0.4, by weight, of the amount of DNA actually present in S II/ we obtained: P III, containing the considerably pure G3 fraction /60 %/ and only traces of the remaining ones, and S III, composed of ca. 42 % G4, 35 % G3, 15 % G2 and of traces of the G1 and M fractions. The composition of the last precipitate, P IV, does not differ much from that of S III /fig. 1-3/.

Table I

Characteristics of calf - thymus DNA fractions

DNA fraction[a]	ρ estimated by ultracentrifugation	$\delta T = T_{max} - T^{o}_{max}$	$\rho = 1.714 + \frac{\delta T}{418}$	mole % of GC pairs	% fraction content in total DNA	data from literature	sources Ref. No.
A 2	1.687	-	-	27,6	1.2	unpublished	-
A 1	1.692	-9.4	1.692	32.6	2.7	unpubl.	-
M	1.699	-6.5	1.698	38.8	30	1.699 1.698	1,2, 3,
G 1	1.706	-3.5	1.706	46.9	32	unpubl.	-
G 2	1.715	0	1.714	55.6	20	1.713 1.714	1,2, 4,
G 3	1.719	+3.6	1.723	62.2	12	1.719 1.721	2, 4,
G 4	1.728	+7.0	1.731	71.4	2	unpubl.	-

[a] M - main DNA

G - fractions more GC rich than main
A - fractions more AT rich than main

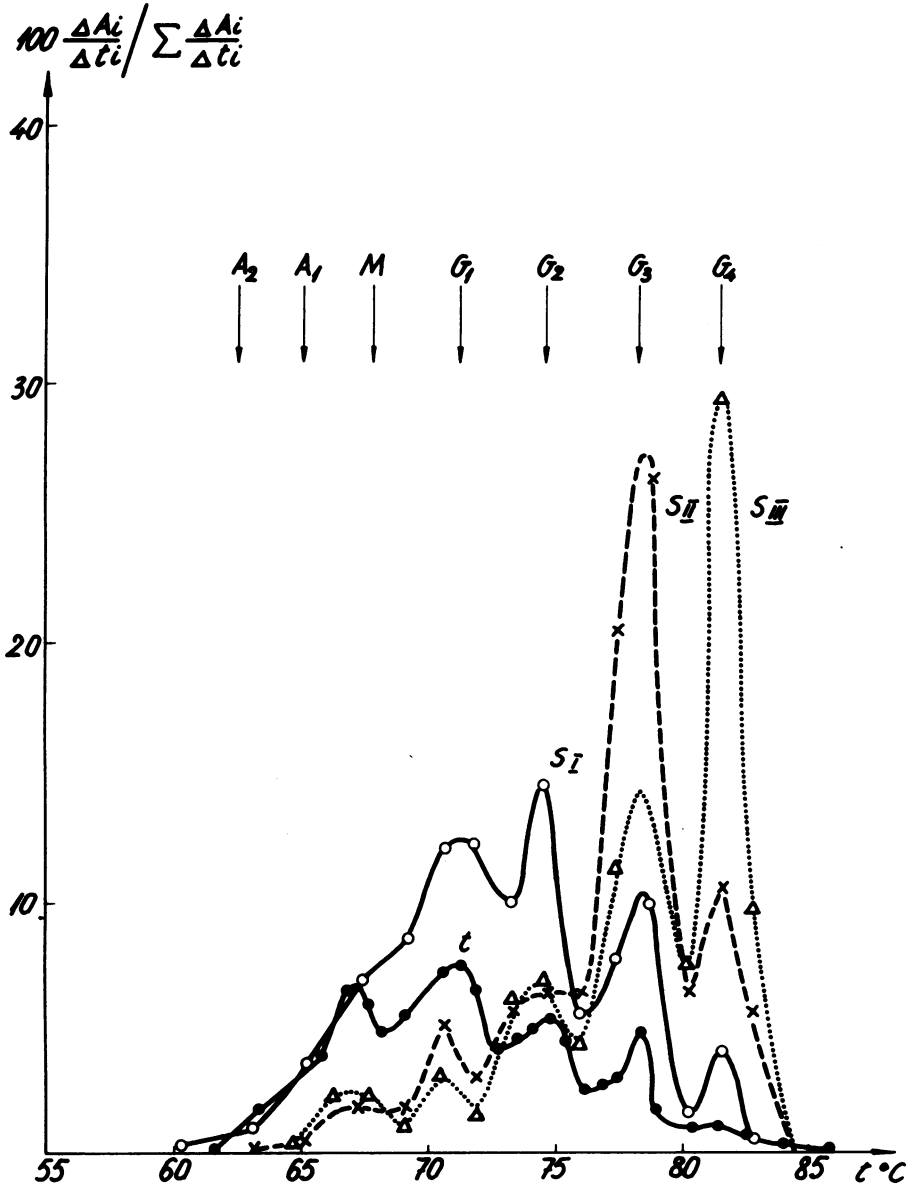


Fig. 2 The derivative melting curves of total DNA and of DNA present in the supernatans obtained by means of complex formation with histone KAP. Details of the procedure - see "Materials and Methods"
——— total DNA, —o— S I, --x-- S II, ... Δ ... S III

The total of 8 full fractionation series performed in this work gave highly reproducible results. The only variable was the ratio of histone to DNA applied during the first precipitation: in the series No 6-8 the initially used ratio 0.4 was lowered down to 0.3 in order to minimize the coprecipitation of a part of the GC rich fractions into P I. At this ratio, P I contained only 50 % of total DNA and the major part of the G1-G4 fractions remained in S I. Independent of the histone/DNA ratio used during the first precipitation, P III always contained 60-70 % of the G3 fraction and P IV - 40-50 % of G4. P III and P IV seem to be excellent materials for CsCl density gradient centrifugation. A single preparative run of P III was sufficient to obtain the G3 fraction contaminated only with 1 % of E, 2 % of G1 and 5 % of G2 /fig. 4, 5/.

The data shown on fig. 2 and summarized in Table No 1 prove that the selectivity of the derivative melting curves is at least as high as that of the CsCl density gradient centrifugation method and the results obtained by means of those two methods are closely comparable. The fractions A1, G1 and G4, so far undescribed, were identified in this work by means of two independent methods. The existence of the minor A2 component requires further confirmation.

Densities of the two newly identified fractions A1 and A2, closely resamble those of human and chimpanzee satellite DNA fractions described by Corneo et al.[23] and Prosser et al.[9]. Thus, the so far observed qualitative species-specific differences among satellite DNAs may appear of quantitative character.

Factors conditioning the observed fractionation require separate consideration. There are no sufficient reasons to state that histone KAP shows for sure higher affinity toward AT-rich DNA fractions. The observation that AT-rich DNAs are the first which form precipitates with histone KAP may merely justify the opinion that the nature of the bonds between DNA and histone KAP is dependent on the base composition of DNA. Possibly, complexes of histone KAP with the GC rich satellite molecules are unable to form crosslinks conditioning the aggregation and sedimentation ability. This view seems to be supported by the

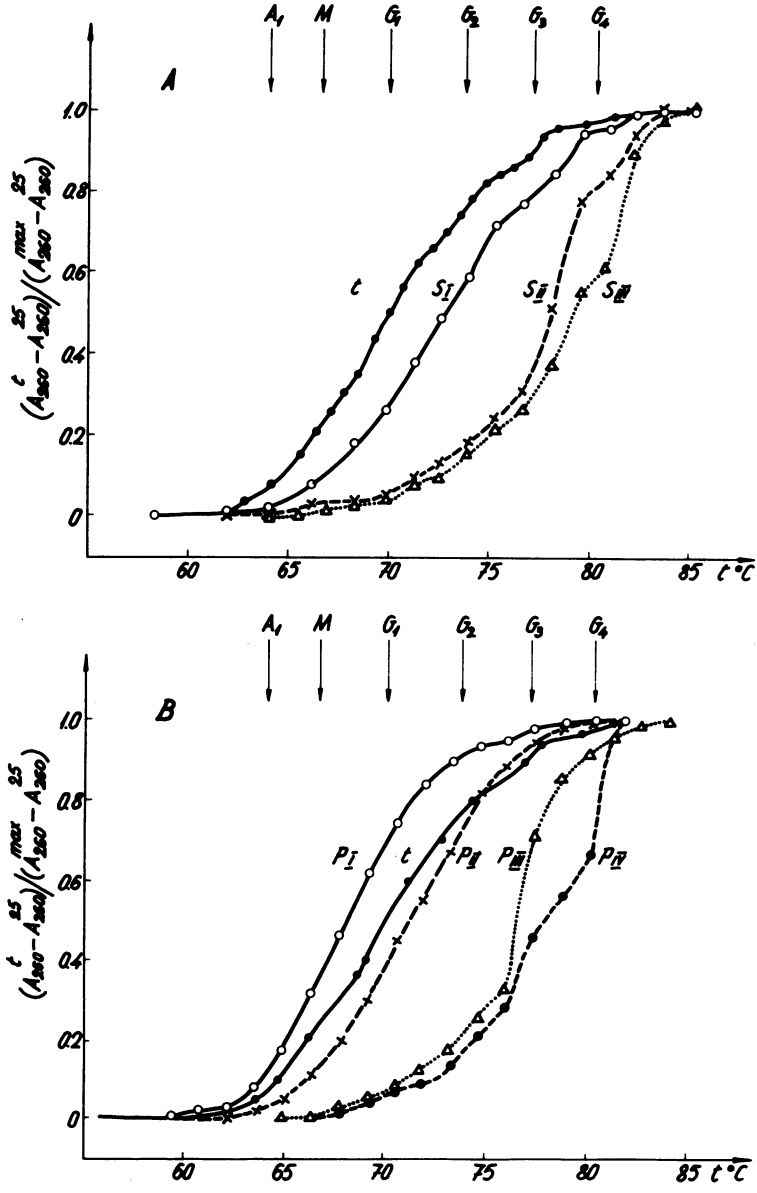


Fig. 3 The melting curves of total calf thymus DNA and of DNA fractions obtained by means of complex formation with histone KAP. A-supernatants, B-precipitates
A: ——— total DNA, —o— S I, --x-- S II, ...△... SIII
B: ——— total DNA, —o— P I, --x-- P II, ...△... PIII

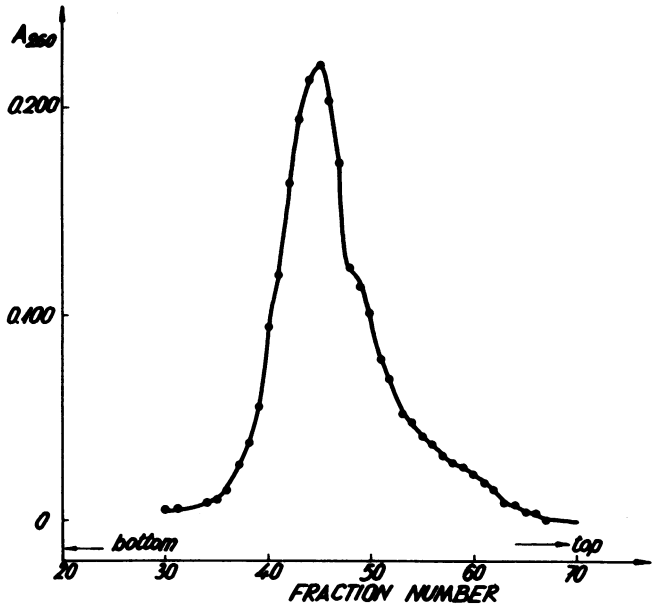


Fig. 4 Preparative centrifugation in CsCl density gradient of DNA from P III. Details of the procedure - see "Materials and Methods".

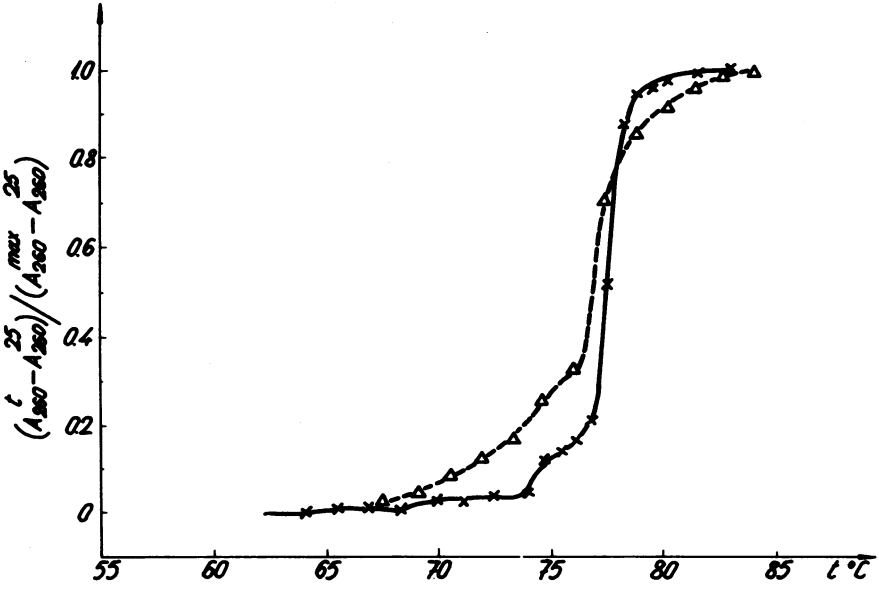


Fig. 5 The comparison of melting curves of DNA from P III before / ...Δ... / and after / ——— / ultracentrifugation /fractions No 45 + 46 from the preparative run shown on fig. 4/.

results of the experiments illustrated on fig. 6 and 7.

The subsequent supernatants show a rapid increase of the histone KAP/DNA ratio depending on the percentage of DNA precipitated or, in other words, on the increase in the number of GC pairs in the DNA in solution /fig. 6/. Thus, in the supernatants remain soluble histone KAP-DNA complexes[24]. At the Histone KAP/DNA ratios found in some of the S III, artificial nucleohistones obtained from calf thymus DNA and histone KAP in 0.15 M NaCl would undergo quantitative precipitation[25,26]. Despite such an extraordinarily high ratio value, even more histone KAP must be added to S III in order to force a further part of DNA to precipitate. There are no evident data available determining whether all histone KAP molecules in S III are bound to DNA or some of them remain free in solution. The comparison of the derivative melting curves of S III before and after deproteinisation, however, reveals that the presence of histone KAP causes a distinct shift of melting temperatures of the GC-rich fractions G3 and G4, whereas it does not influence the T_m values of the M and G1 fractions /fig. 7/. Similar observations on the influence of deproteinisation were made by comparing the corresponding T_m curves of S I and S II. M and G1 fractions, independent of the presence or absence of histone, melted within the same intervals of temperature, whereas the G2 and G4 fractions showed a distinct thermal stabilization. The more GC-rich was the investigated fraction the higher was its stabilization. These thermal stabilization effects supply an indirect proof for the formation of soluble complexes between histone KAP and the GC-rich G2, G3 and G4 fractions. The lack of thermal stabilization of the M and G1 fractions, manifested by the constancy of their melting temperatures before and after deproteinisation, suggests that no or only very little protein is attached to these molecules at the conditions existing in the supernatants. The second alternative - the formation of complexes which would not cause thermal stabilization of DNA - seems rather unlikely. Further investigations on the complexes formed by purified GC-rich fractions and histone KAP are necessary to clarify the mechanism leading to the fractionation of DNA by means of its interaction with histone KAP.

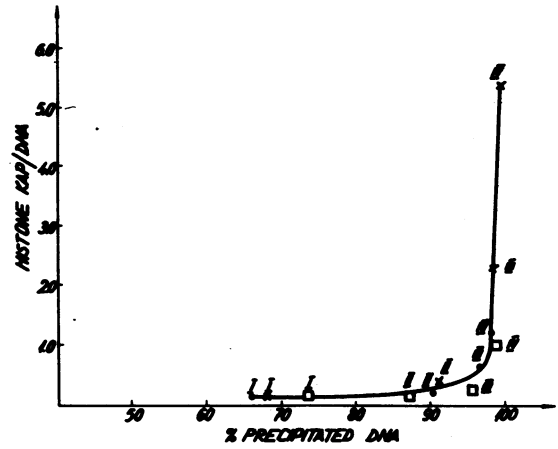


Fig. 6 Histone KAP/DNA weight ratio relations in the supernatants in dependence on the percentage of precipitated DNA. The results of three experimental series /o, x, □/ are shown. Roman numerals are used to designe the numbers of supernatants.

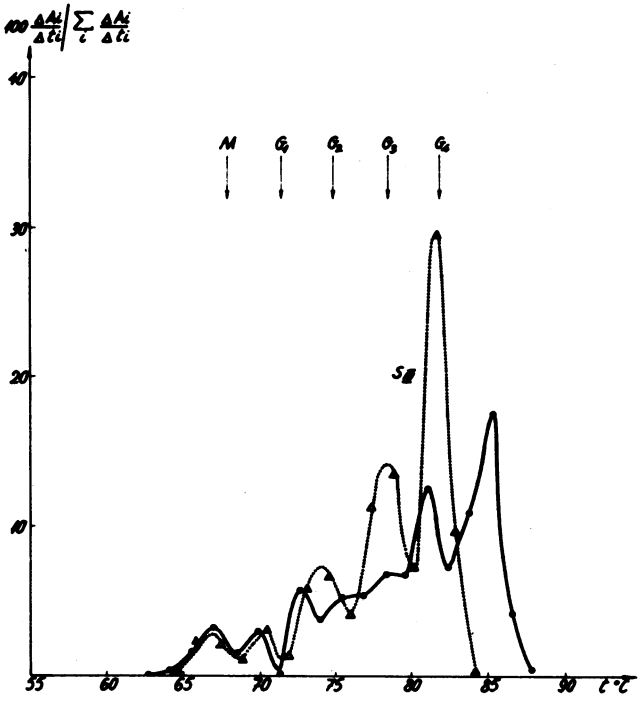


Fig. 7 The comparison of the derivative melting curves of DNA from S III before /——/ and after /... △ .../ deproteinisation.

In our previous work we used as fractionating agent histone KAP immobilized on activated Sepharose[27]. Some GC-rich fractions were the first to be eluted from the histone - Sepharose columns. Even though the attachement to Sepharose by means of some basic residues might have changed the ability of histone KAP to form specific complexes with DNA and although the best resolution was obtained in that work for DNA degraded by sonication, the comparison of fractionation effects of the two methods leads to the following remarks: 1. The basis for fractionation in the present work is the difference between the particular histone - DNA complexes in their ability to form aggregates sedimentable at ca. 25000 g, whereas in the previous work fractionation was based on the differences in the resistance of complexes toward increasing ionic strength. 2. The complexes between the GC-rich fractions and histone KAP molecules show worse aggregation and higher susceptibility to the splitting effect of the ionic strength than those of the AT-rich M and G1 fractions.

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