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Note

Molar mass characterization of DNA fragments by gel permeation chromatography using a low-angle laser light-scattering detector

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Recently, gel permeation chromatography (GPC) has been proposed as an alternative means of separating DNA restriction fragments in addition to the usually employed method of gel electrophoresis[1–3]. The present work has shown that GPC is enhanced by monitoring continuously the molar masses of the fragments leaving the columns. This additional option, commonly applied to synthetic polymers, requires the installation of a low-angle laser light-scattering (LALLS) detector in the gel permeation chromatograph. As far as organic solvents are concerned this coupling technique does not give rise to serious problems, but this is not so with water-soluble polymers. Electrostatic interactions between polymer and gel cause undesirable repulsion or attraction effects which affect the mechanism of size exclusion[4]. In addition there are the common purification problems associated with light-scattering measurements in aqueous solutions.

In spite of these two sources of problems, we recently succeeded in performing combined GPC LALLS measurements on dextrans dissolved in 0.1 M sodium nitrate solution[5]. It was shown that salt must be added to this system to suppress an interfering charge-exclusion mechanism resulting from the repulsion of weakly negatively charged polymer chains by the equally charged gel matrix. Further, it was necessary to observe careful precautions with respect to the purification of the solutions and the LALLS detector. Only in this way were the values of the weight-average molar mass ($\bar{M}_w$), being precisely the average value which is measured on-line, reproducible. In water also DNA fragments are eluted prematurely by charge exclusion without a significant separation according to size. This effect is greater than that found with dextrans because of their polyelectrolyte character. Again, on adding sufficient salt (0.1 M sodium nitrate solution) size exclusion will prevail. Therefore, using conditions identical with those for the elution of dextrans some DNA samples were studied, including three isolated from chicken erythrocytes (A, B and C) and one of the calf thymus type (D).

EXPERIMENTAL

Samples A and B were prepared by a micrococcal nuclease digestion according to the method of Shindo *et al.*[6] and isolated by a procedure described elsewhere[7].
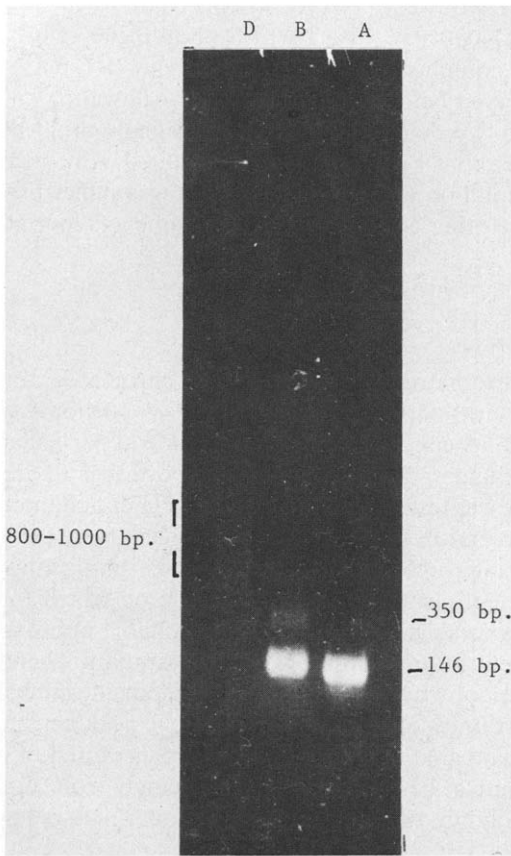
Fig. 1. 1% agarose gel electrophoresis pattern of DNA samples A, B and D.

Special care was taken to remove Mg^{2+} ions by an exhaustive EDTA treatment followed by an atomic absorption spectrometric test, as they may enhance the interaction between DNA and the gel[1].

With sample A the digestion was more complete (67 min at an enzyme concentration of 5.5 units/mg DNA). From the gel electrophoresis pattern (Fig. 1), it can be seen that sample A contains one type of fragment consisting of 147 base pairs and that in sample B two types of fragments are present, which consists of approximately 180 and 350 base pairs. A third sample (C) was cleaved in a Waring blender[8] for 10 s and then digested with micrococcal nuclease for 3 days at an enzyme concentration of 124 units/mg DNA. This sample was treated further in the same way as samples A and B.

Sample D was obtained by sonication (250 ml of 0.44 g/l DNA at 4°C for 45 min) of purified calf thymus DNA (Worthington Biochemical, 2106 DNA) and isolated as described previously[9]. Gel electrophoresis indicated a number of base pairs between 400 and 800 (Fig. 1).

The GPC–LALLS measurements were carried out on a Waters Assoc. Model

150 HPLC instrument equipped with Toyo Soda (Tokyo, Japan) columns of type GWPH, G6000 PW, G5000 PW and G4000 PW. The LALLS photometer (Chromatix KMX 6) was placed between the columns and the refractive index (RI) detector. Hence the LALLS signal was observed before the RI response. Allowance was made for the dead volume between the two detector cells, so both signals could be considered simultaneously. For one run about 1 mg of DNA was required. An eluent ionic strength of 0.1 M sodium nitrate solution was chosen because it was sufficiently high for the type of columns used[2]. The other conditions were the same as reported previously[5].

RESULTS AND DISCUSSION

Every GPC run yielded an RI chromatogram and a LALLS chromatogram, as depicted in Fig. 2 for a specific run on sample A. Apart from a proportionality factor, the RI peak heights reflect the DNA concentrations in the eluate at particular elution times. Consequently, the area under the RI curve is proportional to the amount of DNA injected. This property was tested with only the GWPH pre-column, without any further column, in order to ensure that no adsorption effects were present. The LALLS chromatogram can be interpreted likewise when the scattering angle and the DNA concentration are sufficiently small (*i.e.*, the principle on which the detection method was based). Then the peak heights are, to a reasonable approximation, proportional to the DNA concentration multiplied by its molar mass. Therefore, there is a very plausible explanation of why the curves in Fig. 2 coincide almost exactly. Obviously the molar mass contributes only as a constant to the peak heights in the LALLS chromatogram. This means that sample A is mainly constituted of a single type of fragment. This conclusion can be drawn more convincingly from Fig. 3 where, in addition to the RI chromatogram, the logarithm of the molar masses has
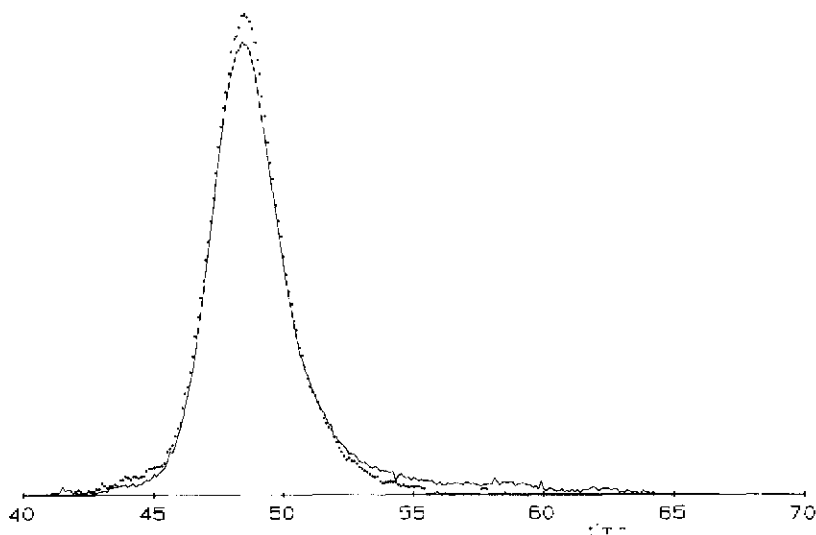

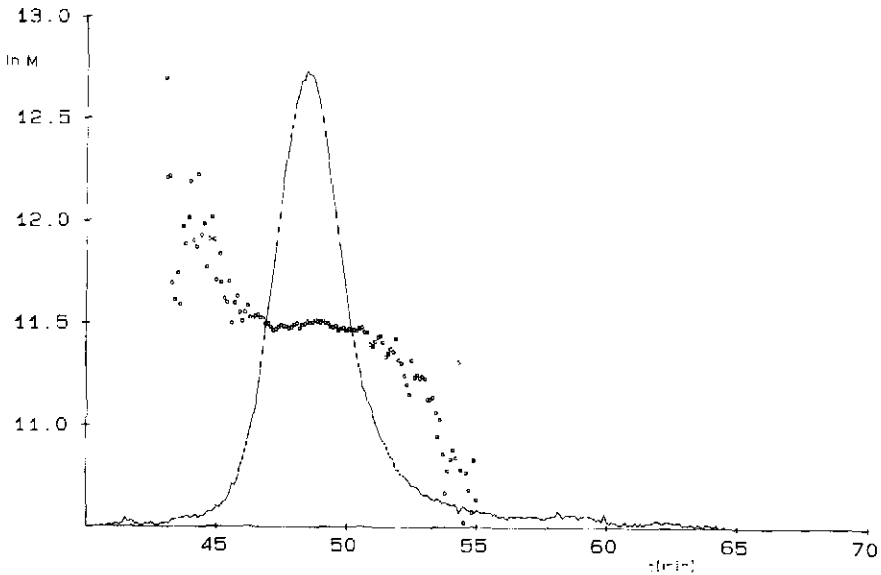Fig. 2. RI (———) and LALLS (......) chromatograms of DNA sample A obtained in experiment 3.

Fig. 3. Experimental values of ln $\bar{M}_w$ vs. elution time (t) together with the RI elution curve obtained for DNA sample A in experiment 3.

been plotted against the elution time. On the latter curve a plateau is clearly observed, which illustrates clearly uniform masses of the fragments appearing in the elution range covered by the RI peak. Curves of ln $\bar{M}_w$ vs. t can be accurately calculated from the RI and LALLS responses if the refractive index increments (dn/dc) and the

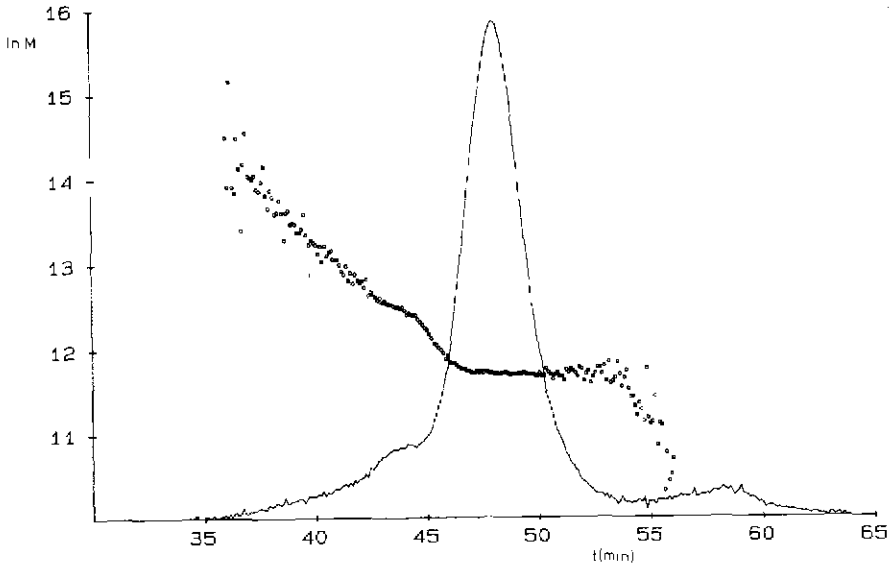Fig. 4. Experimental values of ln $\bar{M}_w$ vs. t together with the RI elution curve obtained for DNA sample B in experiment 8.
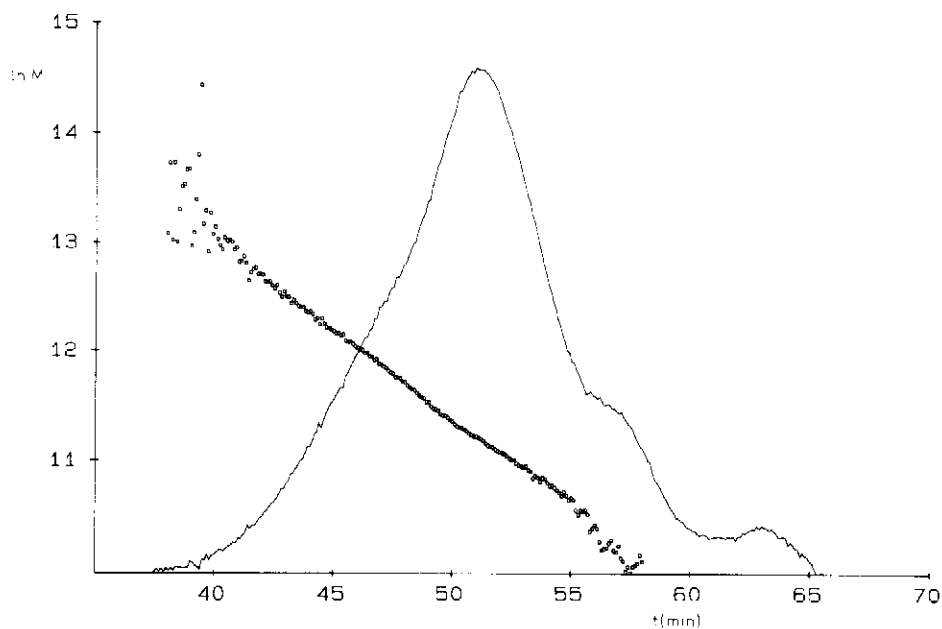
Fig. 5. Experimental values of ln $\bar{M}_w$ vs. t together with the RI elution curve obtained for DNA sample C (Waring blender) in experiment 10.

second virial coefficient (A_2) are previously known. In our calculations we used dn/dc = 0.168 ml/g (λ = 633 nm) and A_2 = 0.6 · 10^{-3} ml/mol · g^2. Both values, measured in our laboratory, refer to 25°C and to 0.1 M sodium chloride instead of to 0.1 M
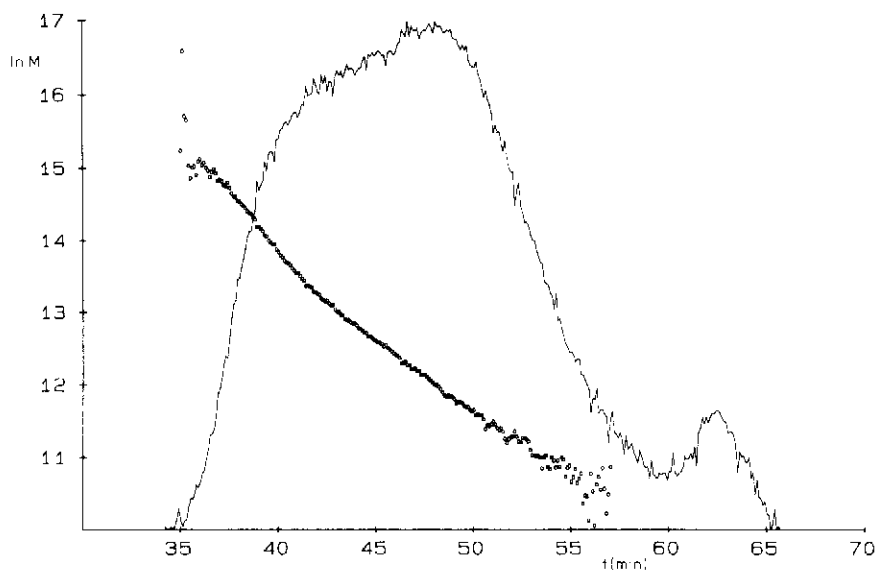

Fig. 6. Experimental values of ln $\bar{M}_w$ vs. t together with the RI elution curve obtained for DNA sample D (sonicated) in experiment 14.

sodium nitrate. The latter does not have a serious effect on the value of $\bar{M}_w$ (as was indicated by an independent light-scattering measurement).

The elution behaviour of sample B can be considered in a similar way (Fig. 4). Again a plateau appears ($48 < t < 50$), but at higher molar mass than for sample A. In addition, larger fragments become manifest as shown by a poorly defined plateau perceptible in the elution range $43.5 < t < 44.5$. The elution curves of samples C and D are shown in Figs. 5 and 6, respectively. Here no plateaus appear and the eluted fragments are more distributed over the elution range than in the former instances. Evidently these samples are heterodisperse with respect to the molar masses of the constituting fragments.

More quantitative conclusions can be drawn from Table I, which gives values of the weight-average molar masses and numbers of base pairs. Apart from a single exception (B), reasonable reproducibility of the results is found. It may also be concluded that the base pair values in Table I for samples A, B and D are consistent with the positions of the bands in the gel electrophoresis pattern (Fig. 1). The plateau value of sample A and its overall sample value are close to the expected values of 147 base pairs[10]. Owing to contamination by smaller fragments, the sample values are smaller than the plateau values. The reverse is seen for sample B, where there is interference from large fragments.

The question remains of why for sample B the plateau value is larger than that of sample A. The key to the answer must be sought in the incomplete digestion of the former sample. The situation is parallel to the case described by Sollner-Webb

TABLE I

WEIGHT-AVERAGE MOLAR MASSES AND NUMBERS OF BASE PAIRS (IN PARENTHESES) OF MONODISPERSE AND HETERODISPERSE DNA FRACTIONS

Sample	Expt. No.	$M \times 10^{-3}$ (plateau)	$\bar{M}_w \times 10^{-3}$ (sample)
A	1	102 (154)	98 (148)
	3	98 (148)	92 (139)
	5	103 (156)	98 (148)
	6	103 (156)	98 (148)
B	2	114 (172)	132 (199)
	4	107 (162)	132 (199)
	7	119 (180)	156 (236)
	8	119 (180)	157 (236)
B'	2	248 (375)	— —
	4	229 (346)	— —
	7	261 (394)	— —
	8	263 (397)	— —
C	9	Not observed	90 (136)
	10	Not observed	94 (142)
D	11	Not observed	408 (616)
	12	Not observed	418 (631)
	13	Not observed	438 (662)
	14	Not observed	429 (648)

and Felsenfeld[11], who studied the kinetics of the digestion of duck reticulocyte nuclei. In the first stage of the digestion they observed significant amounts of fragments of 180 and 370 base pairs. We also found fragments of about the same length, as can be seen from the two plateau values (Table I, B and B′).

Finally, we shall discuss briefly the phenomenon of spreading or dispersion, which particularly emerges from Figs. 2, 3 and 4. The peak widths observed in Figs. 3 and 4 have nothing to do with a separation on the basis of molar masses but represent the broadening of the spreading functions[12] due to the limited resolution of the GPC units. For samples A and B the spreading functions are almost Gaussian. In Fig. 2 it can be seen that the spreading of the RI and LALLS responses are identical, as it ought to be[5]. The above broadening effect does not influence the values of $\bar{M}_w$, as shown in Table I[5]. However, it cannot be ignored if other molar mass averages are concerned.

CONCLUSIONS

GPC extended with a molar mass detector is a useful technique for determining the size of DNA fragments. The virtue of LALLS detection is its generality, *i.e.*, the molar mass is determined irrespective of the form of the macromolecule. In this study the eluted DNA fragments were rod-like or sometimes worm-like chain elements. Extension to flexible-chain DNA seems to be possible. The only serious limit will be the restricted separation range of the columns. Another extension may be scaling up to a preparative routine. The analytical GPC–LALLS method already provides favourable prospects for such applications.

REFERENCES

1 M. E. Himmel, P. J. Perna and M. W. McDonnell, *J. Chromatogr.*, 240 (1982) 155.
2 Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, *J. Chromatogr.*, 266 (1983) 341.
3 Y. Kato, Y. Yamasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, *J. Chromatogr.*, 320 (1985) 440.
4 B. G. Stenlund, *Adv. Chromatogr.*, 14 (1976) 38.
5 J. A. P. P. van Dijk, F. A. Varkevisser and J. A. M. Smit, *J. Polym. Sci.*, in press.
6 H. Shindo, J. D. McGhee and J. S. Cohen, *Biopolymers*, 19 (1980) 523.
7 L. van Dijk, M. L. H. Gruwel, W. Jesse, J. de Bleijser and J. C. Leyte, *Biopolymers*, in press.
8 J. Granot, J. Feigon and D. R. Kearns, *Biopolymers*, 21 (1982) 181.
9 Th. Vreugdenhil, F. van der Touw and M. Mandel, *Biophys. Chem.*, 10 (1979) 67.
10 T. J. Richmond, J. T. Finch and A. Klug, *Cold Spring Harbour Symp. Quant. Biol.*, 47 (1982) 493.
11 B. Sollner-Webb and G. Felsenfeld, *Biochemistry*, 14 (1975) 2915.
12 L. H. Tung, *J. Appl. Polym. Sci.*, 10 (1966) 375.