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Note

Purification of spin-labelled DNA by hydroxyapatite chromatography

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Electron paramagnetic resonance (EPR) spectroscopy is widely used for studying the structure and conformation of RNA and proteins, but it is a relatively new method for investigation of DNA. On the basis of the few articles available[1–5], it can be concluded that the EPR spectra of spin-labelled DNA are only weakly immobilized[1,4]. This limits the application of the method when studying the fine structural and conformational changes in DNA molecules. Our experience in spin-labelling of DNA with alkylating spin-labels shows that in many cases the "weakly immobilized"[1,4] EPR spectrum may be due to the presence of unbound spin probe in the measured DNA preparation. It should be also that conventional method for separation of DNA and low-molecular-weight substances do not give satisfactory results when applied to the purification of spin-labelled DNA from the unbound spin-label.

In the present study we compare the efficiency of different methods for purification of spin-labelled DNA from low-molecular-weight spin-labelled compounds.

MATERIALS AND METHODS

Rat liver DNA was isolated as described previously[6]. The synthesis of hydrazine mustard spin label (HMSL) has been published[7].

The spin-labelling of DNA was carried out by mixing 200 μg of HMSL previously dissolved in dry dioxan (10 mg/ml) with 1 ml of DNA solution (1 mg/ml) in either 0.01 × 0.15 *M* sodium chloride–0.015 *M* sodium citrate (SSC) or 1 × SSC. The mixture was then vortexed and incubated at 37°C for 24 h. Spin-labelled DNA was purified from unbound spin-label by: (a) precipitation with ethanol at room temperature or at −20°C; (b) dialysis against 0.01 × SSC; (c) gel filtration on Sephadex G-50 and (d) hydroxyapatite (HAP) chromatography.

HAP chromatography was performed as follows. The incubation mixture was

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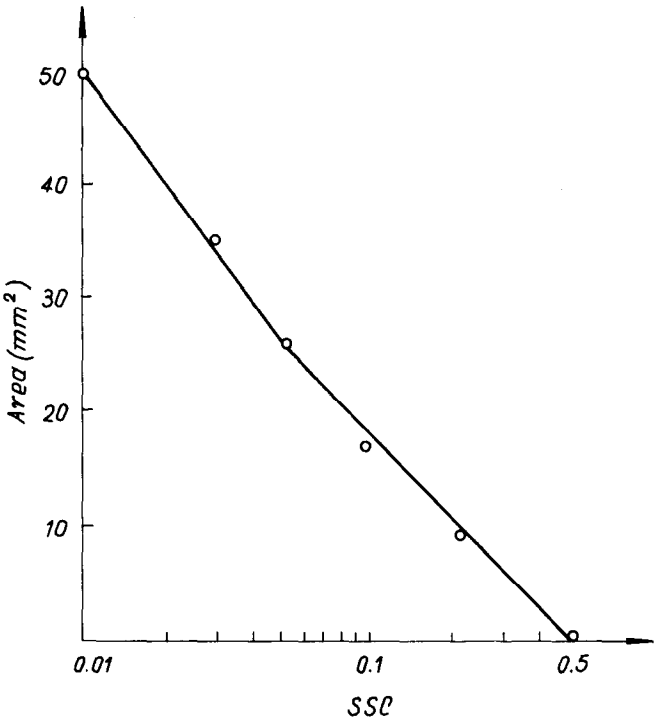

Fig. 1. Effect of SSC on the alkylation of DNA with HMSL. DNA samples were spin-labelled with HMSL (see Materials and Methods) by incubating in various concentrations of SSC, then purified by HAP chromatography at 50°C. After hydrolysis with DNase I (50 μg/ml), the EPR spectra were recorded as described.

applied to a HAP water-jacketed column (4–5 ml bed volumes per 1 mg of DNA) pre-equilibrated with 0.03 *M* sodium phosphate buffer (PB) pH 6.8 at 50°C. When the incubation of DNA with HMSL was carried out in 1 × SSC the samples were diluted ten times with 0.03 *M* PB before loading on HAP (SSC causes the adsorption capacity of HAP to deteriorate). The column was washed with 0.03 *M* PB (3–4 bed volumes) at 50°C and the DNA was eluted with 0.48 *M* PB at the same temperature.

To obtain the EPR spectra, 2 mg/ml DNA samples in 0.01 × SSC were prepared. EPR spectra were recorded on a ESR-220 (D.D.R.) spectrometer at room temperature under the following conditions: magnetic field, 3450 G; microwave power, 12.5 mW; modulation amplitude, 1 G; scan time, 27 min; microwave suppress, between 6 and 36 dB. The area under the spectra, S (mm²), was calculated from

$$S = I(\Delta H)^2$$

where I is the intensity of the EPR signal and ΔH the width of the resonance line[8]. The area of the sample was related to that of the free spin-label as a standard.

RESULTS AND DISCUSSION

The alkylation of DNA with HMSL is strongly dependent on the salt concentration. While 0.01 × SSC is optimal for spin-labelling, the alkylation reaction is completely suppressed in 1 × SSC (see Fig. 1). This made it possible to study the efficiency of different methods for purification of spin-labelled DNA from the unbound spin-label by using two different experimental approaches: by carrying out the reaction in (a) 0.01 × SSC (ionic strength optimal for alkylation) and (b) 1 × SSC (alkylation is suppressed). In the first case a part of the spin probe is covalently bound to DNA, while in the second all the molecules of spin-label are free in the reaction mixture. The presence of any EPR signal in the latter case is an indication of the presence of free spin-label in the measured DNA preparation, *i.e.*, indication of incomplete purification. It is more difficult to estimate the efficiency of the DNA purification by the first approach (spin-labelling in 0.01 × SSC).

The analysis of the EPR spectra of double-stranded DNA preparations spin-

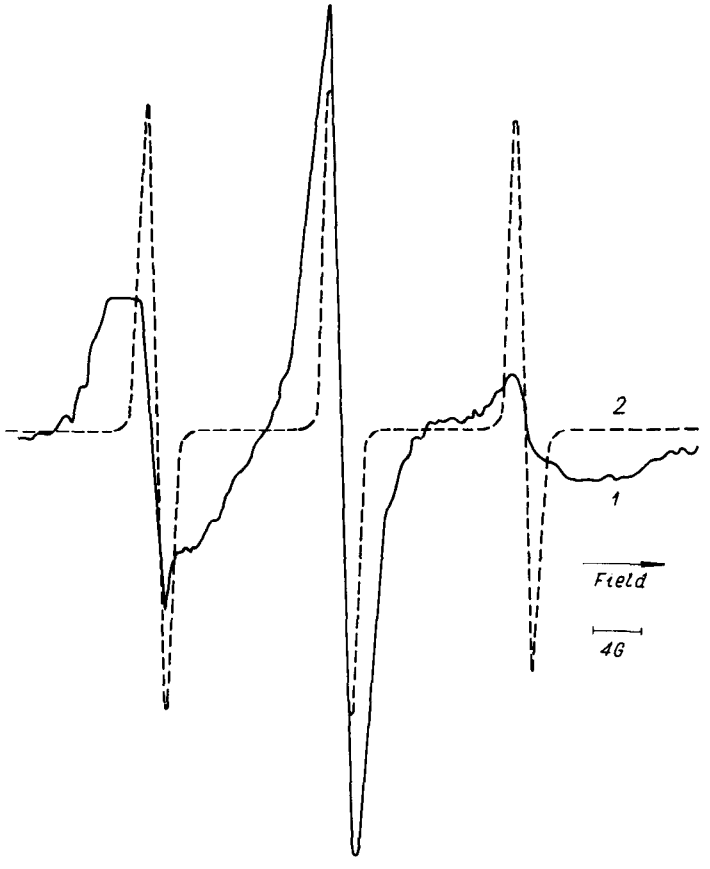

Fig. 2. EPR spectra of double-stranded DNA spin-labelled with HMSL and purified on HAP at 50°C (1), and of free HMSL in dioxan–water (1:1) (2).

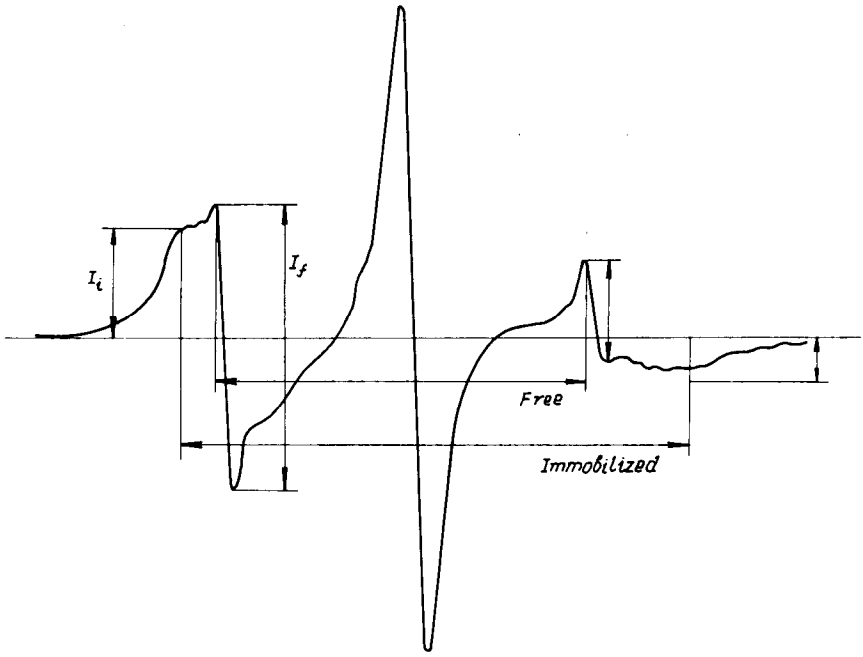

Fig. 3. EPR spectrum of double-stranded DNA spin-labelled with HMSL and purified on HAP at room temperature. The spectrum consists of the superposition of two (or more) components, one of which is free and the other immobilized. The ratio $R_i = 2I_i/I_f$, as calculated from the deformation of the resonance signal at low magnetic field, reflects the relative contribution of each component to the resulting spectrum.

labelled with HMSL showed that they consist of at least two different components, one with the same EPR spectrum as the free spin probe and another whose absorption lines are substantially broadened (Fig. 2 and 3). The contributions of these two signals to the resulting spectrum depend on the secondary structure of DNA (perfectness of the double helix), its base composition, molecular weight, etc.[9]. The relative contributions are best reflected in the resonance signal at low magnetic field. Thus, only this part of the spectrum was used for the further estimation of DNA purification. A roughly quantitative evaluation of the data obtained was achieved using the relationship

$$R_i = 2I_i/I_f$$

where R_i is the ratio of immobilization, I_i is the intensity of the signal corresponding to the immobilized spin-label and I_f the intensity of the signal of the free spin-label (Fig. 3).

When a double-stranded high-molecular-weight DNA with a GC content of 40–50% is used the value of R_i varies from 0 to 1. As is seen in Fig. 4 and Table I, R_i reaches a maximum value ($R_i = 1$) only for DNA preparations purified on HAP at 50°C. Even in the case of HAP chromatography at room temperature R_i is less than 1. It is also lower for DNA preparations purified by dialysis, gel filtration and ethanol

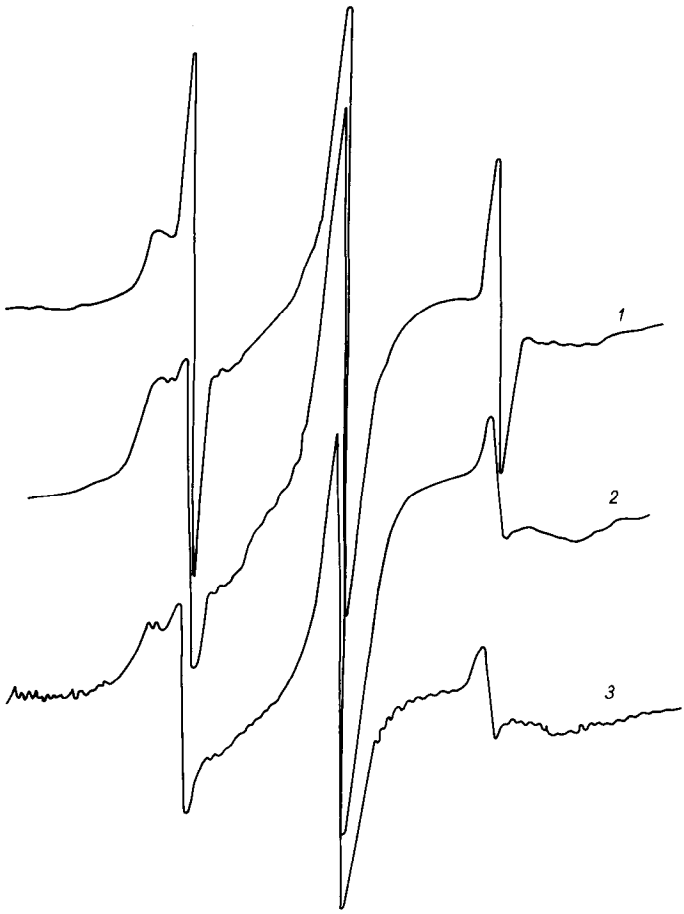

Fig. 4. EPR spectra of double-stranded DNA preparations spin-labelled with HMSL in 0.01 × SSC. The spin-labelled DNA was purified by ethanol precipitation (1), gel filtration on Sephadex G-50 (2) or dialysis against 0.01 × SSC (3). The EPR spectra were recorded as described in Materials and methods with the following suppression: 24 dB for sample 1, 18 dB for 2 and 12 dB for 3.

TABLE I

RATIO OF IMMOBILIZATION OF HMSL IN DOUBLE-STRANDED DNA PREPARATIONS SPIN-LABELLED IN 0.01 × SSC AND PURIFIED BY DIFFERENT METHODS

Method	R_i
HAP chromatography at 50°C	1.00
HAP chromatography at room temp.	0.85
Dialysis against 0.01 × SSC	0.80
Gel filtration on Sephadex G-50	0.69
Ethanol precipitation	0.30

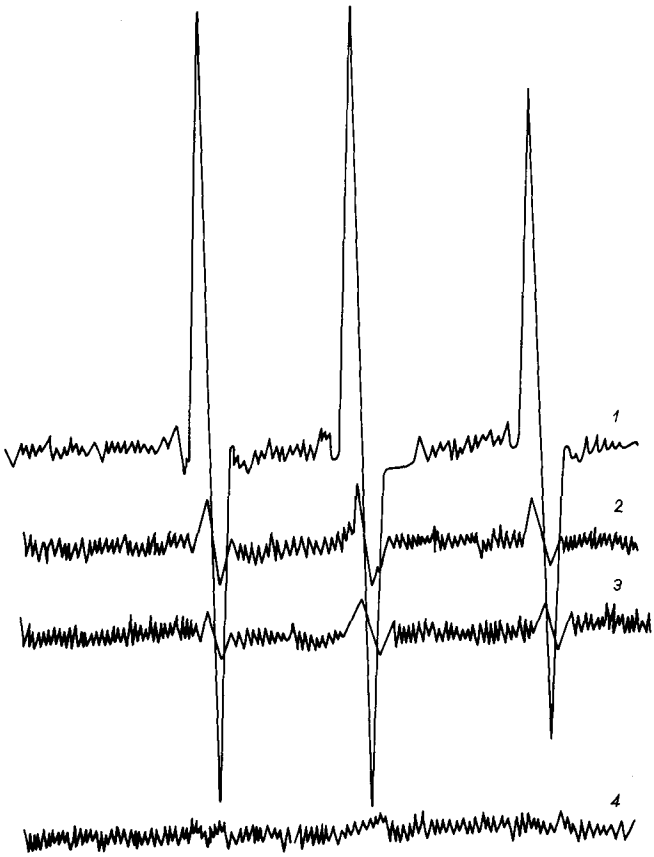

Fig. 5. EPR spectra of double-stranded DNA preparations spin-labelled with HMSL in 1 × SSC (no alkylation). DNA samples were incubated with HMSL (see Materials and methods) in 1 × SSC and purified by ethanol precipitation (1), gel filtration on Sephadex G-50 (2), dialysis against 0.01 × SSC (3) or HAP chromatography at 50°C (4).

TABLE II

EFFICIENCY OF PURIFICATION OF DOUBLE-STRANDED DNA SPIN-LABELLED WITH HMSL IN 1 × SSC (NO ALKYLATION)

DNA samples were spin-labelled with HMSL, purified, hydrolysed with DNase I and the EPR spectra recorded as described in Materials and methods. The area under the spectra was measured and related to a constant amount of DNA.

Method	*Area under the EPR spectrum (arbitrary units)*
HAP chromatography at 50°C	0.0
HAP chromatography at room temp.	20.0
Dialysis against 0.01 × SSC	28.0
Gel filtration on Sephadex G-50	33.0
Ethanol precipitation	211.0

precipitation. In the last case, R_i varies irreproducibly over a very wide range and depends on many factors such as DNA concentration, temperature and manner of precipitation (with or without glass rod).

The results presented are supported by data obtained from spin-labelling of DNA with HMSL in 1 × SSC (no alkylation). In this case the area under the spectrum corresponding to a constant amount of DNA was used as a measure of the amount of free spin-label in the DNA preparations. From Fig. 5 and Table II, it is seen that only the DNA sample purified on HAP at 50°C exhibited no EPR signal.

HAP chromatography is applicable also to the purification of single-stranded DNA labelled with alkylating spin-labels, but in this case R_i cannot be used as a criterion for purification. (The EPR spectrum of single-stranded DNA is very similar to that of the free spin-label.) In the present study the efficiency of HAP for purification of single-stranded DNA was tested by using DNA preparations treated with HMSL in 1 × SSC. No EPR signal was detected.

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