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THE BEHAVIOUR OF NUCLEIC ACIDS IN MEMBRANE CHROMATOGRAPHY AND ELECTROPHORESIS ON NITROCELLULOSE FILTERS

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SUMMARY

Nitrocellulose membrane filters of various types, both intact or impregnated with Tween or serum albumin, were used as carrier material for small-scale chromatography and electrophoresis of native and denatured DNA, RNA and proteins. DNA isolated from calf thymus, and from *B. subtilis*, total RNA from chick myeloblasts and human serum albumin and their mixtures, in various binary combinations, served as model samples in the experiments. Horizontal chromatography and electrophoresis of 0.5–2 $\mu g/\mu l$ samples was completed within 1–30 min on 3–4 cm long strips in citrate, veronal or acetate buffers pH 7, 8.6 or 4.5. Nucleic acids were stained with toluidine blue, proteins with nigrosin. Characteristic behaviour of different samples under different conditions is described and optimal conditions are found for the separation of most substances under study. In general the model substances to be separated either remained at or moved from the start in single spots. However, the sieving effect of small-pore membranes permitted the convenient detection of the presence of three components of total RNA by electrophoresis.

The micromethod also appears to extend the applicability of membrane chromatography and electrophoresis on nitrocellulose filters into the field of nucleic acids, especially where rapid information and simple characterization of minute samples is needed.

Nitrocellulose membranes have been found to be a suitable carrier for microchromatography and microelectrophoresis of various substances[1,2]. Under appropriate conditions they show a specific adsorptivity towards proteins, which makes it possible to achieve a series of rapid chromatographic separations and determinations especially in the field of protein analysis[3,4].

Nitrocellulose membranes have also been used in the investigation of nucleic acids, especially of RNA–DNA and DNA–DNA hybrids[5–10]. These experiments were

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based on the fact that denatured (single stranded) DNA can be bound firmly to the membranes under appropriate (rather severe) conditions of drying, whereas the adsorption of RNA to the membranes is negligible. When filtering a solution of labelled RNA through the membranes "loaded" with denatured DNA, annealing of RNA and DNA molecules takes place and the extent of hybridization can be estimated by measuring the radioactivity of RNA remaining on the filter disc. A chromatographic column filled with nitrocellulose also permits the separation of native DNA, RNA and denatured DNA by column chromatography[11].

In a previous communication we referred to the microchromatographic separation of RNA and serum proteins on nitrocellulose membranes[12]. In the present work we wanted to investigate in more detail the behaviour of different preparations of nucleic acids on various types of commercial nitrocellulose membranes in small scale chromatography and electrophoresis under mild conditions, avoiding drying of the samples and preferably using common staining procedures for detection.

MATERIALS AND METHODS

Nitrocellulose membranes: Synpor 1, 2, 3, 4, 5, 6 and 8 (Chemapol, Prague, Czechoslovakia), Sartorius 11001, 11002, 11004, 11006, 11007, 11008, 11009, 11011, 11307 and 11308 (Membranfiltergesellschaft, Göttingen, Germany), Millipore HA (Millipore sa., Malakoff, France) and Selectron BA 83 and BA 85 (Schleicher & Schuell, Dassel, Germany) were used. Acetylcellulose membranes Oxoid (London) and Sartorius 50 A were also used in comparative experiments.

Nucleic acids: RNA samples were prepared from chick leukaemic myeloblasts by the extraction with the two phase system phenol–0.1 *M* acetate (pH 6), using successively increasing temperatures[13]. The following RNA samples were used:

(a) A fraction of cytoplasmic RNA containing ribosomal RNA (28 S, 16 S) and soluble RNA (4 S)

(b) 1 *M* NaCl-insoluble part of the RNA preparation composed from 28 S and 16 S ribosomal RNA and only minutely contaminated by soluble RNA. The RNA samples used were not significantly contaminated with proteins or DNA[13].

A preparation of calf thymus DNA and three different preparations of DNA from *B. subtilis* (a gift from Dr. S. ZADRAŽIL, Czechoslov. Acad. of Sciences, Prague) were used. The samples were dissolved in the "SSC" buffers (mostly 0.15 *M* NaCl and 0.015 *M* Na citrate), pH 7.0 and contained between 0.5 mg and 2 mg of nucleic acid per ml. Denaturation of nucleic acids was performed by heating the solution to 98–100° for 15 min and then cooling rapidly by immersing the test tube in an alcohol bath of −27°[11,14,15].

Human and bovine serum albumin samples were prepared in our Institute. They were denatured by heating 1% solutions at pH 8.6 for 3 min at 98–100°. Chromatography and electrophoresis were carried out horizontally on strips 1–2 cm × 3–4 cm in simple plexiglass chambers. Nucleic acids were applied on the wet membranes in 0.1–1 μl amounts by glass capillaries. Chromatography and electrophoresis, respectively, were begun immediately after the samples had been absorbed. Citrate buffers SSC, pH 7.0 were used in most experiments, veronal–citrate–oxalate buffer pH 8.6, $\mu = 0.1$ and 0.1 *M* acetate buffer pH 4.5, were also used in some comparative experiments. For electrophoresis, the veronal and acetate buffers were diluted 1:1

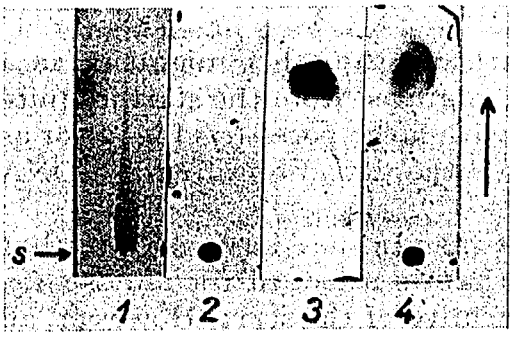
(v/v), the SSC buffer 1:3 (v/v), by adding an aqueous solution of 10% glycerine. Chromatography usually lasted 1–30 min, the time being dependent mainly on the porosity and mean pore size of the membranes[2,1]. Electrophoresis was usually performed at 20–22 V/cm and 0.4–0.5 mA/cm and lasted 10 min. Impregnation of the membranes by Tween 20, 60 or by proteins was carried out as described before[4]. Nucleic acids were stained by toluidine blue or methyl green as mentioned previously[12]. The membranes were not dried before staining. (Detection by U.V. light was not suitable since nitrocellulose membranes have a very high absorption themselves. Autoradiography seemed neither convenient nor necessary in the present investigation.)

RESULTS

Chromatography

Typical results of chromatography on membranes with mean pore size below 0.9 μm are summarized in Fig. 1. Very similar results to those obtained with Sartorius 11007 (Fig. 1) were achieved on Sartorius 11004, 11005, 11006, 11008, 11009, and in some experiments on Synpor 6 and 8, on Millipore HA and Selectron BA 83 and 85 also. However, on the latter 3 membranes a disturbing tailing was usually observed between the spots of denatured DNA, at the start, and of RNA at the front. The spots of denatured DNA were usually adsorbed on the upper side of the membrane. In some samples of denatured DNA a slight second spot was visible at the front, which was probably due to degradation products[14,15]. The separation of RNA and denatured DNA was similar with DNA from thymus as well as from *B. subtilis*. However, native DNA from thymus formed more prolonged spots and tailing than did bacterial DNA.

As is shown schematically in Fig. 2, the behaviour of denatured DNA was found to be quite different on membranes having a mean pore size greater than 5 μm, *e.g.*



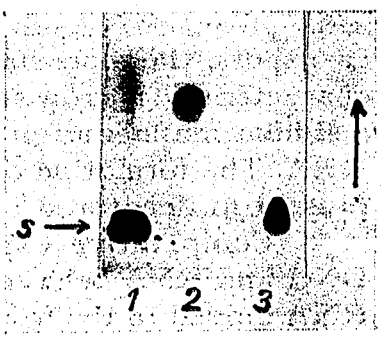


Fig. 1. Chromatography of total RNA and DNA on membranes Sartorius 11007. (1) Native calf thymus DNA; (2) heat denatured calf thymus DNA; (3) total RNA from chick leukaemic meyloblasts; (4) mixture of samples 2 and 3. (The chromatograms of both native and denatured DNA from *B. subtilis* were similar to that of sample 2.) SSC buffer pH 7.0; chromatographed for 20 min. Stained with toluidine blue; s = start.

Fig. 2. Chromatography of denatured DNA and denatured human serum albumin (HSA) on membranes Sartorius 11001. (1) Mixture of denatured DNA from *B. subtilis* and denatured HSA; (2) denatured DNA; (3) Denatured HSA. SSC buffer pH 7.0; chromatographed for 1 min; proteins stained with nigrosin, DNA with toluidine blue.

Synpor 1 or on Sartorius 11001 or 11002. Denatured DNA (both from calf thymus and *B. subtilis*) migrated with the front similarly to RNA, so that a separation of RNA and denatured DNA was not possible on these membranes. However, this permits a simple separation of denatured DNA from the remnants of denatured proteins, which stay at the start. Similarly, at pH 4 even native proteins were separated from denatured DNA.

A separation of denatured and native DNA by chromatography was not successful although in separate runs both types of DNA migrated differently. A similar difficulty, caused presumably by the formation of a complex, was encountered when a mixture of RNA and DNA was chromatographed.

Marked changes in the chromatographic behaviour of DNA were found on membranes having smaller pores (*e.g.* Sartorius 11007) after they had been impregnated with Tween (Fig. 3). Native DNA usually formed a slight streak and sometimes even

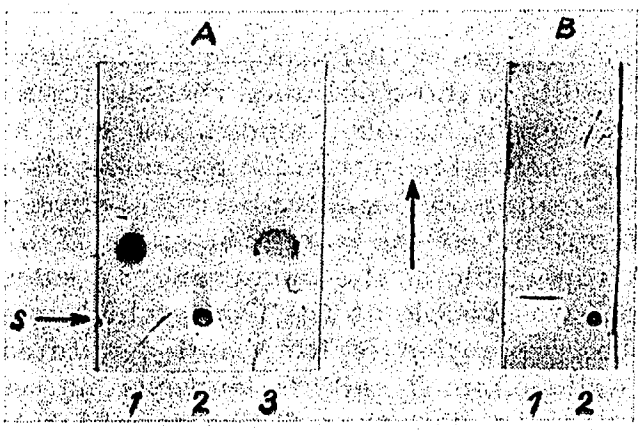


Fig. 3. Chromatography of RNA, native and denatured DNA on membranes Sartorius 11007 impregnated with 2% Tween 60. A: (1) RNA; (2) native DNA *(B. subtilis)*; (3) denatured DNA. B: (1) Denatured DNA applied into the limited area impregnated with Tween 60 (that area was significantly less coloured); (2) a comparative sample of denatured DNA applied on the non-impregnated part of the membrane. SSC buffer pH 7.0; stained with toluidine blue.

two spots, the more intense one remaining at the start and the other migrating in a characteristic narrow streak, whereas denatured DNA migrated from the start in quite a similar manner as on non-impregnated membranes with large pores (*cf.* Fig. 2) or as RNA. The formation of the spot at the start permitted the detection of native DNA present in denatured DNA.

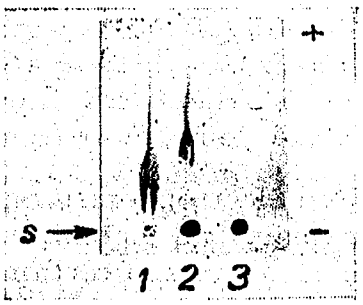
Comparative experiments with acetylcellulose membranes have shown that DNA and RNA behaviour was, in general, similar to that on intact nitrocullulose, *i.e.* RNA moved to the front and DNA remained in the vicinity of the start. However, all spots were very elongated and showed much tailing. This fact and also an intense staining of the background of the chromatograms did not favour the use of acetylcellulose.

Electrophoresis

Electrophoresis of the samples of nucleic acids performed at different pH values and on membranes having different mean pore sizes led to the following results.

In general, electrophoresis permitted more rapid runs than chromatography on

membranes with pores smaller than about 0.5 μm. The electrophoretic patterns of native DNA isolated from calf thymus differed in some respects from DNA isolated from *B. subtilis*. While calf thymus DNA moved as a characteristic narrow streak towards the anode (Fig. 4) on membranes with a mean pore size about 0.4 μm at pH 7 and 8.6, the bacterial DNA remained predominantly at the start and only a minute portion migrated to the anode (Fig. 5). On membranes with smaller pore sizes both types of native DNA remained at the start and formed only a slight streak towards the anode.



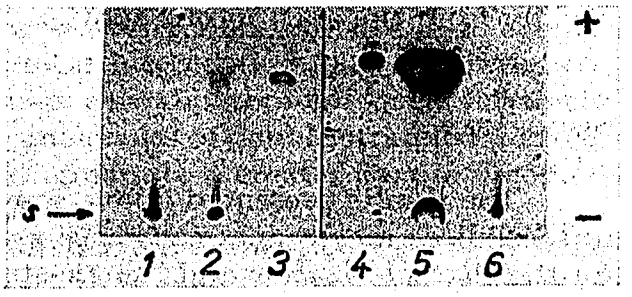


Fig. 4. Electrophoresis of native and denatured DNA on Synpor 6 membranes. (1) Native DNA (calf thymus); (2) Mixture of native and denatured DNA; (3) Denatured DNA. Veronal-citrate-oxalate buffer, pH 8.6, μ = 0.05.

Fig. 5. Electrophoresis of RNA, native and denatured DNA on Synpor 6 membrane impregnated with Tween 60. (1) Native DNA (*B. subtilis*); (2) mixture of native and denatured DNA; (3) denatured DNA; (4) RNA; (5) mixture of RNA and native DNA; (6) native DNA (*B. subtilis*). SSC buffer pH 7.0.

Denatured DNA from both thymus and *B. subtilis* did not move from the start, especially on membranes having smaller pores than 0.2 μm (*e.g.* Synpor 8 and Sartorius 11011). This permitted a rapid separation of denatured DNA from RNA, which moved very rapidly towards the anode at pH 7 and 8.6 on all types of membranes.

"Total" RNA samples were separated into three zones at pH 8.6 on membranes having a mean pore size less than 0.4 μm, which was probably due to the sieving effect of those membranes (Fig. 5(4) and Fig. 6).

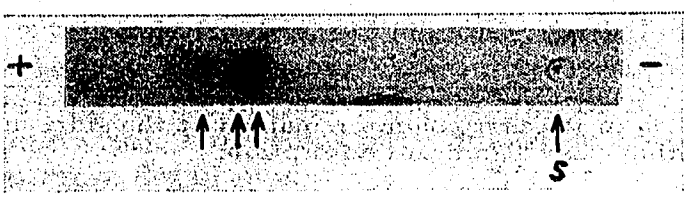


Fig. 6. Electrophoresis of RNA on Synpor 6 membranes. Veronal-citrate-oxalate buffer, pH 8.6, with 5% glycerin, μ = 0.05.

Marked changes in electrophoretic behaviour, especially of denatured DNA (similar to the changes in chromatographic behaviour), were found on nitrocellulose membranes having smaller pores than about 0.5 μm at pH 7 after they had been impregnated with Tween 60. Thus, denatured bacterial DNA migrated as a compact spot towards the anode rather like RNA, whereas native DNA remained at the start.

Under these conditions it was possible to separate native DNA from denatured DNA and RNA, respectively (Fig. 5). However, this was not always possible at pH 8.6, since native DNA usually formed two spots during electrophoresis, one of which interferred with the spots of denatured DNA and RNA.

Investigation of suitable conditions, whereby RNA would be immobilized at the start, showed that only electrophoresis (and to a lesser extent also chromatography) at pH 4.5 on nitrocellulose membranes impregnated with native or denatured bovine serumalbumin led to satisfactory results. The chromatographic variant of this procedure seems to offer the separation of RNA from free amino acids, which migrate rapidly from the start in one spot[4].

DISCUSSION

The results presented above show that chromatography and electrophoresis on nitrocellulose membranes can serve as a simple and rapid microanalytical technique for the characterization and "single-step" separation of very small samples of certain preparations of nucleic acids and proteins. As is shown in the figures and summarized in Table I it was possible to separate, in model experiments, the individual components of various binary combinations of the given samples under suitable conditions.

TABLE I

SUITABLE CONDITIONS FOR THE SEPARATION OF CERTAIN BINARY COMBINATIONS OF NUCLEIC ACIDS AND PROTEINS ON NITROCELLULOSE MEMBRANES

Substances to be separated	*Chromatography*			*Electrophoresis*		
	Mean pore size (μm)	*pH*	*Impregnation*	*Mean pore size (μm)*	*pH*	*Impregnation*
DNA + RNA	—	—	—	0.1–0.5	7	Tween 60
Denatured DNA + RNA	0.1–0.9	7	—	0.1–0.3	7; 8.6	—
Serum proteins + RNA	0.1	7	—	—	—	—
DNA + serum proteins	0.4	7.5; 5.4	Tween 20	—	—	—
DNA + denatured DNA	0.3	7	Tween 60	0.4	7	Tween 60
Denatured serum proteins + denatured DNA	5	7	—	—	—	—
RNA + amino acids	0.4	4.5	serum albumin	—	—	—

The substances quoted on the left hand side in the first column remained at the start or in its vicinity, the other substances migrated without any significant adsorption.

Some of our results were in general agreement with the observations made during filtration experiments of nucleic acids on nitrocellulose membranes[5–10] or during chromatography on nitrocellulose columns[11]. Thus RNA usually migrated quite freely during chromatography, native DNA moved only to a limited degree and denatured DNA remained either at the start (on membranes with smaller pores) or moved predominantly from the start (on membranes with larger pores). The latter observation, that different values of the mean pore size of the membranes made them more or less suitable for the separation of DNA samples, seems to indicate that a filtration

mechanism might be decisive for the immobilization of denatured DNA at the start during electrophoresis. However, experiments on membranes with small pores, impregnated with Tween 60, where denatured DNA migrated, indicates that other factors apart from a simple mechanical hindrance play a role on intact membranes. Since a massive splitting of DNA molecules by the detergent to smaller filterable fragments does not seem probable (*cf.* Fig. 3B) we think that the following explanation is possible: The long thin fibres of denatured DNA might be intercepted both mechanically and by adsorption in the rigid microporous labyrinth of the membrane. After impregnation with Tween the previously rigid inner surface is assumed to be covered by a rather flexible, slippery and to a certain degree even mobile film of the detergent. As a result of this both adsorption and mechanical interception may be prevented, and the flexible single stranded DNA molecules can slip through even relatively narrow channels in the membrane. By contrast, molecules of double stranded (native) DNA, which are presumably less flexible, might remain to a greater part intercepted mechanically even on the impregnated membranes.

The finding that native as well as denatured DNA formed two spots in some experiments may be explained by the known fact that even purified preparations of nucleic acids are neither quite stable nor homogeneous, so that native, denatured and renatured forms, as well as molecules differing in their molecular weight and shape, must be expected to be present to a certain extent in each sample[14,15]. The specific differences between bacterial and thymus DNA may, of course, also have a role here.

As for the different behaviour of native DNA during chromatography and electrophoresis (*cf.* Figs. 1 and 4), it may be assumed that in the given electric field the DNA molecules are pulled from the start by a force greater than the forces binding them to the membrane. During chromatography the traction is exerted only by the hydrodynamic friction between the DNA molecules at the membrane and the molecules of the slowly migrating developing buffer. That force is evidently too small so that the greater part of DNA does not move from the start.

As was already mentioned, RNA migrated freely on the membranes under most experimental conditions in contrast to DNA. It seems difficult to give an explanation for this on the basis of the present experimental data. However, we are inclined to believe that the explanation might be found in terms of chemical structure of RNA, rather than in terms of molecular weight or size although the latter may also play a certain role (*cf.* Fig. 6). The only condition permitting complete or partial immobilization of RNA in our experiments was both chromatography or electrophoresis at pH 4.5 on membranes impregnated with serum albumin (on intact membranes RNA migrated). This seems to be due to electrostatic interactions and is being investigated in more detail now.

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