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A RAPID PURIFICATION METHOD OF RESTRICTION ENDONUCLEASES FROM *HAEMOPHILUS* STRAINS

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Summary

A simple and rapid method of purification of restriction endonucleases from different *Haemophilus* strains is presented. By this method highly purified and stable enzymes can be obtained. Separation of different restriction activities present in the same strain is possible. This method was so far successfully used with *Haemophilus influenzae*, *Haemophilus parainfluenzae* and *Haemophilus aegyptius* strains.

The main advantages over previously published procedures reside in the simplification of certain purification steps (for instance the BioGel A 0.5 M filtration is replaced by a hydroxyapatite batch step), elimination of exonuclease activity by fractionation with $(NH_4)_2SO_4$, separation of different restriction activities by phosphocellulose chromatography, application of this method to various strains and high purification degree of enzymes.

Introduction

Restriction nucleases isolated from different bacterial strains are highly site-specific endonucleases producing double-strand cleavages in native, non-modified DNA, and releasing well-defined fragments of DNA. For this reason these enzymes are an advantageous tool for the study of the organization of viral, bacterial and eucaryotic genomes.

We describe here a rapid method for the purification of restriction enzymes from different *Haemophilus* strains (*Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Haemophilus aegyptius*); with this procedure, which incorporates some steps of the original methods [1—3], highly purified restriction

Abbreviations: Eco RI, Hin II, Hin III, Hpa I, Hpa II, Hae: nomenclature proposed for restriction modification systems by Smith and Nathans [9]. NaCl/citrate buffer: 0.015 M sodium citrate/0.15 M NaCl.

endonucleases with no contamination by non-specific endonucleases and by exonucleases are obtained, and different restriction activities present in the same strain are separated (ex: Hpa I and Hpa II).

Additional advantages of this method over previous ones are quicker purification, and separation of activities. The purification procedure is greatly simplified by the use of hydroxyapatite batch elimination of most of nucleic acids, instead of the long filtration through BioGel A 0.5 M. This gives a 15-fold purification of crude enzyme extract, instead of a 3-fold purification obtained with the BioGel filtration, and it is much more rapid and simple to handle.

Moreover, step-wise fractionation with $(NH_4)_2SO_4$ allows separation of endonuclease activities from exonucleases. The different restriction activities are then separated from each other by chromatography on a phosphocellulose column. This method can be used without any changes on different *Haemophilus* strains, and enzymes obtained by this method are purified 1000 to 5000 times.

Materials and Methods

Strains and growth conditions

H. influenzae Rd and *H. parainfluenzae* strains were obtained from Professor S. Goodgal; *H. aegyptius* from Institute Pasteur (Paris). Cells were grown in brain heart infusion (Difco Laboratories, Detroit, Michigan, U.S.A.; BD-Merieux, France) 37 g/l, supplemented with nicotinamide-adenine dinucleotide (NAD) (Merck AG, Darmstadt, Germany) 2 μg/ml, and haemin chloride (Calbiochem, San Diego, California, U.S.A.) 10 μg/ml; for *H. parainfluenzae* growth haemin was omitted from the medium. A preculture was prepared by inoculation of 1 l of complete medium with cells in logarithmic phase and growth was continued at 37° C overnight without shaking. Overnight precultures were used as inocula for large scale preparation of cells (15 or 40 l). From large scale preparation we obtained 2.5—3 g of wet weight cells/l in a Biolafitte fermentor (France) with aeration (1 l of air/l of culture per min) and stirring (500 rev./min).

DNA preparation

H. influenzae and *H. parainfluenzae* DNAs were prepared by a modification of the method described by Goodgal and Herriott [4] followed by hydroxyapatite chromatography according to Bernardi [5].

Calf thymus DNA was isolated according to the method of Kay et al. [6]. SV40 DNA was prepared from subcloned CV 1 cell line, infected by large plaque strain SV40, by selective extraction of viral DNA [7]. Eco RI was kindly supplied by Dr P. Yot from this Institute.

Assays of enzyme activity

(a) *Viscosimetry.* Restriction endonuclease activity (Endo R) was determined by following the decrease of DNA viscosity at 37 °C, using an Ostwald viscometer (flow rate of buffer about 60 s^{-1}), with calf thymus, *H. influenzae* or *H. parainfluenzae* DNAs as substrate. To 4 ml of DNA (A_{260nm} = 1.7—1.8) 5—50 μl of enzyme were added and viscosity followed at regular time intervals till a plateau was reached.

Enzyme units are those of Smith and Wilcox [1] at 37°C.

(b) *SV40 DNA digestion followed by electrophoresis in polyacrylamide gels.* 0.02—0.1 A_{260nm} of SV40 DNA was digested with 0.001—0.1 unit of enzyme, at 37°C, in the appropriate buffer; the final digest was incubated 30 min at 37°C in 1% sodium dodecylsulfate; saccharose and bromophenol blue were added to the digest to final concentrations of 7.5 and 0.015%, respectively. Slab gels of different concentration of polyacrylamide (2.2—4%), supplemented by 0.5% agarose (for gels under 3% polyacrylamide), were run at 40 mA and at 4°C, for 12—20 h; the slabs were either 0.4 × 16 × 20 cm or 0.4 × 16 × 40 cm, and the electrophoretic buffer was 0.04 M Tris/acetate, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.8.

Bands were revealed by soaking the gels in the electrophoresis buffer supplemented with 2 μg/ml of ethidium bromide (Sigma, St. Louis, Mo. U.S.A.) for 60 min and visualized in ultraviolet light (short-wavelength lamp) [3]. Exonuclease activity was followed by liberation of acid-soluble products using ^{32}P- or ^{3}H-labelled SV40 or T7 DNAs.

Other methods

(a) Protein concentration was estimated by absorbance at 280 nm.

(b) Electrophoresis of proteins was performed in 7.5% polyacrylamide gels according to the method of Maizel [8].

(c) Hydroxyapatite was prepared by the method described by Bernardi [5].

Results and Discussion

Purification procedure of H. influenzae restriction enzymes

Cells from logarithmic phase of growth (0.7 A_{650nm}) were harvested by centrifugation. 40—45 g of wet weight of cells were obtained from a 15-l culture. Fresh cells were washed once with NaCl/citrate buffer, resuspended in 0.05 M Tris/HCl buffer, pH 7.4, with 0.001 M glutathione (1 g of cells/1.5 ml) and disrupted by sonication (MSE-sonicator, London, England) at 20 kcycles/s, 5—6 times 1 min, in an ice-salt bath to keep the temperature below 5°C. Absorbance at 650 nm of suspension of cells in water (1/200) was followed, the decrease usually was 90% of the initial A_{650nm}.

All further operations were carried out at 0—4°C. Cellular debris were eliminated by 30 min centrifugation at 17 000 rev./min and the supernatant was stored (Fraction I).

Hydroxyapatite batch: about 100 ml of crude enzyme extract (Fraction I) were adsorbed on 300 ml of packed hydroxyapatite equilibrated with 0.001 M potassium phosphate buffer in the presence of 0.01 M 2-mercaptoethanol, pH 6.8, under gentle stirring and kept at 4°C overnight without stirring.

Hydroxyapatite was sedimented by short centrifugation (3 min at 3000 rev./min) and the supernatant was discarded. Hydroxyapatite was then washed many times with 1 M KCl in 0.001 M potassium phosphate buffer/0.01 M 2-mercaptoethanol, pH 6.8, until A_{260nm} was lower than 1. Enzyme activity was then eluted from hydroxyapatite with 0.17 M potassium phosphate buffer/0.01 M 2-mercaptoethanol and 1 M KCl, pH 6.8. Elution with portions of

this buffer was repeated three times, each followed by short centrifugation. Supernatants were stored at 0°C and the activity tested in each of them by viscometry. By this step a 15-fold purification is obtained (Table II). Supernatants with restriction endonuclease activity were pooled and diluted with one volume of 0.001 M sodium phosphate buffer/0.01 M 2-mercaptoethanol, pH 7.4, to decrease salt concentration (Fraction II). To 1840 ml of this fraction 575.9 g of solid $(NH_4)_2SO_4$ (0.5 saturation at 20°C) were slowly added (in a time interval of 20 min) and mixed gently for 30 min. The precipitate was removed by 15 min centrifugation at 8000 rev./min. To 2000 ml of the supernatant 132 g of solid $(NH_4)_2SO_4$ were added (0.6 saturation) with slow stirring. The precipitate was sedimented by 15 min centrifugation at 8000 rev./min and stored. The supernatant (2020 ml) was saturated to 0.7 by addition of 139.4 g of solid $(NH_4)_2SO_4$ and after 30 min stirring the precipitate was sedimented by centrifugation. By this step most of the exonuclease activity is eliminated and is recovered in the 0.7 saturation precipitate of $(NH_4)_2SO_4$.

$(NH_4)_2SO_4$ precipitate between 0.5 and 0.6 saturation was dissolved in 20 ml of 0.01 M sodium phosphate buffer with 0.001 M 2-mercaptoethanol, pH 7.4 (Fraction III).

Enzyme solution (Fraction III) was loaded on a Sephadex G-25 (medium) column (1.6 × 50 cm) equilibrated with 0.01 M sodium phosphate buffer in the presence of 0.001 M 2-mercaptoethanol, pH 7.4. Fractions with enzyme activity eluted in one peak were pooled (35 ml) (Fraction IV) and loaded on the phosphocellulose column (1.2 × 18 cm, Whatman P11) equilibrated with 0.01 M sodium phosphate buffer/0.001 M 2-mercaptoethanol, pH 7.4. The column was washed with the same buffer and then a gradient of NaCl molarity (0—0.5 M) in the same buffer was applied. Restriction activity was eluted between 0.26 and 0.3 M NaCl. Fractions having activity higher than 1 unit/ml were pooled (Fraction V) and concentrated about three times with Lyphogel (Gelman, France) (Fraction VI). The purification is summarized in Table I. Enzyme was stored several months at 0°C without any loss of activity. Restriction activity so obtained is not contaminated by detectable exonuclease, as no acid-soluble products from ^3H- or ^{32}P-labelled SV40 or T7 DNA digests are

TABLE I

PURIFICATION OF *H. INFLUENZAE* RESTRICTION ENDONUCLEASE (Hin II + III)

The activity is estimated by viscosimetry.

Fraction		Activity		$A_{280\ nm}$	Specific activity (units/$A_{280\ nm}$)
		Total units	Units/ml		
I.	Crude enzyme extract	434 *	4.2	255	0.016
II.	Hydroxyapatite	—	—	—	—
III.	$(NH_4)_2SO_4$ precipitate	40 **	3.0	22.6	0.13
IV.	Sephadex G-25 filtration	84	2.4	7.0	0.34
V.	Phosphocellulose chromatography	36.0	1.44	0.09	16.6
VI.	Lyphogel concentration	36.0	3.75	—	—

* Units of activity are overestimated because crude extract contains exonucleases and probably also non-specific endonucleases.

** Activity is underestimated because it is inhibited by the presence of $(NH_4)_2SO_4$.

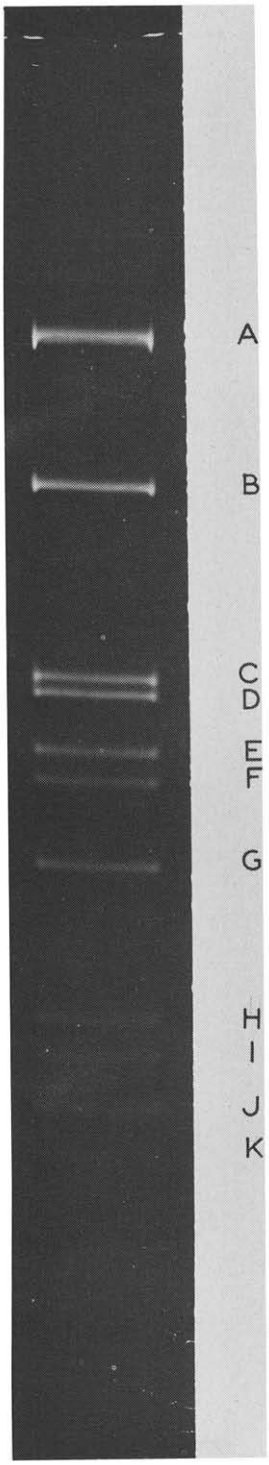


Fig. 1. Degradation of SV40 DNA by restriction endonuclease Hin II + III. 2.5 μg SV40 DNA from large plaque stock virus were digested with 5 μl of Lyphogel concentrated fraction of Hin II + III for 16 h at 37°C; made 1% in sodium dodecylsulfate, incubated for 30 min at 37°C, made 7.5% saccharose and 0.025% bromophenol blue. Migration was 16 h in 3% polyacrylamide/0.5% agarose gel at 4°C and 40 mA.

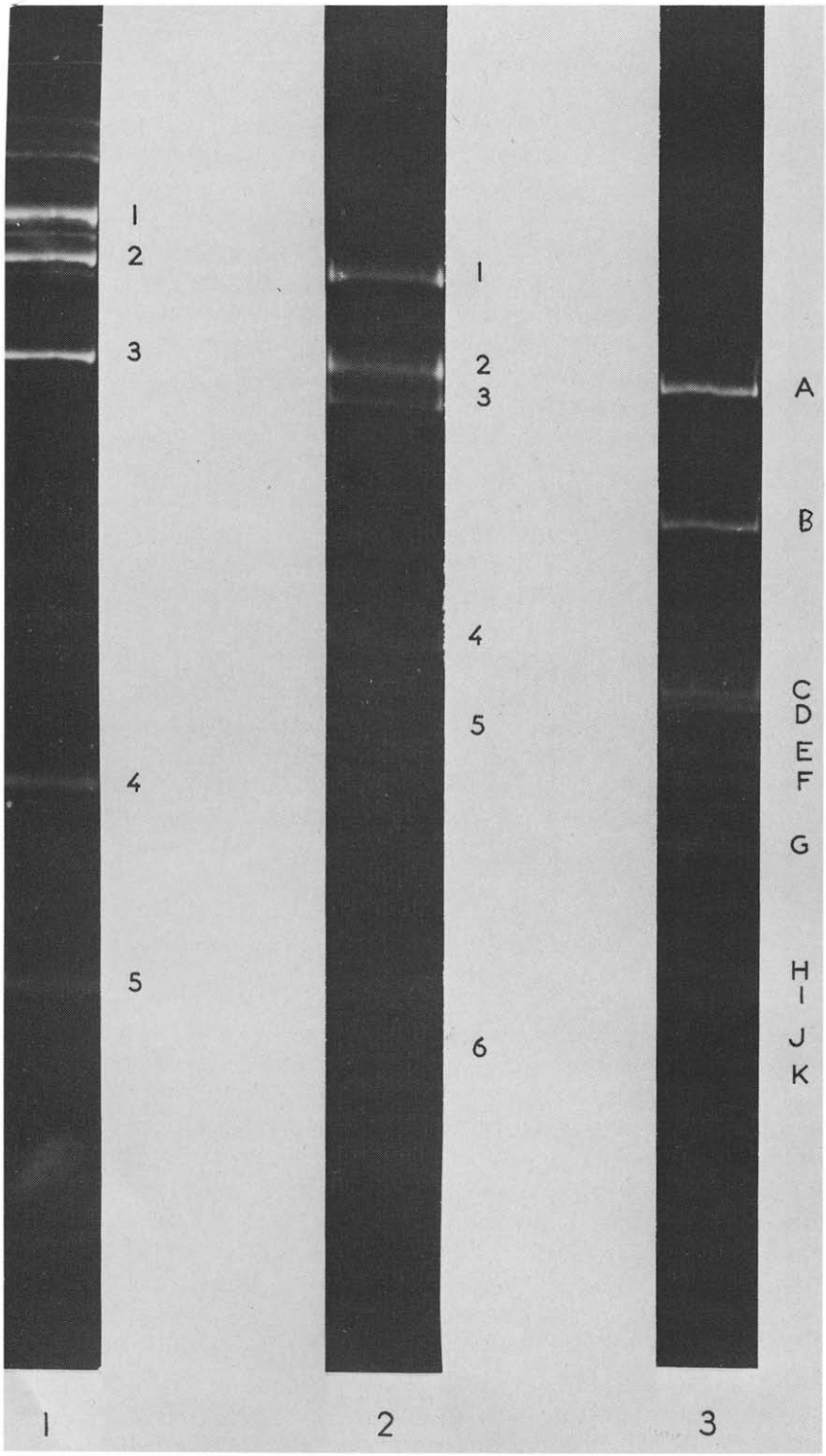


Fig. 2. Degradation of SV40 DNA by restriction endonuclease Hin II, by Hin III and by the mixture of both activities. 2.5 μg of SV40 DNA were digested with 20 μl of Hin II (lane 1), 2.5 μg of SV40 DNA digested with 40 μl of Hin III (lane 2) and 2.5 μg of SV40 DNA digested with Hin II + III (lane 3). Treatment and electrophoresis were the same as for Fig. 1.

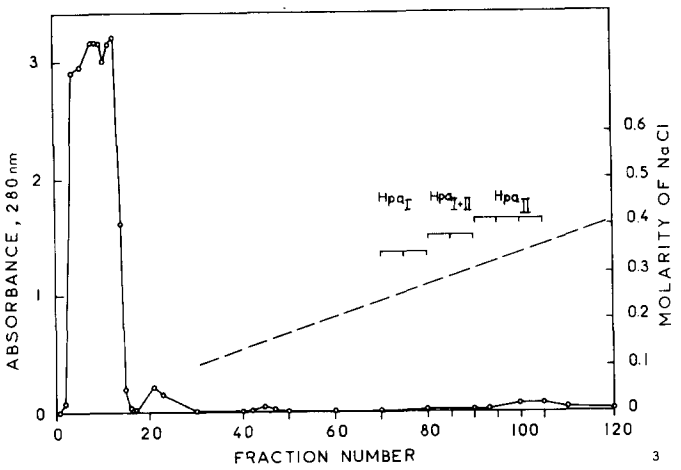


Fig. 3. Phosphocellulose chromatography of Hpa restriction endonuclease. 35 ml of Fraction IV was loaded on the phosphocellulose column (1.2 X 18 cm), washed with buffer (0.01 M sodium phosphate, 0.001 M 2-mercaptoethanol, pH 7.4) and a NaCl linear molarity gradient (0—0.5 M) in the same buffer was applied. Fractions of 2.5 ml were collected.

obtained, even after incubation for 24 h at 37°C. Non-specific endonuclease activity is not found either, as *H. influenzae* DNA is not at all degraded by the enzyme, while *H. parainfluenzae* DNA is degraded and reaches a plateau. (These degradations were followed by viscometry). The enzyme is strongly sensitive to low temperatures as its activity is lost rapidly at −20°C, even in the presence of 20—50% glycerol.

Restriction activities from *H. influenzae* Rd strain so far obtained represent mainly two of the known restriction Hin activities, Hin II and Hin III according to the nomenclature suggested by Smith and Nathans [9], since SV40 DNA (form I) is degraded by Hin II + III into 11 fragments separated by

TABLE II

PURIFICATION OF *H. PARAINFLUENZAE* RESTRICTION ENDONUCLEASE (Hpa I AND Hpa II)

The activity is estimated by viscosimetry.

Fractions		Activity		$A_{280\ nm}$	Specific activity (units/$A_{280\ nm}$)
		Total units	Units/ml		
I.	Crude enzyme extract	440 *	4.0	240	0.016
II.	Hydroxyapatite batch	532	0.7	2.84	0.25
III.	$(NH_4)_2SO_4$ precipitate	—	—	—	—
IV.	Sephadex G-25 filtration	23	0.64	3.4	0.19
V.	Phosphocellulose chromatography				
	Hpa I	10.4	0.8	0.02	40
	Hpa I + II	5.1	0.32	0.02	25
	Hpa II	7.0	1.6	0.02	80
VI.	Lyphogel fraction				
	Hpa I	5.6	0.8	—	—
	Hpa II (after dialysis)	2.95	0.64	—	—

* Units of activity are overestimated because crude enzyme extract contains exonucleases and probably also non-specific endonucleases.

electrophoresis in polyacrylamide gels, as was shown by Danna and Nathans [10] (Fig. 1). Hin II can be separated from Hin III by a gentle slope molarity gradient on the phosphocellulose column, and activities are eluted at 0.230—0.245 M NaCl for Hin II and 0.320—0.340 M NaCl for Hin III. The fragments of SV40 DNA obtained by digestion with these enzymes and separated by electrophoresis in a 3% polyacrylamide slab gel are shown at Fig. 2. Five main fragments are formed with Hin II (lane 1), six main fragments with Hin III (lane 2) and 11 fragments with the mixture of both activities (lane 3). Some extra bands (in lane 1), Hin II digest, are probably due to incomplete digestion or contamination by other restriction activity.

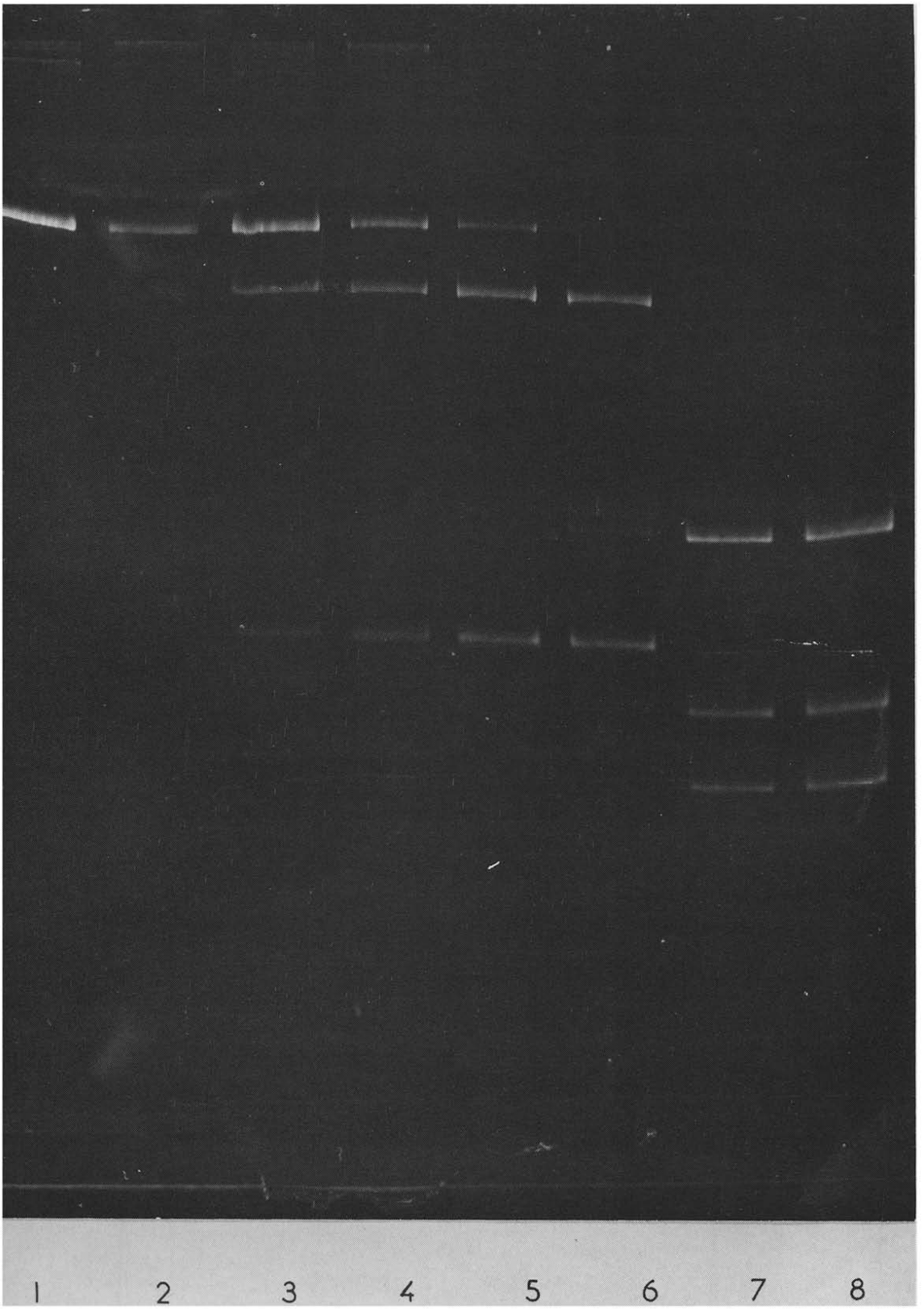
Purification of H. parainfluenzae restriction enzymes

The procedure just described for *H. influenzae* Rd enzymes was used to purify *H. parainfluenzae* restriction enzymes. We were able to separate the two restriction activities by chromatography on phosphocellulose. Hpa I and Hpa II (nomenclature of Smith and Nathans [9], are eluted between 0.25 and 0.27 M NaCl and between 0.31 and 0.35 M NaCl, respectively (Fig. 3); intermediate fractions correspond to the mixture of both activities. SV40 DNA, (in a linear form) degraded by different fractions from phosphocellulose chromatography, and separation of fragments by electrophoresis in polyacrylamide gel, is shown in Fig. 4. Both activities (Hpa I and Hpa II) were pooled separately, concentrated with Lyphogel and Hpa II was dialyzed against 0.01 M Tris, 0.001 M dithiothreitol and 3% glycerol. No exonuclease activity was detected in this enzyme preparation. Endo Hpa activities are stable at 0°C for many months. Purification of these enzymes is summarized in Table II.

The method described for *H. influenzae* restriction enzyme purification was also successfully applied for *H. aegyptius* restriction enzymes and the pattern of ten main fragments obtained from a SV40 DNA digest is shown in Fig. 5 [11].

The purity and molecular weight of purified enzymes were determined by electrophoresis in 7.5% acrylamide slab gels [8]. Molecular weights were estimated by plotting the log of molecular weight against the mobility. As shown in Fig. 6 Hpa I + II shows only one band, corresponding to a molecular weight of 75 000 (lane 1); Hpa I forms one band which corresponds to a very close molecular weight (lane 2); Hpa II also forms a similar band, corresponding to a

Fig. 4. Electrophoresis of SV40 DNA in composed 2.2% polyacrylamide/0.5% agarose gel. 18 μg of super-coiled SV40-DNA (form I) was converted into the linear form (form III) by 20 μl of Eco RI by digestion in 0.09 M Tris/HCl, 0.01 M $MgSO_4$, pH 7.4, for 3 h at 37°C. The digest was then diluted five times with 0.01 M Tris/HCl, 0.007 M $MgCl_2$, 0.01 M NaCl and 0.001 M dithiothreithol, pH 7.4; to 30 μl of this digest (2.2 μg DNA), were added *H. parainfluenzae* enzymes, purified on phosphocellulose and this was left to incubate 16 h at 37°C. All samples were made 1% in sodium dodecylsulfate and treated as described in Materials and Methods at 37°C, and run in a 2.2% polyacrylamide/0.5% agarose slab gel. Fragments were visualized as described in Materials and Methods. Lanes 1 and 2 show SV40 DNA treated by Eco RI (form III). Lanes 3 and 4, show SV40 DNA (form III) treated by pooled fractions Hpa II (90—105) obtained after phosphocellulose chromatography; in lane 3, 20 μl of this pool were added and in lane 4 μl of the same pool were added. Lane 5 shows SV40 DNA (form III) treated by 15 μl of pooled fractions (86—90). The sixth lane shows the same DNA digested by 15 μl of pooled fractions (81—85); lanes 5 and 6 represent the activity of Hpa I + II. Lanes 7 and 8 show the fragments obtained by digestion with 15 μl of pooled fractions (76—80 and 71—75, respectively) and represent the activity of Hpa I.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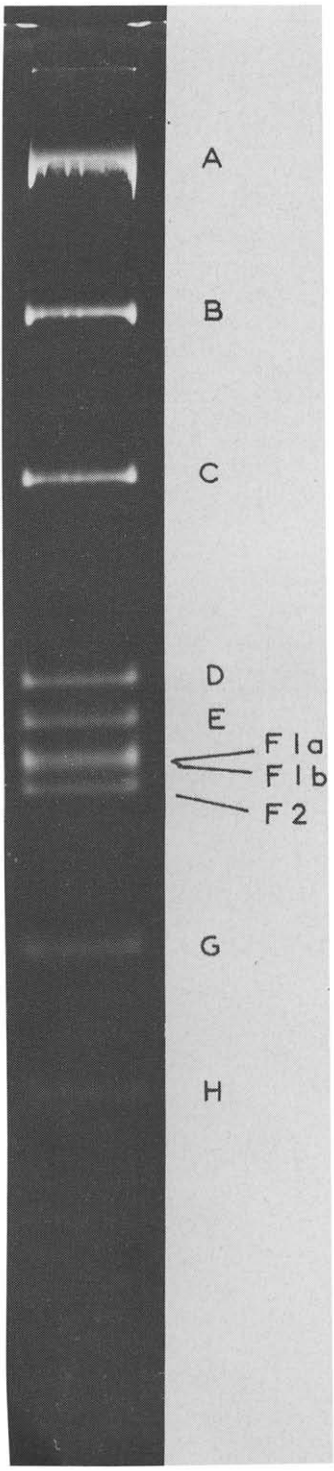

Fig. 5. SV40 DNA digestion pattern obtained by Hae restriction endonuclease. 2.5 μg of SV40 DNA were digested with 5 μl of Hae in conditions described for Hin restriction endonuclease. Electrophoresis was done in 4% polyacrylamide gel slab for 16 h at 4°C, 50 mA.

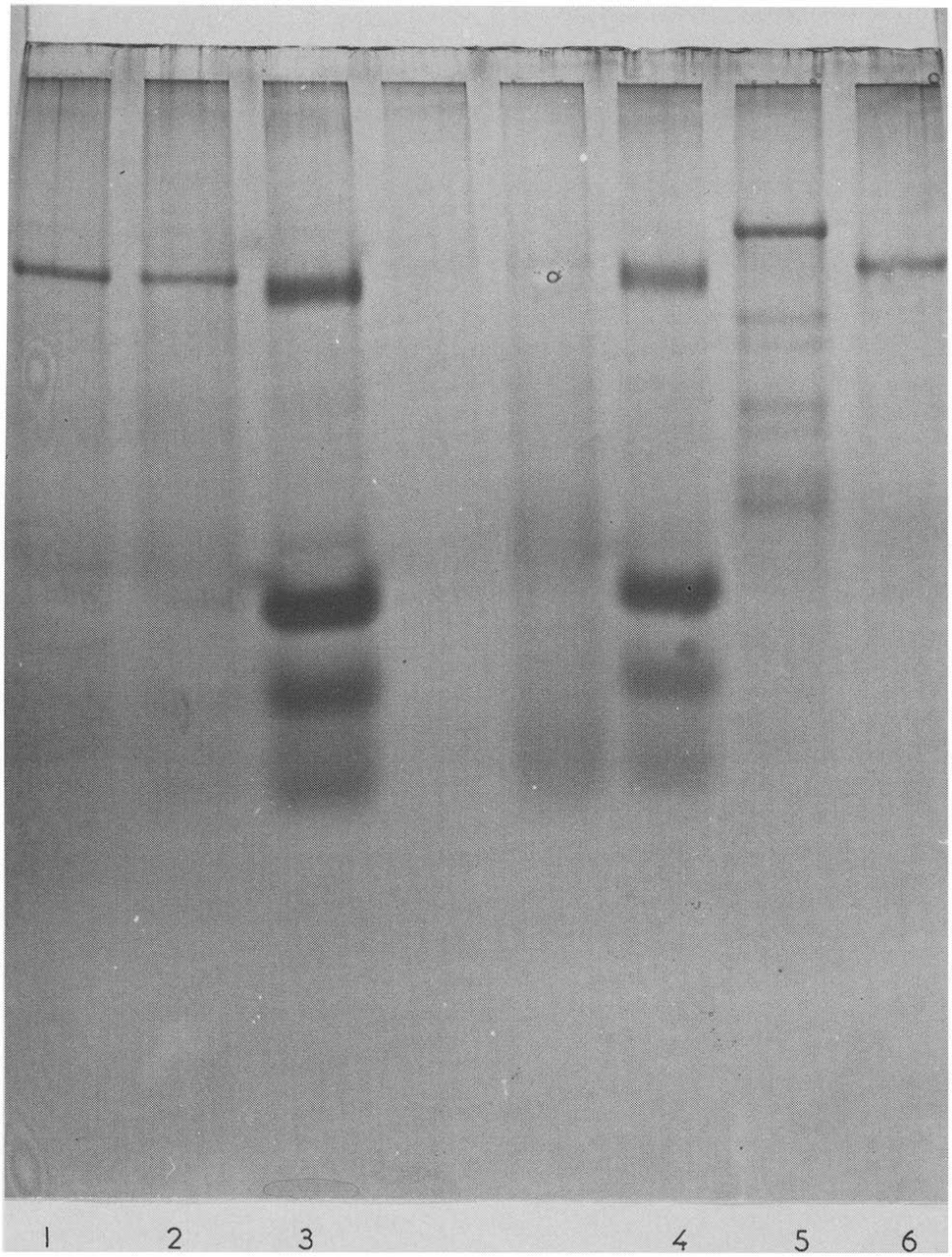

Fig. 6. Electrophoresis of proteins in 7.5% polyacrylamide gel. 200—300 µl of each purified enzyme were dialyzed, concentrated to 20 µl, made 1% in sodium dodecylsulfate, 0.01 M sodium phosphate, pH 7.1, 0.1% in 2-mercaptoethanol, stored overnight at 20°C, heated 2 min at 100°C; 5 µl of 60% solution of sucrose and 0.2% bromophenol blue were added and loaded on a 7.5% polyacrylamide slab gel, and run for 24 h at 40 mA at 20°C with a 0.1 M sodium phosphate buffer, pH 7.1, 0.1% sodium dodecylsulfate. The gel was then fixed in 10% trichloroacetic acid and stained for 3 days in a solution of 0.125 g of Coomassie blue in 40% methanol, 5% acetic acid, 2.5% glycerin. Destaining was done in solution of 20% methanol, 5% acetic acid and 2.5% of glycerin. Bovine serum albumin, lysozyme, and L5 (ribosomal protein) were used as molecular weights markers, corresponding to 68 000, 14 400 and 21 400, respectively. Lane 1, Hpa I + II; Lane 2, Hpa I; Lanes 3 and 4, markers (bovine serum albumin, lysozyme, L5); Lane 5, Hin II + III; Lane 6, Hpa II.

molecular weight of 70 000—75 000 (lane 6); Hin II + III shows one main band, estimated at 80 000 daltons and six small contaminating bands (lane 5). Our estimation of molecular weight of Hin is in good agreement with data obtained by Smith and Wilcox [1]. The values reported by Sharp et al. [3], by using chromatography on Sephadex G-100 and agarose A 0.5 M are slightly lower for Hpa I (mol. wt 68 000), and very different for Hpa II (mol. wt 13 370).

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