GEL CHROMATOGRAPHY AS A MEANS FOR DIFFERENTIATION BETWEEN DEHYDROGENASES

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1. Introduction

In a recent paper Jaenicke et al. [1] demonstrated the identity of molecular weights of LDH-H (pig) and LDH-M (rabbit) in various types of experiments. They came to a value of 142 000 ± 3600 which is in line with most of the earlier findings (for references cf. ref. [1]). There was no significant difference between LDH-H and LDH-M with such methods as sedimentation velocity, light scattering, and osmotic pressure. In the present investigation we intend to reconfirm our preliminary results [2,3] of differences in the behaviour of various LDH's in gel chromatography.

2. Material and methods

Cytochrome c, α-chymotrypsinogen, GAPDH, aldolase, catalase and LDH isoenzymes were obtained from Boehringer Mannheim GmbH, and, in one case, (LDH-M, pig) from Dr. Holbrook of this University. Ovalbumin (Serva Entwicklungslabor, Heidelberg) and bovine serum albumin (Behringwerke AG, Marburg) served as further molecular weight standards. Sephadex G-200, superfine, for thin-layer experiments as well as G-150 and G-200 for column chromatography were purchased from Deutsche Pharmacia GmbH, Frankfurt (Main).

Thin-layer gel chromatography was performed in the sandwich technique as described earlier [12]. Sephadex G-200, superfine, was allowed to swell in the appropriate buffer for one week. The glass plates were stored after spreading with a 0.5 mm gel layer ready for use for 12 hr (minimum) and 8 days (maximum) in a dessiccator whose inside was covered with wet filter paper. 1–5 μl each of the samples was applied with a capillary as spots of 1–3 mm diameter. The sample concentration was 10 mg/ml if not indicated otherwise; 10 mg/ml cytochrome *c* was added to most of the samples as internal standard. The thin-layer plate was covered at little distance with a glass plate (sandwich) and fixed under an inclination angle of 15–30° to the horizontal line. A wick of filter paper (Schleicher & Schüll 2316) connected the upper end with a buffer reservoir. A dry paper was mounted at the lower end to take up the eluant. After 5–8 hr (depending on the inclination and the age of the layer) a piece of dry filter paper (Macherey & Nagel 212) was pressed tightly onto the wet gel layer for several minutes, and for lactic dehydrogenases the moist paper was sprayed with a solution of sodium pyruvate (25 mg) and NADH (3 mg) in water (10 ml). The spots of LDH-activity could be observed under the UV-lamp (350 mμ) where the bright fluorescence of NADH disappeared. Higher concentrations of LDH and reference proteins were visualized by spraying with Pauly's reagent (0.01% diazotized sulfanilic acid in 10% sodium carbonate). The proteins appeared as orange spots on yellow background.

Column gel chromatography was performed with Pharmacia Laboratory Columns K 25/100 equipped with an upward flow adapter. The appropriate amount of gel (G-150 and G-200) was allowed to swell in the eluting buffer for at least 3 days at room temperature and carefully packed [12] to obtain a gel bed of 440–460 ml. Only 20–30 cm of hydrostatic pressure was applied during the entire operation to avoid clogging of the column. Under these conditions the upward

flow rate was kept at 30–35 ml/hr during several days. The outlet of the column was connected with a LKB-Uvicord 4701 A recording UV-absorbance (254 mμ). Sample solutions with concentrations down to 1 mg protein per ml could be detected. LDH of lower concentration was located by its enzymatic activity in the collected fractions (LKB Radirac).

3. Results and discussion

Fig. 1 represents one of hundreds of experiments, illustrating the faster migration of LDH-H as compared with LDH-M on a Sephadex tl-plate. It is of great interest that the hybrid enzyme H_2M_2 lies between the two isoenzymes H_4 and M_4. Fig. 2 compiles the elution diagrams of 3 runs of H_4-, M_4- and H_2M_2 LDH-isoenzymes on the same column of Sephadex G-200. The significant difference of 20 ml in elution volume between the two maximum activities of LDH isoenzymes is in good agreement with the thin-layer experiments.

In fig. 3 the average R_{cyt}-values of 62 runs of several reference proteins were plotted against the logarithm of molecular weights giving a perfectly straight calibration line. Cytochrome *c* was admixed also to both lactic dehydrogenases as well as to GAPDH, and more than 60 runs resulted in the average R_{cyt}-values

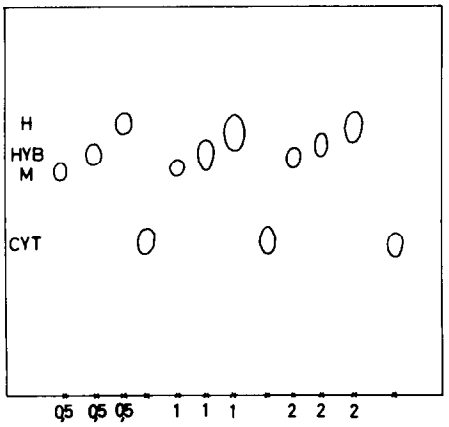

Fig. 1. Thin-layer gel chromatography of LDH-H, pig (= H); LDH-M, rabbit (= M); and LDH-H_2M_2, pig (= HYB) in 0.2 M phosphate buffer (pH 6.0) containing 0.8 M NaCl, inclination 20°, 5 hr. The protein concentration (mg/ml) is given under the starting point.

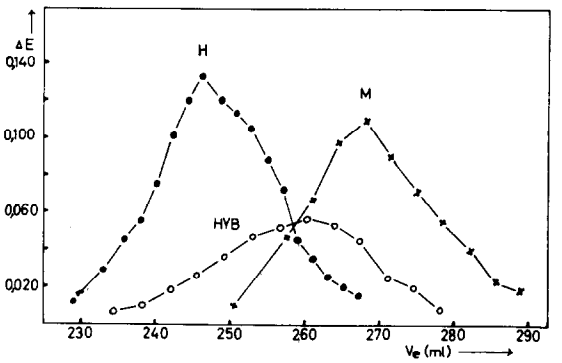

Fig. 2. Column gel chromatography of the samples of fig. 1 (1 mg/ml) on a gel bed (2.5 × 90 cm = 440 ml) of Sephadex G-200 in phosphate buffer (pH 6.65, I = 0.2). Sample volume 2 ml, flow rate 20 ml/hr. Ordinate: enzymatic activity, abscissa: elution volume.

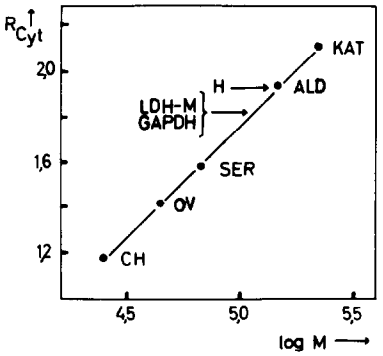

Fig. 3. Calibration line from thin layer experiments. The average migrations of the reference proteins (in proportion to cytochrome *c*) are plotted on the ordinate against the logarithm of their molecular weight on the abscissa. CH = α-chymotrypsinogen; OV = ovalbumin; SER = serum albumin (bovine); ALD = aldolase; KAT = catalase.

of table 2, from which molecular weight values could be deduced.

Furthermore, thin-layer experiments were performed in the range of 0.5 to 10 mg enzyme per ml in order to check earlier reports of a dissociation depending on concentration [4,5] of the protein. Fig. 4 shows that there is no significant difference whether the enzyme migrates in low or high concentration.

The behaviour of LDH-H on Sephadex G-200 is in full agreement with the calibration work of other

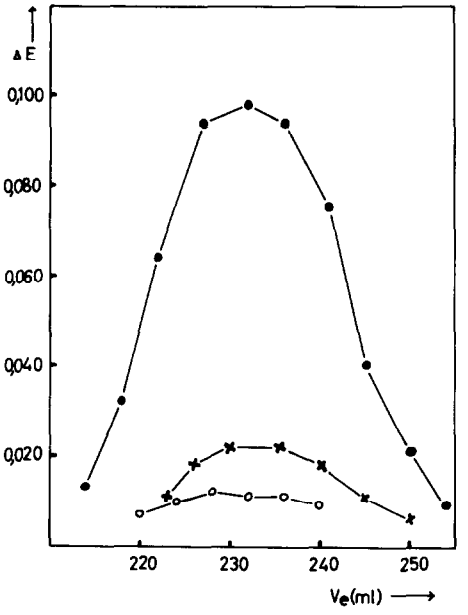

Fig. 4. Elution diagrams of LDH-H (pig) of various concentrations (● 0.5, x 0.1, ○ 0.05 mg/ml) from Sephadex G-150 (2.5 X 92 cm = 450 ml) in 0.2 M phosphate buffer (pH 6.0) plus 0.8 M NaCl.

Table 1
Relative migration rates of reference proteins in thin-layer gel chromatography (averages of 62 determinations).

Protein	Abbreviation	Mol. wt.	R_{cyt}
α-chymotrypsinogen	CH	25 000	1.17 ± 0.03
Ovalbumin	OV	45 000	1.41 ± 0.04
Bovine serum albumin (monomer)	SER	67 000	1.58 ± 0.03
Aldolase	ALD	147 000	1.93 ± 0.06
Catalase	KAT	225 000	2.11 ± 0.10

Table 2
Apparent molecular weights of dehydrogenases from migration rates relative to cytochrome c.

Enzyme	R_{cyt}	Mol. wt.
LDH-H, pig	1.93 ± 0.06	147 000
LDH-M, rabbit	1.83 ± 0.06	118 000
GAPDH	1.84 ± 0.06	120 000

laboratories [6]. The values for LDH-M and GAPDH, however, are differing from those obtained by other methods. The work of Jaenicke and Knof [1] clearly demonstrates the very similar physical behaviour of both the dehydrogenases. In gel chromatography, however, they show big differences, GAPDH and LDH-M being retarded like proteins of molecular weight of 120 000 and 118 000, respectively. A similar molecular weight (117 000) was attributed to GAPDH formerly by Elias and coworkers [7] but has been corrected later to 145 000 ± 6000 [8]. In the meantime the latter value has been confirmed by sequence analysis [9]. A much lower value (120 000) results not only from our but also from former chromatographic investigations [6,10,11]. Two reasons for an anomalous behaviour of proteins on Sephadex gels are known till now [12]: the specific retardation of carbohydrate splitting enzymes and the enhancement due to a high carbohydrate content. Additionally a retardation may be due to a reversible dissociation of protein subunits. Considering the independence of elution rate from protein concentration it is unlikely that dissociation is the reason for the retardation of GAPDH and LDH-M in chromatographic experiments.

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