ADDITIONAL ELECTROPHORETIC DIFFERENTIATION OF LDH_5 AND MITOCHONDRIAL GOT IN IONIC STRENGTH AND pH GRADIENTS *

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1. Introduction

Many electrophoretic and ion-exchange chromatography procedures are available for the separation of isoenzymes. They depend primarily upon the nature and resultant charge on the protein fraction in the buffer solution used. The pH and ionic strength have a profound effect on the rate of migration [2,3]. Ressler et al. [4] have shown that, with buffers of low ionic strength, LDH_5 isoenzyme tends to migrate towards the anode, whereas with buffers of higher ionic strength it migrates towards the cathode. Secondly, the pH and ionic strength are of prime importance in examining various factors which influence the solubility of proteins, e.g. factors of the isolating medium involved in determining the amount of enzyme activity associated with subcellular fractions [5,6].

The present communication is connected with both points mentioned. It reports the importance of the pH and ionic strength for the electrophoretic mobilities of LDH and GOT isoenzymes isolated from the soluble fraction of rat tissues. Setting out from low ionic strength values, the electrophoretic mobility of the enzymes increases with the increasing amount of salts in the electrophoretic medium. In this process, several subfractions of individual isoenzymes are formed which, in a certain point ("turning-point"), again form a single zone which migrates regularly. The "turning-point" is determined by the ionic strength and pH value.

* See ref. [1].

Abbreviations used: LDH, L-lactate:NAD oxidoreductase (EC 1.1.1.27); GOT, L-aspartate:2-oxo-glurarate aminotransferase (EC 2.6.1.1); $NADH_2$, nicotinamide adenine dinucleotide reduced; NAD, nicotinamide adenine dinucleotide; NBMT, 2-(*p*-nitrophenyl)-5-phenyl-3-(3,3-dimethoxy-4-diphenylyl) tetrazolium chloride.

2. Methods and materials

2.1. *Enzyme preparation*

Liver and brain were obtained from adult male Wistar rats immediately after death, cut into pieces, rinsed with 0.15 M sodium chloride and added to 5×10^{-2} M barbital buffer at pH 8.3, containing 10^{-4} M NADH (Boehringer) [7]. The sample was homogenized at 4°C at 600 rpm for 1 min in a Potter-Elvehjem homogenizer with a glass piston and tube and centrifuged for 60 min at 40,000 rpm in the No. 50 rotor of a Spinco Model L ultracentrifuge. A 20% (w/v) homogenate was prepared. LDH_5 isoenzyme was separated after the electrophoresis (at pH 8.3 and $\mu = 3 \times 10^{-2}$) of the supernatant fraction of liver by cutting the active zone out of the agar plate and centrifuging it for 30 min at 20,000 rpm. The isoenzymes LDH_1–LDH_4 from brain were separated by the same way. The subfractions of LDH_5 were also separated by cutting out and centrifuging of the active electrophoretic zone. The GOT isoenzymes were examined in the whole liver homogenate.

2.2. *Electrophoresis*

The author's modification of quantitative microelectrophoresis in agar gel [8] was employed. Purified Difco Bacto agar was used for the separation. The separation was done for 60 min at 30 V/cm and 4 mA/cm, the temperature of the agar layer during the separation was + 3°C. Barbital buffer at pH 7.6,

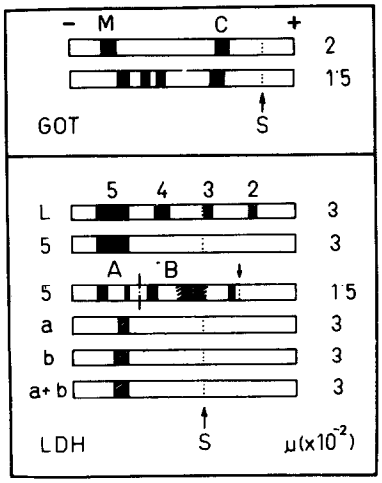

Fig. 1. Influence of ionic strength variations on electrophoretic separation of GOT and LDH. Separations were done at pH 8.3. →S, start; M, mitochondrial GOT; C, cytoplasmic GOT, both from rat liver homogenate; L, LDH isoenzymes of rat liver homogenate; 5, LDH_5 eluate at an ionic strength of 3×10^{-2} and 1.5×10^{-2}; a, eluate of the cathodic part A of the preceding electrophoreogram; b, eluate of its anodic part B; a+b, their mixture.

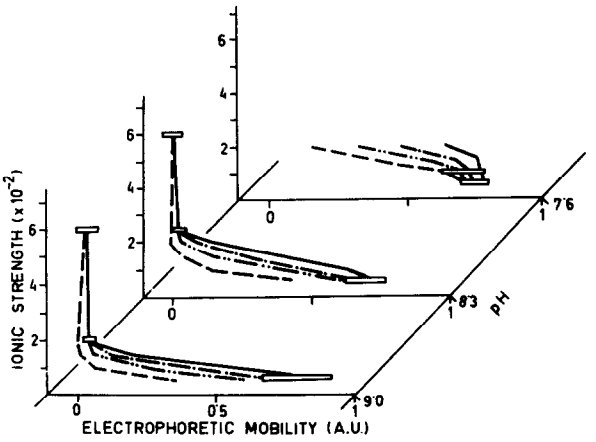

Fig. 2. Dependence of variations in the electrophoretic mobility of LDH_5 isoenzyme isolated from rat liver homogenate on the ionic strength and pH variations. The ionic strength values used: 6×10^{-2}, 2×10^{-2}, 1.5×10^{-2}, 10^{-2}, 6×10^{-3}. 0, electrophoretic mobility of starch; 1, mobility of human albumin at an ionic strength of 6×10^{-2}.

8.3 and 9.0 was used with ionic strength decreasing from 6×10^{-2} to 6×10^{-3}. The relative electrophoretic mobility was calculated from the distance of the fraction to starch. The electrophoretic mobility of starch was considered to be zero, the mobility of human albumin at an ionic strength 6×10^{-2} was taken as unity. In the electrophoreograms the LDH isoenzymes were detected by 20 min incubation in the following mixture: 10^{-2} M sodium L-lactate (Lachema), 10^{-3} M NAD (Boehringer), 10^{-4} M phenazine methosulfate (Calbiochem) and 10^{-3} M NBMT [9] in 5×10^{-2} M barbitone buffer at pH 8.3. GOT isoenzymes were detected by incubation of electrophoreograms at 37°C for 60 min in the mixture reported by Babson et al. [10], using Echtblausalz BB (Hoechst) as the diazonium salt. After incubation, the electrophoreograms were washed for 60 min in 3% acetic acid and dried.

3. Results

The starting point of this work was the finding demonstrated in fig. 1. The upper part of the figure shows the influence of ionic strength variation from 2×10^{-2} to 1.5×10^{-2} on the mitochondrial isoenzyme GOT [11]. This slight change of ionic strength is the reason, for which three activity bands are found instead of the original single fraction of the mitochondrial isoenzyme GOT. Similarly in the second part of fig. 1, LDH_5 isolated from the liver homogenate is interesting in being, at an ionic strength of 3×10^{-2}, the only fraction which forms at least five to six activity regions at half the ionic strength. The cathodic and anodic fraction groups were isolated. Eluates from them were again separated by agar gel electrophoresis, this time at the double ionic strength. Under those conditions LDH_5 is again a single fraction having the same electrophoretic mobility as the two eluates and their mixture.

A three-dimensional graph in fig. 2 expresses the dependence of variations in the electrophoretic mobility of the LDH_5 isoenzyme on the ionic strength and pH variations. A decrease of ionic strength causes disintegration of the originally unified isoenzyme into several subfractions. Four subfractions, which are present always, are illustrated in the graph. A further decrease of ionic strength first leads to a rise in electrophoretic mobility of each of the subfractions.

When the ionic strength is decreased even more, the activities unite in a single band in the start region, the electrophoretic mobility of which decreases sharply. Like with disintegration, joining of the subfractions takes place at a higher ionic strength already with lower pH values.

The analogous study of the dependence of variations in the electrophoretic mobility of LDH_1-LDH_4 isoenzymes on ionic strength and pH variations has shown that in the pH and ionic strength range studied, the LDH_4 isoenzyme disintegrates into three subfractions, while the other isoenzymes do not. Similar changes take place in the mitochondrial isoenzyme GOT. On decrease of the ionic strength, the originally unified isoenzyme forms three subfractions at first, which on further decrease of ionic strength form again a single fraction in the start region. This phenomenon again depends on pH. Under the conditions employed, the cytoplasmic isoenzyme GOT does not change.

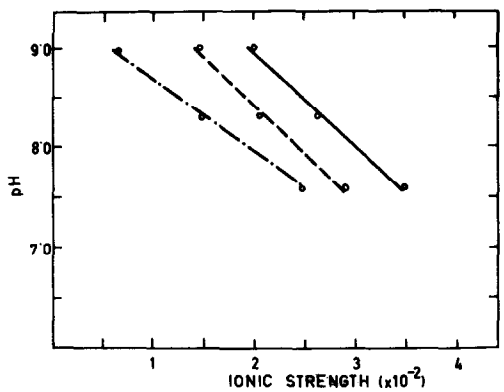

Fig. 3. The pH and ionic strength values for the "turning-point" where a decrease of one of them leads to disintegration of the isoenzyme. —, LDH_5; –.–, LDH_4; - - -, mitochondrial isoenzyme GOT.

4. Discussion

It was stated, that several subfractions of isoenzymes unite to form a single activity zone at very low ionic strength values in the case of LDH_5 as well as mitochondrial GOT. The same effect is to be expected in the case of LDH_4 at lower ionic strength (or pH) values. This zone is always located in the start region and electrophoretic mobility does not rise any more when the ionic strength is decreased even more. A salting-in effect is probably involved. The reason is that, in the inverse view (i.e. setting out from extremely low ionic strength values), ionic strength increases with the increasing amount of salts in the electrophoretic medium and therefore the protein solubility increases. In their study of the distribution of LDH between the particulate and soluble fractions of homogenized muscle, Hultin and Westort [6] found, that the amount of LDH released into the soluble fraction increased with increasing pH and ionic strength. Their results show that solubilization of the enzyme as a function of pH begins at pH 6.8 (using 1.5×10^{-4} M imidazole buffers) and at an ionic strength of 5×10^{-3} (using 5×10^{-4} M imidazole buffer at pH 7.0). These data are in good agreement with our findings. We showed that in the process of increasing of enzyme solubility, several subfractions of the isoenzyme are formed which, in a certain point, form a single zone which migrates regularly. This point, which we may call the "turning-point" is quite unambiguously determined by the ionic strength and pH values. An analysis of the results obtained up to now showed the relationship of these values, which is illustrated in fig. 3. In the pH and ionic strength range studied, the values for the "turning-point" may be read. The data for LDH_4, LDH_5 and mitochondrial GOT isoenzymes are given. The pH and ionic strength range which has been used in our experiments is a boundary range of the barbital buffer system. The "turning-points" at various pH values were partly determined by extrapolation of the curves obtained for LDH_5 (see fig. 2), LDH_4 and mitochondrial isoenzyme GOT. On account of this the "turning-point" values are rather imprecise and will be studied further.

It would seem that the disintegration of isoenzymes into subfractions after the ionic strength or pH decrease under the "turning-point" cannot be explained by means of the Markert subunit theory [12], since subunits LDH_5 are identical according to this theory. The explanation seems more probable, that the subfractions differ in their tertiary structure. It is also possible to attribute the effect described to association of the enzyme with anionic substances present in the electrophoretic medium [4]. The phenomena described may readily be used to explain some dis-

crepant findings of larger numbers of isoenzymes. There have been several reports that a greater number than five LDH isoenzymes [13–15] and also GOT [16] may be detected.

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