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# Clustering of Sequential Enzymes in the Glycolytic Pathway and the Citric Acid Cycle

**Sonia Beeckmans, Edilbert Van Driessche, and Louis Kanarek**

*Laboratorium voor Chemie der Proteïnen, Vrije Universiteit Brussel, B-1640 Sint-Genesius-Rode, Belgium*

In recent years, evidence has been accumulating that metabolic pathways are organized in vivo as multienzyme clusters. Affinity electrophoresis proves to be an attractive in vitro method to further evidence specific associations between purified consecutive enzymes from the glycolytic pathway on the one hand, and from the citric acid cycle on the other hand. Our results support the hypothesis of cluster formation between the glycolytic enzymes aldolase, glyceraldehydephosphate dehydrogenase, and triosephosphate isomerase, and between the cycle enzymes fumarase, malate dehydrogenase, and citrate synthase. A model is presented to explain the possibility of regulation of the citric acid cycle by varying enzyme-enzyme associations between the latter three enzymes, in response to changing local intramitochondrial ATP/ADP ratios.

**Key words: multienzyme cluster, complex, affinity electrophoresis, metabolic regulation, channeling**

Accumulating evidence tends to indicate that metabolic pathways are organized in vivo as multienzyme clusters: Consecutive enzymes are supposed to be physically in contact with each other, so that metabolites can directly be transferred among enzymes without first equilibrating with the bulk phase. Such a strict organization of metabolic processes, both in space and in time, allows a cell to interrupt, slow down, or speed up very accurately a biochemical pathway according to its needs at any time (see [1] and references mentioned therein [2–7]).

Two principally different approaches have been used to study enzyme-enzyme interactions in vitro. A) One option consists of breaking up cells or cell-organelles in a very gentle way, thereby avoiding as much as possible disruption of the original structures and associations. B) Alternatively, enzymes and cell constituents are fully purified and cluster formation is assessed by studying the behaviour of mixtures of these components by using some physico-chemical technique. It has been shown on several occasions that it is often essential to modify the physical properties of water around the enzymes, in order to mimic the real intracellular conditions which are characterized by huge protein concentrations, large excluded volumes, and low content of water which,

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consequently, appears to be organized in layers, at least to a certain extent [8]. One way to bring an enzyme in an environment where diffusion is limited consists in immobilizing it onto a solid support, e.g., Sepharose [9].

Such immobilized enzyme preparations can be embedded in agarose gels and used in affinity electrophoresis. This technique was recently described by us to visualize specific interactions between fumarase and mitochondrial malate dehydrogenase [1]. In this paper we report associations between the citric acid cycle enzymes citrate synthase, malate dehydrogenase, and fumarase on the one hand, and between the glycolytic enzymes aldolase, triosephosphate isomerase, and glyceraldehydephosphate dehydrogenase on the other hand. These results have been presented at the UCLA Symposium "Structural and Organizational Aspects of Metabolic Regulation" [10].

## MATERIALS AND METHODS

Pig heart fumarase (EC 4.2.1.2) was purified according to Beeckmans and Kanarek [11]. Pig heart citrate synthase (EC 4.1.3.7) and malate dehydrogenase (both the mitochondrial and the cytoplasmic isoenzymes) (EC 1.1.1.37) as well as rabbit muscle aldolase (EC 4.1.2.13), triosephosphate isomerase (EC 5.3.1.1), and glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) were obtained from Sigma Chemical Co. The purity of all these enzymes was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [12] and by isoelectric focusing. Bovine serum albumin was obtained from Sigma Chemical Co., whereas bovine gamma globulins were from Miles Laboratories Inc.

Enzyme activities of fumarase, citrate synthase, and malate dehydrogenase were assayed as described previously [13], while aldolase and glyceraldehydephosphate dehydrogenase activities were determined according to Bergmeyer [14]. The enzymes fumarase, citrate synthase, aldolase, and glyceraldehydephosphate dehydrogenase were immobilized onto Sepharose-4B as described previously [1]. During coupling, which was allowed to proceed for 18 h at 4°C, the enzymes were protected by their substrates, i.e., respectively 20 mM L-malate (fumarase), 20 mM citrate (citrate synthase), 2 mM D-fructose-1,6-bisphosphate (aldolase), and a mixture of 2 mM glyceraldehydephosphate + 120 mM $P_i$ + 2 mM dihydroxyacetone phosphate + 170 mM D-fructose-1,6-bis-phosphate (glyceraldehydephosphate dehydrogenase). The latter was prepared from D-fructose-1,6-bis-phosphate by the action of aldolase ($K_{eq} = 6.7 \times 10^{-5}$ M) as follows. To 8 ml of 0.2 M sodium phosphate (pH 8.0) were added 560 mg ($Na_3$)-fructose-1,6-bis-phosphate and 1 g Sepharose containing 100 units of covalently attached aldolase. This suspension was incubated during 2 h at 37°C with shaking. The Sepharose beads were then removed and 7.5 ml of the supernatant fraction was added to 5 ml of glyceraldehydephosphate dehydrogenase solution (which was dialyzed against 0.1 M $NaHCO_3$ buffer pH 8.2) to obtain a final enzyme concentration of 2 mg/ml. The amount of enzyme immobilized was estimated from the activity recovered in the washings. It was determined that respectively 9.5 mg fumarase, 5.6 to 6.2 mg of citrate synthase, 6.0 to 7.8 mg of aldolase, and 6.0 mg of glyceraldehydephosphate dehydrogenase were bound per gram (wet weight) of Sepharose.

Affinity electrophoresis was performed as described previously [1]. The immobilized enzyme strips contained respectively 390 $\mu g/cm^2$ fumarase, 240–260 $\mu g/cm^2$ citrate synthase, 250–320 $\mu g/cm^2$ aldolase, 250 $\mu g/cm^2$ glyceraldehydephosphate dehy-

drogenase, 210 $\mu g/cm^2$ bovine serum albumin, and 390 $\mu g/cm^2$ gamma globulins. Before electrophoresis free enzymes were dialyzed twice during 1.5 h against electrophoresis buffer at 4°C. In each of the lanes (1–10) 5 $\mu$l of free enzyme, at a concentration of 2 mg/ml, was applied.

For citric acid cycle enzymes 92 mM Veronal (pH 8.6) was used both as electrophoresis and as gel buffer (as described in [1]). Electrophoresis was performed overnight at 80 volts and 22°C. Since the pI of mitochondrial malate dehydrogenase is rather high, electrophoresis with this dehydrogenase as the free enzyme was allowed to proceed for another 24 h at 120 volts.

For glycolytic enzymes a 100 mM Tris + 4.5 mM EDTA buffer (pH 9.0–9.3) was used as electrophoresis buffer. A $^1/_{10}$ dilution was used as gel buffer. Electrophoresis was performed overnight at 50 volts and 22°C.

After electrophoresis the gels were histochemically stained for either fumarase or malate dehydrogenase according to Brewer and Singh [15], or for glyceraldehydephosphate dehydrogenase, triosephosphate isomerase, or aldolase according to Shaw and Prasad [16]. Eventually the gels were post-stained for total protein with Coomassie brilliant blue R-250 as in [1].

## RESULTS AND DISCUSSION

### Affinity Electrophoresis Involving Citric Acid Cycle Enzymes

In Figure 1, the electrophoretic migration of either free mitochondrial malate dehydrogenase (1A) or the cytoplasmic isoenzyme (1B) through an intermediate gel containing fumarase immobilized onto Sepharose-4B (lanes 6–10) is compared with the migration of these enzymes through underivatized Sepharose (lanes 1–5). It can be concluded that part of the mitochondrial isoenzyme applied in lanes 6–10 firmly sticks to the immobilized fumarase. On the other hand, the cytoplasmic isoenzyme, which is known not to be involved in the citric acid cycle, shows no affinity for fumarase. These

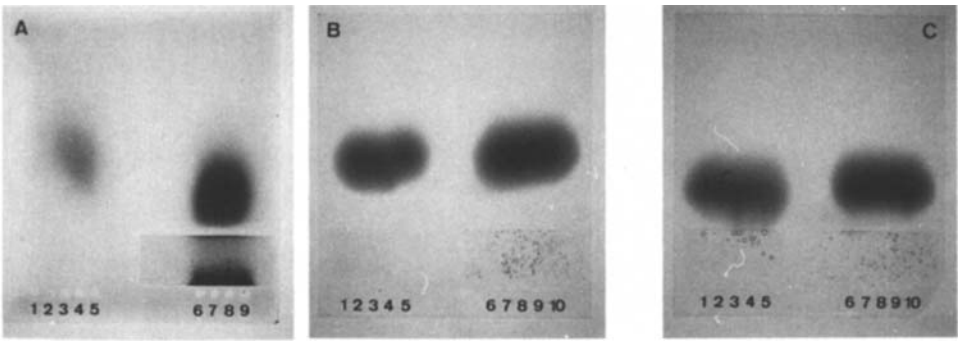
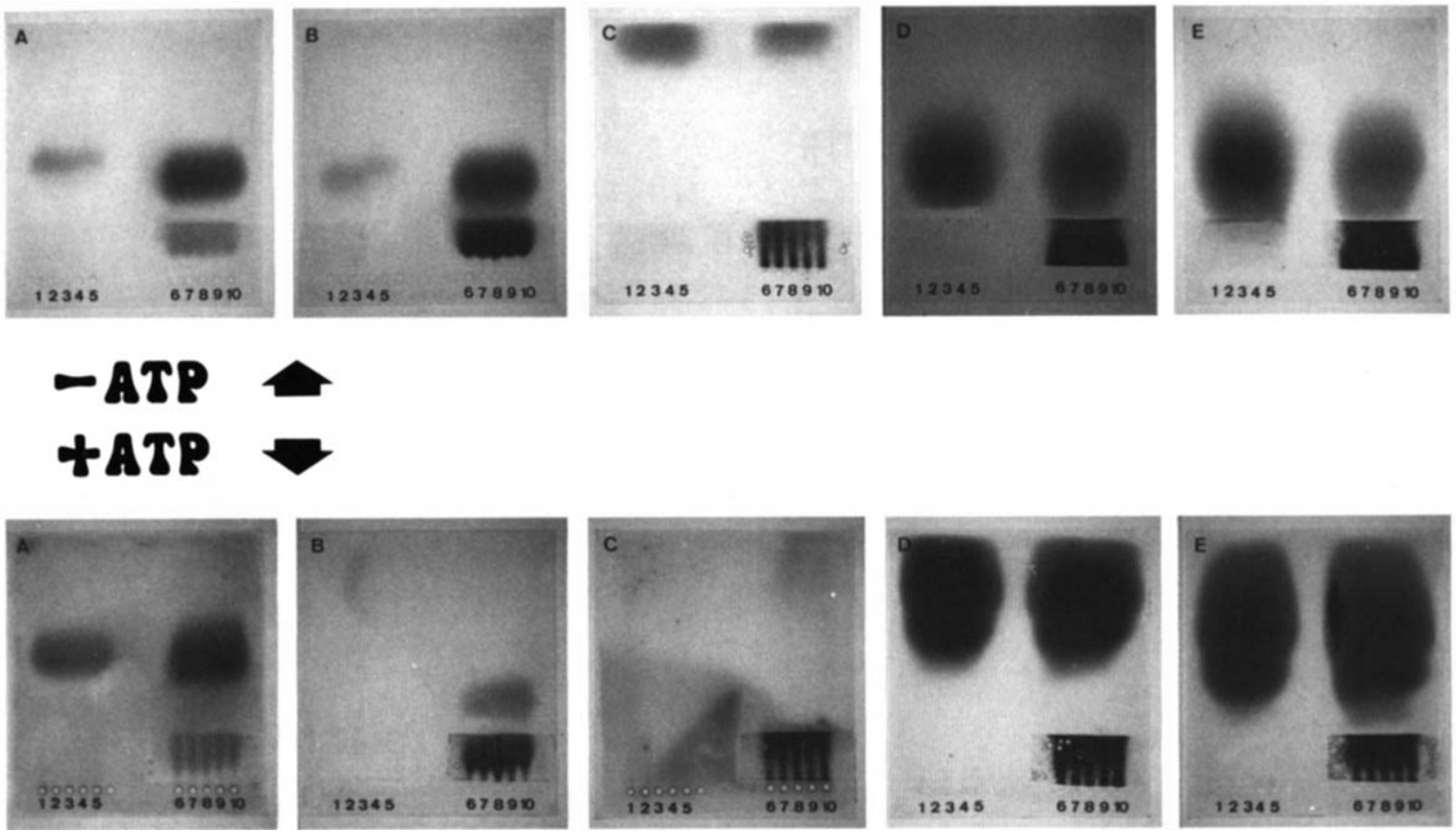



Fig. 1. **A:** Electrophoretic migration of free mitochondrial malate dehydrogenase through an intermediate gel containing fumarase immobilized onto Sepharose-4B (**lanes 6–9**), in comparison with its migration through underivatized Sepharose (**lanes 1–5**). **B,C:** Electrophoretic migration of free cytoplasmic malate dehydrogenase through an intermediate gel containing respectively immobilized fumarase (B) or citrate synthase (C) (**lanes 6–10**), in comparison with its migration through underivatized Sepharose (**lanes 1–5**). Bromophenol blue was used as tracking dye. Electrophoretic conditions were as described in Materials and Methods. All three gels were stained for malate dehydrogenase activity.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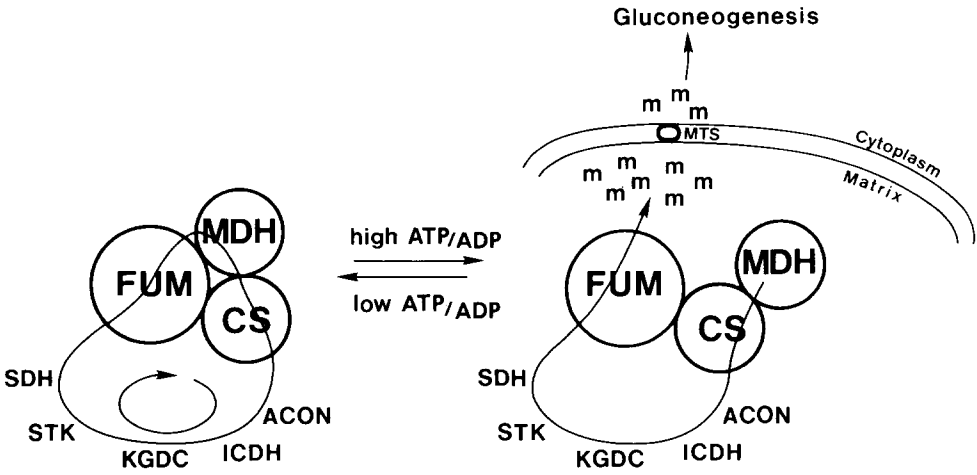


Fig. 3. Hypothetical organization of part of the citric acid cycle in the mitochondrial matrix (see text for further details). The following abbreviations are used: FUM: fumarase, MDH: malate dehydrogenase, CS: citrate synthase, ACON: aconitase, ICDH: isocitrate dehydrogenase, KGDC: α-ketoglutarate dehydrogenase complex, STK: succinate thiokinase, SDH: succinate dehydrogenase, m: L-malate, MTS: mitochondrial L-malate transport system.

results clearly show that the adsorption observed between enzyme couples is highly selective. Our data are in agreement with our previously published observations where the adsorption of fumarase onto immobilized malate dehydrogenases was assayed by the same technique [1].

Migration of either fumarase, malate dehydrogenase, or a mixture of both through immobilized citrate synthase is shown in Figure 2 (above). Both enzymes, either alone or in combination, recognize the immobilized citrate synthase. The associations seem to be rather persistent since they even survive a 40 hours electrophoresis time. The specificity of the observed interaction is again confirmed by the lack of adsorption of cytoplasmic malate dehydrogenase onto immobilized citrate synthase (Fig. 1C).

The presence of 1 mM ATP during electrophoresis of fumarase or malate dehydrogenase through immobilized citrate synthase has no obvious influence on the associations (Fig. 2, below). The latter observation is in agreement with fluorescence anisotropy data from Tompa et al. [18]. On the contrary, 1 mM ATP or $Mg^{++}$-ATP was shown to prevent completely the association between fumarase and mitochondrial malate dehydrogenase [1].

In spite of the fact that the results described here make use of an in vitro system (which was, however, designed to mimic intracellular environmental conditions; i.e., the physical properties of water around the enzymes are altered as was discussed in [1] and

Fig. 2. Electrophoretic migration of free fumarase (**A**), mitochondrial malate dehydrogenase (**E**), or a mixture of both (**B–D**) through an intermediate gel containing immobilized citrate synthase (**lanes 6–10**), in comparison with their migration through underivatized Sepharose (**lanes 1–5**). Electrophoresis was performed in the absence (**above**) or in the presence (**below**) of 1 mM ATP. Gels A and B were run overnight at 80 volts, whereas gels C–E were run for an additional 24 h at 120 volts. Gels A–C were histochemically stained for fumarase, whereas gels D and E were stained for malate dehydrogenase activity.

[17]), it is tempting to formulate the following hypothesis concerning citric acid cycle organization in situ (see Fig. 3). At high intramitochondrial ATP/ADP ratios the citric acid cycle is interrupted at the fumarase/malate dehydrogenase level. Under these conditions direct delivery of malate, generated by fumarase, to the next enzyme, malate dehydrogenase, is impaired, leading to accumulation of malate. This metabolite can then be exported to the cytoplasm where it can be used as a starter product for gluconeogenesis (discussed in [1]). Since, on the other hand, neither the interaction between malate dehydrogenase and citrate synthase nor the association between fumarase and citrate synthase appears to be prevented by the presence of ATP, the consecutive cycle enzymes fumarase and malate dehydrogenase will remain in close vicinity, ready to reassociate as soon as the ATP/ADP ratio decreases. In view of this hypothesis the extremely unfavourable equilibrium displayed by the malate dehydrogenase reaction for the citric acid cycle direction is no longer a disadvantage (see discussions in [1] and [17]), but on the contrary turns out to be profitable. Indeed, when the ATP/ADP ratio is high (i.e., the fumarase/malate dehydrogenase interaction is broken), the majority of the malate molecules generated by fumarase will most likely be picked up by a malate transport system [19,20] and not by malate dehydrogenase, and this in spite of the fact that the latter enzyme remains in the neighbourhood of fumarase. These malate transport systems may be presumed to reside in the vicinity of the citric acid cycle clusters since the enzymes of this cycle are known to be associated with the inner mitochondrial membrane [17]. When the ATP/ADP ratio is low (i.e., fumarase and malate dehydrogenase form a cluster) it might be suggested that malate is directly passed (channeled) to malate dehydrogenase without first being delivered to the bulk phase.

Such a type of citric acid cycle regulation would be very advantageous indeed for an organism since it allows a fast and efficient adaptation of metabolic flow to fluctuating cellular conditions.

## Affinity Electrophoresis Involving Glycolytic Enzymes

The migration of either glyceraldehydephosphate dehydrogenase or triosephosphate isomerase through a gel containing immobilized aldolase is shown in Figure 4 (A–C). Both enzymes readily recognize the immobilized aldolase.

Especially glyceraldehydephosphate dehydrogenase is almost quantitatively adsorbed: Only a minor fraction of the dehydrogenase is, under the conditions used, apparently not retained by the aldolase, as is shown by activity staining in Figure 4A, and by post-staining for total protein in Figure 4B. Moreover, the migration distance of this unadsorbed fraction clearly differs from that of glyceraldehydephosphate dehydrogenase which did not pass through immobilized aldolase.

The migration of either aldolase or triosephosphate isomerase through a gel containing immobilized glyceraldehydephosphate dehydrogenase is shown in Figure 4 as well (D–F). Both enzymes recognize the immobilized dehydrogenase and also here the migration distance of the unadsorbed aldolase fraction differs from that of the same enzyme which did not pass through the immobilized dehydrogenase. Since aldolase apparently inactivates quite easily, a Coomassie blue post-stain was performed (Fig. 4F).

The migration of aldolase and glyceraldehydephosphate dehydrogenase through immobilized bovine serum albumin or gamma globulins is shown in Figure 5. From this figure it is clear that no associations are formed and that both enzymes pass unretarded

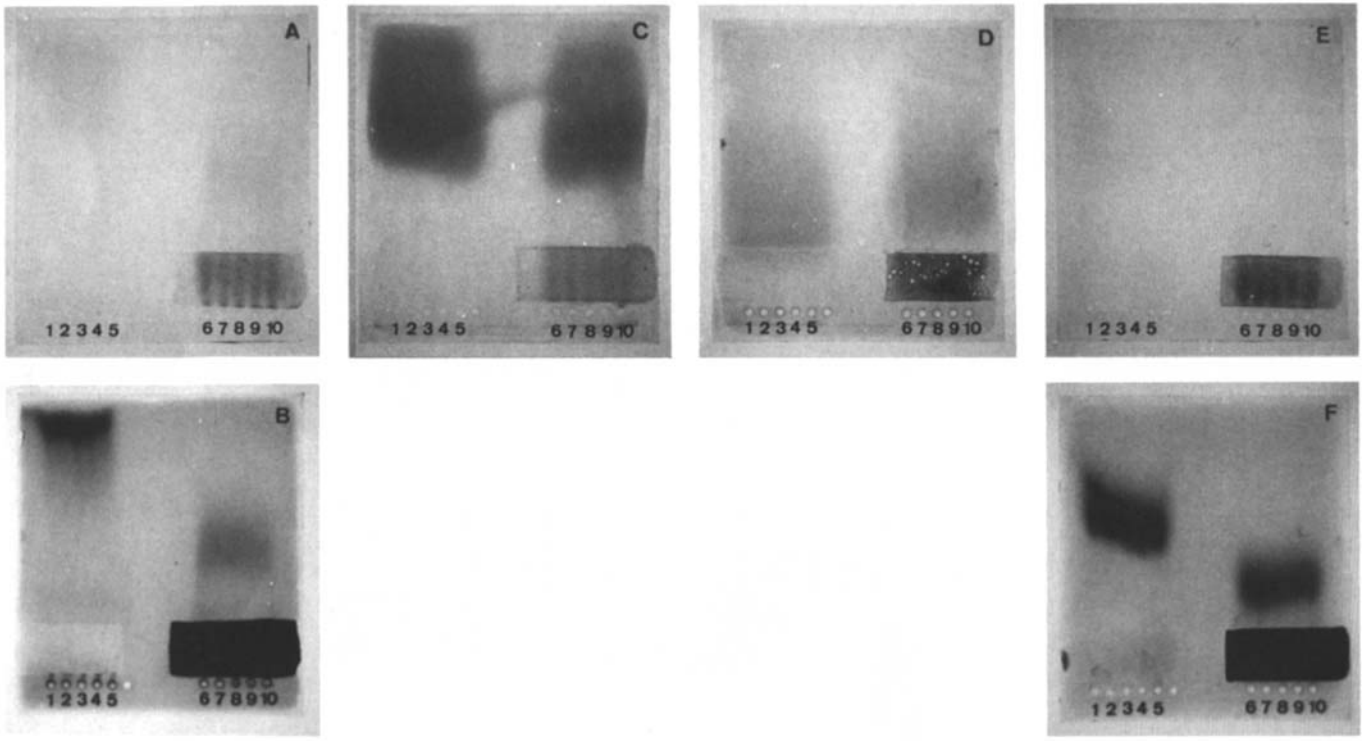


Fig. 4. Electrophoretic migration of free glyceraldehydephosphate dehydrogenase (**A, B**), triosephosphate isomerase (**C, D**), or aldolase (**E, F**) through an intermediate gel containing (in **lanes 6–10**) respectively immobilized aldolase (A–C) or immobilized glyceraldehydephosphate dehydrogenase (D–F), in comparison with their migration through underivatized Sepharose (**lanes 1–5**). Electrophoretic conditions were as described in Materials and Methods. Gel A was stained for glyceraldehydephosphate dehydrogenase activity, whereas C and D were stained for triosephosphate isomerase and E for aldolase activity. Gels B and F are respectively gels A and E, which were post-stained for total protein with Coomassie brilliant blue.

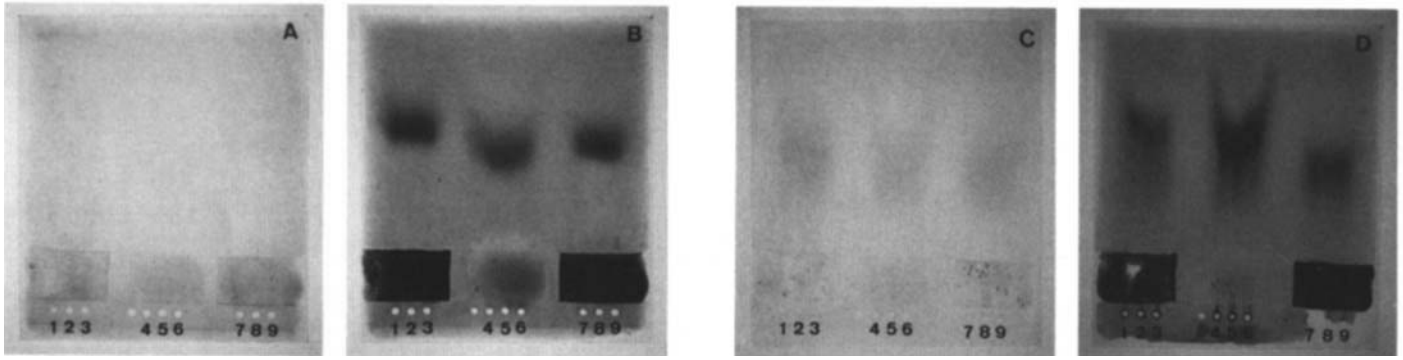


Fig. 5. Electrophoretic migration of, respectively, free aldolase (**A** and **B**) and free glyceraldehydephosphate dehydrogenase (**C** and **D**) through immobilized bovine serum albumin (**lanes 1–3**) and gamma globulins (**lanes 7–9**), in comparison with their migration through underivatized Sepharose (**lanes 4–6**). Gels A and C were respectively stained for aldolase and glyceraldehydephosphate dehydrogenase activity. Staining time was twice as long as for the gels in Figure 4. Gels B and D are respectively gels A and C, which were post-stained for total protein with Coomassie brilliant blue.

through these immobilized proteins. We conclude from these observations that the above-described associations among glycolytic enzymes are indeed specific.

## CONCLUSIONS AND EVALUATION OF THE METHOD

The results presented in this paper and elsewhere [1] clearly show that affinity electrophoresis can be successfully used to demonstrate specific enzyme-enzyme interactions which may be of physiological importance in the regulation of both the glycolytic pathway and the citric acid cycle. Consequently it may be reasonably supposed that this technique will prove to be of general use to visualize cluster formation between enzymes of other metabolic pathways as well. Currently we are investigating cluster formation between other glycolytic enzymes, as well as between citric acid cycle enzymes and between glyoxysomal enzymes of fat-storing plant tissues.

Finally it seems appropriate to discuss here some of the characteristics of this new method. It should be stressed that, in its actual concept, the affinity electrophoresis technique is an exclusively qualitative one. Indeed, the enzymes are localized on the gels by activity staining, a procedure which is recognized not to provide any information on the amount of enzyme present in the different activity bands or spots. Moreover the kinetics of enzymatic staining will be influenced by the adjacent immobilized enzyme molecules: It was already proven that the latter influence the stability of the free enzyme molecules which are sticking by adsorption (see also discussion in [1]). On the other hand it is known that the immobilized enzyme molecules are linked to the Sepharose beads by "multi-point attachment" (discussed in [13]). As a consequence considerable parts of their surface will not be available for eventual association with free enzyme molecules. It has to be expected that this shielding effect will have a severe but variable influence on the amount of free enzyme which can be adsorbed onto the population of immobilized enzyme molecules. Consequently, affinity electrophoresis provides useful information on the possibility of two enzymes, having complementary surface structures, to associate and to form a multienzyme cluster. However, this technique can not be used to determine the stoichiometry or the association constant in such enzyme cluster.

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