An Electrophoretic Technique to Concentrate and Separate Substances and Its Application to Insect Hemolymph

GUNNAR ERIKSSON

Institute of Biochemistry, University of Stockholm, Stockholm, Sweden

By utilizing the principle of sharp moving boundaries, first described by Lodge [1] and Whetham,[2] an electrophoretic procedure has been developed at this laboratory in cooperation with A. Vestermark.[3,4] This method makes it possible to concentrate and separate substances at the same time, in a manner which may be compared with the displacement development of chromatography. The concentrating effect is achieved by utilizing a discontinuous buffer system, where the sample is placed behind a fast and in front of a slow ion in the direction of migration. Ions in the sample, which are slower than the fast ion and faster than the slow ion of the buffer system, will separate and arrange themselves into contiguous zones with sharp moving boundaries. The order of the ions is decided by their ion mobilities. The length of a zone will be determined by the amount of the ion in the sample, but also by its final equilibrium concentration in the zone. According to Kohlrausch,[5] this concentration is mainly a function of the concentration of the fast ion. Fig. 1 schematically illustrates the appearance of a sample with three negative ions, before (A) and after (B) electrophoretical separation. Fig. 2 shows the correlation between zone length and concentration in the sample for different concentrations of the fast ion.

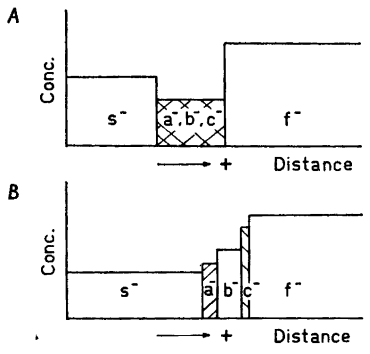

Fig. 1. A sample containing three different negative ions (a^-, b^-, and c^-), which are slower than the fast ion (f^-) and faster than the slow ion (s^-), before (A) and after (B) separation into contiguous zones. The height of each zone represents the concentration of the corresponding ion. The abscissa indicates the distance along the separation path.

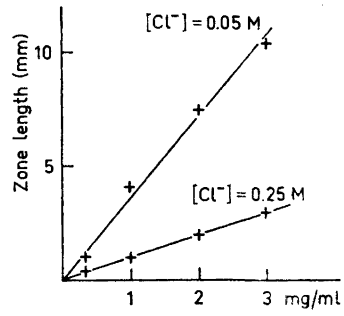

Fig. 2. Final zone length as a function of the original concentration in the sample, for different concentrations of the fast ion. The sample volume has been constant. Chloride functioned as fast ion, while ascorbate (0.05 M) was used as slow ion.

This method of concentrating substances electrophoretically has been suggested by Kendall,[6] who separated some rare earth metals on agar gel columns. The same principle is applied in the concentration step of disc-electrophoresis.[7] The name "displacement electrophoresis" for the method has been suggested by Martin.[8]

To obtain a good final separation, which makes it possible to elute and isolate the separated substances, a suitable stabilizing medium for the electrophoresis is essential. At this laboratory good results were obtained with thin-layers of cellulose powder. The powder (MN 300, Machery and Nagel, Düren, Germany) was washed with 1) 4 M HCl, 2) EtOH containing 3 % (v/v) conc. HCl, and 3) deionized water to neutral pH.

Cellulose thin-layers (0.4 mm thick), prepared on glass plates (120 × 163 mm) according to Nybom,[9] were divided into ten parallel, 9 mm wide, lanes. Thus different samples or samples with different additions (see below) can be run parallel in the same experiment.

Before electrophoresis the thin-layer was moistened with the anodic buffer from one end and with the cathodic buffer from the opposite end, leaving a 2–3 cm dry space for application of the sample. The sample volume has varied from 10 to 20 μl. In the experiment to be described, chloride (0.10 M, at pH 7.0) was used as fast ion and glycinate (0.06 M, at pH 8.3) as slow ion, while the cation of tris-(hydroxymethyl)aminomethane has functioned as a buffering component throughout the system.

This procedure has been applied to the isolation and identification of various compounds in the hemolymph from insects. Crude hemolymph with the addition of a little phenylthiourea to inhibit tyrosinase activity, was analysed. Uric acid, which usually occurs in high concentration in insect hemolymph, was isolated in early experiments (G. Eriksson, unpublished). It is easily detected due to its UV-absorbancy. The incorporation of ^{32}P into phosphate esters was studied by injection of [^{32}P] phosphate into *Lepidoptera* pupae. After electrophoresis labelled phosphorus compounds will give rise to radioactive zones, detected by autoradiography. By adding a known phosphate ester to the sample an enlarged zone will be obtained, if the ester is originally present (Fig. 3). After incubation *in vivo* for 3 to 24 h with [^{32}P] phosphate the presence of *P*-ethanolamine in the hemolymph from *Amorpha populi*, *Dicranura vinula*, and *Acronycta megacephala* was indicated in this manner. For further proof of identity, the zone with the anticipated *P*-ethanolamine was eluted with water and analysed by thin-layer chromatography on cellulose and silica gel. The chromatographic systems were worked out for the separation of the related esters, *P*-choline and *P*-ethanolamine. For cellulose thin-layers, washed with formic acid (25 %), *t*-BuOH:-formic acid:water (11:5:4, by vol.) and for silica gel (Kieselgel H nach Stahl, not activated) ethanol:water (1:4, by vol.) were used. The samples were run parallel as well as in a mixture with the reference. For *P*-choline and *P*-ethanolamine, R_F-values of 0.78 and 0.51, respectively (on cellulose) and 0.37 and 0.76, respectively (on silica gel) were found. Ninhydrin and $FeCl_3$-sulfosalicylic acid [10] were used for detection of the substances. Additional proof was obtained by enzymatic hydrolysis of the eluted substance with alkaline phosphatase. The ninhydrin-positive product behaved chromatographically as ethanolamine.

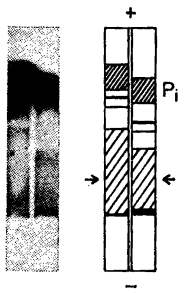

Fig. 3. Autoradiography from electrophoreses of hemolymph samples (*A. megacephala*). The left lane with the addition of 2 μl *P*-ethanolamine (10 mg/ml) and the right lane without any addition. The arrow indicates the *P*-ethanolamine zone. All the radioactive components migrated towards the anode.

By the electrophoretic procedure described, it has been possible to isolate and identify compounds from small sample volumes, due to the simultaneous concentration and separation of the substances. This makes it possible to study the metabolism in single individuals of insects. Furthermore the zone lengths give quantitative estimations of the substances.

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