Die Fig. 2 zeigt das chromatographische Bild bei U.V.-Betrachtung und die gemessenen Fluoreszenzintensitäten im R_F-Bereich des Harmans. Die Flächenwerte der Vergleichspeaks, die wir durch Multiplikation der Höhe und ihrer Breite in halber Höhe erhalten haben, führen in bekannter Weise zu einer für jedes Chromatogramm neuen Eichkurve, die nach unseren Erfahrungen bei Verwendung von Fertigplatten bis 300 ng geradlinig verläuft. Für die zu messenden Proben ergeben sich aufgrund der ermittelten Flächenwerte im vorliegenden Fall 221 und 227 ng Harman/0.01 ml Auftraglösung. Das entspricht einem durchschnittlichen Gehalt von 5.6 mg % Harman in der zu untersuchenden Lösung. Die relative Standardabweichung beträgt $\pm$ 1.9 %.

In schwächeren Harmanlösungen sind bei entsprechenden Lichtintensitäten des Fluorometers und reduzierten Vergleichsmengen Messungen bis zu 10 ng mit einer relativen Standardabweichung von etwa 10 % bei zwei Werten durchführbar. In harmanarmen Passiflorendrogen ist erst nach Anreicherung der Base in den durch Kaltextraktion mit 1 %iger Salzsäure erhaltenen Auszügen eine Bestimmung in der oben beschriebenen Weise durchführbar.

Frau E. M. Hoernes danke ich für bewährte experimentelle Mitarbeit.

Kontroll-Laboratorium der Dr. Willmar Schwabe GmbH, W. MESSERSCHMIDT
75 Karlsruhe-Durlach (Deutschland)

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Visualization of acyl phosphatase after zone electrophoresis and thin-layer gel filtration*

A variety of techniques is currently available for the visualization of proteins after zone electrophoresis and thin-layer gel filtration. The method reported here for the specific detection of fractions with acyl phosphatase activity is based on the use of *p*-nitrobenzoyl phosphate as substrate for the enzyme[1], and on the staining of the residual substrate, after a suitable time, by the hydroxylamine–ferric chloride reaction according to LIPMANN AND TUTTLE[2]. In our laboratory this procedure was used in order to localize the fractions with acyl phosphatase activity in crude extracts of tissues, and during the purification of the enzyme from horse muscle.

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Experimental

Materials

Acyl phosphatase was prepared from horse muscle, as described by GUERRITORE *et al.*[3]

A crude extract was obtained by homogenizing horse muscle in 0.1 *M* acetate buffer pH 5.3 (1:4, w/v), for 60 sec, in an Ultra-Turrax apparatus in a refrigerated container. The homogenate was centrifuged at 150,000 × *g* for 30 min and the precipitate was discarded.

p-Nitrobenzoyl phosphate was synthesized as previously described by RAMPONI *et al.*[1].

Starch hydrolyzed for gel electrophoresis was purchased from Connaught Medical Research Laboratories (Toronto, Canada), Sephadex G-75 Superfine from Pharmacia (Uppsala, Sweden).

Hydroxylamine hydrochloride, KOH, ferric chloride·$6H_2O$, HCl and ethanol were obtained commercially from E. Merck AG; nigrosin from Schmid & Co.

Reagents

p-Nitrobenzoyl phosphate solution was 140 m*M*.

Solution A: 10 g of hydroxylamine hydrochloride were dissolved in 25 ml of distilled water and made up to 100 ml with ethanol.

Solution B: 20 g of KOH were dissolved in as small as possible a volume of distilled water and then brought to 200 ml with ethanol.

Spray I (*hydroxylamine reagent*). This was prepared fresh each time by mixing one volume of solution A and two volumes of solution B. The precipitate of KCl was removed by filtration.

Spray II (*ferric chloride reagent*). 5 g of ferric chloride were dissolved in 10 ml of concentrated HCl and made up to 100 ml with distilled water.

Methods

Starch gel electrophoresis. This was performed according to the procedure of SMITHIES[4] in a Shandon apparatus at 4°. 0.3 *M* acetate buffer, pH 5.3, was used as such in the buffer vessels, and diluted fifteen times for the preparation of the starch gel plate (5 × 18 × 0.8 cm). The sample (purified acyl phosphatase or crude extract) was placed in the slot with a Pasteur pipette. With a current of 20 mA and 170–150 V, for 6–7 h, a good separation was obtained. Under these conditions acyl phosphatase present in the sample analyzed traveled toward the cathode. After the electrophoretic run, the starch gel was cut in three sections: the first section was discarded, the middle one was used as a control for protein staining with nigrosin and the bottom section for the specific staining of acyl phosphatase fractions as follows:

(1) A strip of paper (Schleicher and Schüll 2043b) wetted with *p*-nitrobenzoyl phosphate solution was placed on the surface of the gel section and allowed to incubate at room temperature for a suitable length of time, depending on acyl phosphatase concentration (about 40 min for muscle extract).

(2) After the incubation, the strip was removed, dried in a current of cold air and sprayed with Spray I. White spots, corresponding to acyl phosphatase activity, on a yellow background appeared immediately.

(3) The paper was completely dried in warm air and sprayed with Spray II, in order to obtain a better contrast: yellow spots on a dark brown background.

Paper electrophoresis. The electrophoretic run was performed on a strip of Whatman paper No. 1 (5 × 8 cm) with a current of 2 mA/cm and 330–200 V, for 2 h, using 0.1 *M* acetate buffer pH 5.3. After electrophoresis, the paper was dried in a stream of cold air and sprayed with *p*-nitrobenzoyl phosphate solution. After incubation at room temperature for a suitable time, the strip was dried in cold air and steps 2 and 3 of the above procedure were followed successively.

Thin-layer gel filtration. This was carried out according to JOHANSSON AND RYMO[5], using Sephadex G-75 Superfine in 0.02 *M* triethanolamine–HCl buffer, pH 7.5, for 3 h on a plate (10 × 20 cm) at an angle of 10° to the horizontal. After chromatography, a strip of filter paper (Schleicher and Schüll 2043 b) was applied to the gel surface. The plate was dried in cold air and then sprayed with a *p*-nitrobenzoyl phosphate solution, and incubated at room temperature for a suitable time. The above procedure was followed. Another plate was used for the control protein staining with nigrosin.

Results and discussion

In Fig. 1 the visualization of acyl phosphatase activity after starch gel electrophoresis of crude extract and purified enzyme is reported and compared with protein staining. Good results were also obtained after paper electrophoresis and Sephadex G-75 Superfine gel filtration. The results obtained with Sephadex G-75 Superfine gel filtration are shown in Fig. 2.

In this method, many acyl phosphates may be used as substrates of acyl phosphatase, but in our experiments *p*-nitrobenzoyl phosphate was preferred. This substrate is, in fact, easily hydrolyzed by the enzyme: the hydrolysis rate is 21 times higher than with acetyl phosphate, the most commonly used substrate for acyl phosphatase[1]. Small amounts of proteins with acyl phosphatase activity may also be detected in this way.

As can be seen from the above results, this technique easily permits the location of fractions containing acyl phosphatase activity among many others that may be

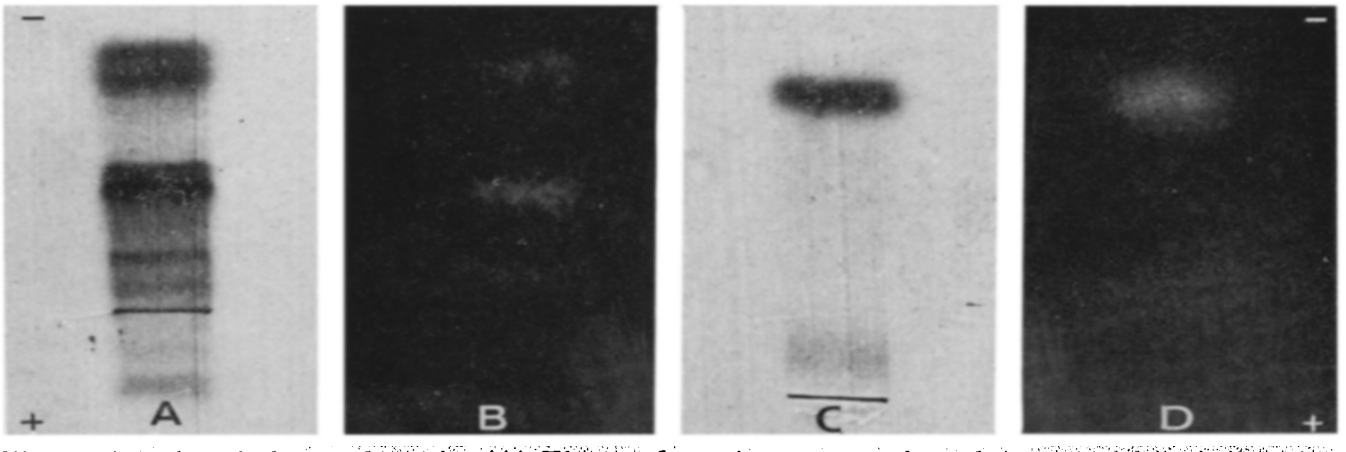

Fig. 1. Starch gel electrophoresis: (A) Electrophoretic pattern of crude extract proteins, stained by nigrosin; (B) specific staining of crude extract proteins with acyl phosphatase activity; (C) purified acyl phosphatase stained by nigrosin; (D) visualization of purified acyl phosphatase by the specific method.

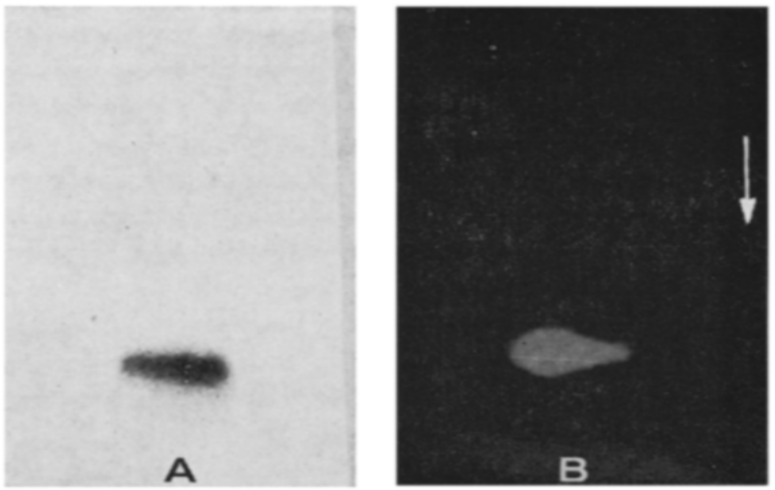

Fig. 2. Thin-layer gel filtration on Sephadex G-75 Superfine: purified acyl phosphatase stained by nigrosin (A) and by the specific staining (B).

isolated by zone electrophoresis or gel filtration. Consequently it is possible to carry out a comparative study in order to determine some of the properties of proteins from different sources with this enzymatic activity and the relationships among eventual isoenzymes. In addition, if preparative applications are envisaged, this method permits the localization of the fractions with acyl phosphatase activity without disrupting or destroying the gels, so that recovery is possible by cutting out the zones with enzyme activity.

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Institute of Biochemistry, University of Florence, Florence (Italy)

P. NASSI
C. TREVES
G. RAMPONI

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