stimulate independently different kind of olfactory receptors. The same analysis was performed for the other compounds (a→a′ and b′→b″ in **3a–3b** series (fig. 2A), and $\alpha \rightarrow \alpha'$ and $\beta' \rightarrow \beta''$ in **7a–7b** series (fig. 2B)], and the independent contributions of the optical isomers to the EAG amplitudes were elucidated in these enantiomeric pairs as well as the case of **8a–8b** series.

It is reasonable to expect that the pheromonally inactive enantiomers interact with receptors other than sex pheromone receptors which are assumed to interact with active (+)-enantiomers[10]. Thus, it has been shown by behavioral and physiological methods that in the American cockroach pheromonally inactive enantiomers of optical chiral pheromonal substances possess no influence on the potency of active enantiomers.

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Rapid characterization and partial purification of various animal amine oxidases[1]

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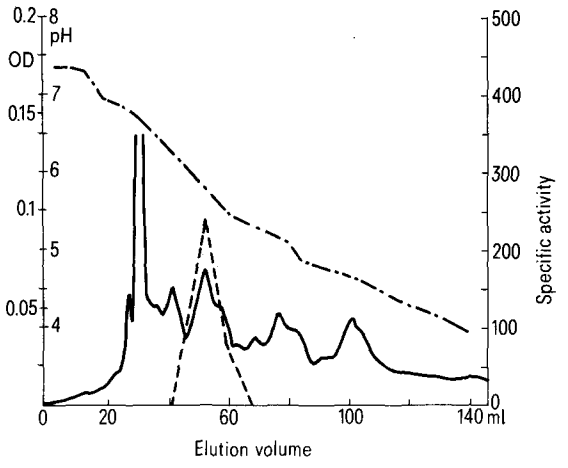
Summary. The use of chromatofocusing to obtain a rapid characterization of tissue amine oxidases from various mammals is proposed. This technique yields partially purified enzymes well suited for immunological studies. Chromatofocusing can be also used in a three-step purification of pig kidney diamine oxidase.
Key words. Amine oxidases, purification; chromatofocusing.

Amine oxidases are widespread throughout living organisms[2–5]. They catalyze the oxidative deamination of various amines according to the equation:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

Circulating and intracellular amine oxidases have been described both in plants and animal tissues[2–6]. The intracellular enzymes are more active on the diamines, particularly putrescine and cadaverine; histamine is often oxidized at an even greater rate[5]. In view of the increasing interest in putrescine as the precursor of polyamines, the level of which has been related to various cellular events and particularly to cellular proliferation, the role of diamine oxidases is at present actively being investigated. The mechanism of action of these enzymes and their localization have been studied[7]. Their presence in various tissues of the same organism, and in different organisms, prompted us to start a thorough investigation on the immunological relationships between some amine oxidases[8,9].
In the present paper we describe a rapid purification procedure using a chromatofocusing technique that has been successfully applied to a number of mammalian tissues. This procedure allows us to obtain purified amine oxidase preparations suitable for comparative kinetic and immunological studies.

Materials and methods. All chemicals used were of analytical grade and used without further purification. Putrescine was purchased from Sigma, St. Louis, Mo., USA. Polybuffer exchanger 94 (PBE 94), polybuffer 74 (PB 74) and Sepharose 4B from Pharmacia, Uppsala, Sweden. Bio-Rad protein assay kit from Bio-Rad Laboratories, Richmond, Cal., USA; 1,4-^{14}C-Putrescine by The Radiochemical Centre, Amersham, England.
Enzyme assay. Protein was determined either with a biuret method[10] or with the Bio-Rad protein assay. Diamine oxidase activity was tested according to Okuyama and Kobayashi[11] by using ^{14}C-putrescine as substrate. After 1 h incubation at 37 °C, the reaction mixture was stopped and extracted twice with toluene. The toluene containing ^{14}C-Δ'-pyrrolidine so formed was mixed with 3 ml of Lipoluma (LUMAC, Swiss) and counted in a liquid scintillation counter.
Antibody production. New Zealand rabbits were injected with diamine oxidase from pig kidney, purified according to Rinaldi et al.[12], in complete Freund's adjuvant. After several boosters, the animals were bled and the antisera pooled. The gamma-globulin fraction was purified by 33% ammonium sulphate precipitation and extensive dialysis against borate-NaCl buffer, pH 8.4. Antigen-antibody reaction was tested either by double diffusion[13] or with a Behring Laser Nephelometer (Behring-



Chromatofocusing elution profile of pig kidney homogenate. A column (0.8 × 10 cm) with flux rate of 20 ml/h was used. OD at 280 nm (——), specific activity (– – – –) and pH gradient (–·–·–·–·–) are shown. OD was measured in an optical path of 0.25 cm. The specific activity towards putrescine was expressed as nanomoles oxidized × h^{-1} × mg $protein^{-1}$ at 37 °C.

werke AG, Marburg/Lahn, GFR)[9]. Part of the purified antibody was conjugated to Sepharose-4B previously activated by cyanogen bromide. The resin was washed and equilibrated with 0.1 M phosphate buffer pH 7.4.

Enzyme purification. Pig kidneys from the slaughterhouse were washed with phosphate buffered saline (PBS) and the cortical zone alone was homogenized in a Waring Blendor in cold 0.1 M phosphate buffer pH 7.4. The homogenate was heated at 60 °C under stirring for 10 min in a termostatic water bath and then centrifuged at 15,000 rpm for 20 min at 4 °C.

After dialysis against 50 mM phosphate buffer pH 7.4, the material was loaded onto a column for the chromatofocusing step. After washing with the starting buffer, a 7–4 pH gradient elution was performed using PB 74, pH 4. Optical density at 280 nm was continuously recorded and all the fractions tested for diamine oxidase activity. Active fractions were pooled and dialyzed again.

This pool was finally loaded onto a column for the immuno affinity chromatography. After washing with 0.1 M phosphate buffer pH 7.4, diamine oxidase was eluted by 2 M KCl containing 0.1% Triton X-100. Diamine oxidase activity in the eluate was tested after dialysis.

Results and discussion. The determination of mammalian diamine oxidase activity in crude extracts from mammalian tissues is of considerable interest because this enzymatic activity seems to be correlated with tissue proliferation or hypertrophy[15–18] thus representing a useful indicator of these important cellular events. However, the methods in use suffer from several drawbacks[14,15]. We have used chromatofocusing as a tool to overcome these problems. The figure shows the elution profile from the chromatofocusing of pig kidney diamine oxidase, an enzyme already purified to homogeneity[19–21]. The enzymic activity was recovered in a distinct protein peak with an apparent isoelectric point of 6.0. Several other diamine oxidases have been partially purified in the same way. They were always eluted in the same pH range with similar elution profiles while their apparent isoelectric points ranged from 5.4 to 6 (table 1). The purification factor obtained with this single chromatographic step was about 60–100-fold and the recovery was near to 80% (see below). A heat treatment (60 °C for 10 min) of the homogenate before chromatography drastically reduced the amount of irreversibly adsorbed material without affecting the total enzymic activity and the selectivity of the column. These data show that despite the antigenic difference[9], diamine oxidases from largely different organsims and tissues gave quite similar chromatographic patterns. The chromatofocusing procedure alone can yield from a limited amount of starting material ($\simeq$ 50 mg of tissue) partially purified preparations, well suited for kinetic and immunochemical studies. It was confirmed in this way that human placenta diamine oxidase does not cross-react with anti-pig kidney diamine oxidase antibodies[9], as determined both by immunoprecipitation and by laser nephelometry.

Table 1. Apparent isoelectric points of DAOs from different mammalian sources obtained by chromatofocusing

Tissue	Apparent pI
Human kidney	6.0
Human placenta	5.4 ± 0.2
Pig kidney	6.0 ± 0.1
Beef kidney	5.6

An immuno-affinity chromatography column for pig kidney diamine oxidase was prepared by conjugating the antibodies against this enzyme to Sepharose 4B. The diamine oxidase-containing fraction resulting from chromatofocusing as above was passed directly through the immuno-affinity column. All the enzymic activity was retained by the column. Various attempts to resolve the antigen-antibody complex bound to the column, like increasing the ionic strength up to 2 or varying the pH of the elution buffer in the range 5–9 failed. Finally the enzymatic activity was released from the column using a 0.1 M phosphate buffer pH 7.4 containing 2 M KCl and 0.1% Triton X-100. After dialysis the eluted protein showed about 80% of the total activity adsorbed onto the column. Table 2 shows a purification pattern for pig kidney diamine oxidase on a preparative scale. The purification factor obtained with only 3 steps compares favorably with most published purification procedures[19–21]. However, recent reports have shown that with conventional methods requiring 8–10 purification steps a greater specific activity was obtained[12].

In conclusion, chromatofocusing appears to be an important tool for rapid characterization of amine oxidases and a useful purification step in the isolation of these proteins, particularly considering the speed, flexibility and reproducibility of this technique.

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Table 2. Partial purification of pig kidney diamine oxidase

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)*	Yield (%)	Purification (fold)
Crude extract	9,000	50.4	0.0056	100	1
Heat treatment	1,260	27.3	0.0217	53.9	3.9
Chromatofocusing	19.2	25.0	1.306	49.3	233
Affinity chromatography	3.0	19.6	6.345	38.4	1,133

The starting material was 40 g of kidney cortex. * The specific activity is expressed as nmoles of putrescine transformed × h^{-1} × mg^{-1} of protein at 37 °C.

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Isoelectrically focused carboxyesterases as a biological marker in chimeras

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Summary. Species-specific multiple forms of carboxyesterases (CE) were determined in zymograms obtained by isoelectric focusing (IEF) using homogenized wing zeugopodal tissues of chick, quail and quail-chick chimeras. The validity of the CE pattern of chimeric tissues was verified by the nuclear marker technique. Analytical IEF of CE was found to be useful for investigation of the origin of tissues in chimeras.
Key words. Chimeras, quail-chick; multiple enzyme forms, carboxyesterases, nuclear marker technique; zymograms.

The quail-chick marker technique[1] represents an important tool in experimental embryology. Since the interphase nuclei of Japanese quail cells are characterized by a large mass of perinucleolar heterochromatic DNA which does not exist in chick nuclei, it is possible to use quail cells as stable histological markers in interspecific grafting experiments. By the use of this technique various systems of migrating cells have successfully been analyzed in chimeric embryos; for example the migration of limb muscle precursor cells[2]. The employment of such a technique is limited to those species which exhibit distinctly different patterns of heterochromatin.

In order to solve some problems of morphogenesis it may be necessary to produce chimeras of species whose cells cannot be distinguished by nuclear markers. The analysis of such chimeras would require the application of non-histological techniques which could also help to elucidate biochemical aspects of developmental processes. It has been shown that the cellular genotypes in allophenic mice can be distinguished, for example, on the basis of electrophoretic variants of the enzyme glucosephosphate isomerase[3,4]. To achieve a more general employment of such a method one has to look for enzymes which occur ubiquitously and which are characterized by distinct species-specific features. It is well known that the number of multiple forms of carboxyesterases (CE) varies greatly between different species, even when they are phylogenetically closely related[5].

The aim of this study is a) to look for the differences of CE pattern of chick and quail tissues; b) to analyze the origin of tissues within chimeras using the species-specific patterns of CE and to prove the validity of this method. Different molecular forms of CE were separated by isoelectric focusing (IEF) of homogenized chick, quail and chimeric wing muscles. Zymograms were obtained by subsequent staining for CE. The validity of this method was tested using the quail-chick marker technique on sections from parallel tissue samples.

Material and methods. White Leghorn (Velaz, Praha) and Japanese quail (own hatchery) were used throughout the investigations. Eggs were incubated at 38 ± 1 °C. In order to obtain chimeric wings unilateral replacements of chick brachial somites by those of quail were performed. Two-day-old embryos at stages 13 and 14 according to Hamburger and Hamilton[6] were used. The details of experimental procedures and their results have been described previously[2]. The chimeric wing muscle contains muscle cells of quail origin and connective tissue of chick origin. Such wings represent a well-defined model where cells from two different species are intermingled. Embryos were sacrificed 13–17 days after somite replacement. Altogether 15 chimeras were evaluated. Muscles of the wing stylopodes were removed and fixed in Serra's fluid, embedded in paraplast, sectioned, treated with the Feulgen-reaction[7] and post-stained with light green. Zeugopodal muscles containing muscle fibers, connective tissue, vessels and nerves were processed for detection of CE by zymograms. Corresponding muscles of the contralateral wing were used as controls. Muscle samples ranging from 20 mg to 50 mg were homogenized and diluted with distilled water containing 1% Triton X-100