10 Khomutov, R.M., Zavalova, L.L., Siirku, V.I., Artamonova, E.Y., and Khomutov, A.R., Bioorg. Chem. (Russ.) *9* (1983) 130.
11 Hölttä, E., Jänne, J., and Pispa, J., Biochem. biophys. Res. Commun. *47* (1972) 1165.
12 Gashel, M., and Gallant, J., Nature *221* (1969) 838.
13 Khomutov, R.M., Hyvönen, T., Karvonen, E., Kauppinen, L., Paalanen, T., Paulin, L., Eloranta, T., Pajula, R.-L., Andersson, L.C., and Pösö, H., Biochem. biophys. Res. Commun. *130* (1985) 596.
14 Wickner, R.B., Tabor, C.W., and Tabor, H., J. biol. Chem. *245* (1970) 2132.
15 Karvonen, E., Kauppinen, L., Partanen, T., and Pösö, H., Biochem. J. *231* (1985) 165.
16 Wu, W.H., and Morris, D.R., J. biol. Chem. *248* (1973) 1696.
17 Kallio, A., McCann, P.P., and Bey, P., Biochemistry *20* (1981) 3163.

0014-4754/87/020174-03$1.50 + 0.20/0

A practicable variant of the ion exchange method for the radiometric estimation of ornithine decarboxylase activity

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Summary. A known ornithine decarboxylase assay working with ion exchange separation of [^{3}H]ornithine and [^{3}H]putrescine has been revised. The assay can be performed in disposable 1.5 ml vessels with a total of four pipetting steps. The separation of enzyme substrate and product, respectively, requires 3 h per 50 samples. The detection limit is about 50 pmoles [^{3}H]putrescine formed.
Key words. Ornithine decarboxylase; radiometric enzyme assay; ion exchange separation; Michaelis constants; pyridoxal phosphate.

Ornithine decarboxylase activity (L-ornithine carboxy-lyase, EC 4.1.1.17) is usually estimated with the $^{14}CO_2$-trapping method proposed by Morris and Pardee[1]. The preparation of this enzyme assay requires considerable experimental expenditure and equipment for the release and quantitative absorption of $^{14}CO_2$ in one incubation vessel. Numerous variants of the procedure have been published. Alternative methods are based on the separation of ornithine and putrescine by ion exchange procedures. They have the advantage of measuring exclusively the product formed by the action of ornithine decarboxylase. One method, originally published by Clark[2] and improved by Djurhuus[3], works with cation exchange paper and is suitable for small volume samples. Maderdrut and Oppenheim[4] presented another radiometric method in which non-reacted enzyme substrate, [^{3}H]ornithine, is separated from the reaction product, [^{3}H]putrescine, by an acidic ion exchange resin. Although this method works reliably with simple laboratory equipment and little experimental expenditure for the enzyme assay, it has not come into use. This may be due to some shortcomings of the procedure which can easily be overcome, as will be detailed in the following.

Materials and methods. Brains of young Wistar rats (typically 10 days old, weighing 850–1000 mg) were homogenized with 10 strokes of a Potter-Elvehjem homogenizer in 10 vols of 50 mM sodium phosphate buffer pH 7.4, 2 mM dithiothreitol. Homogenates were centrifuged for 30 min at 45,000 xg or for 12 min at 10,000 xg, resulting in protein concentrations of about 2.5 mg/ml and 3.5 mg/ml, respectively. 0.4-ml aliquots of the supernatants were combined in 1.5-ml reaction vessels with 0.1 ml of an assay medium to result in final concentrations of 0.1 mM ornithine hydrochloride, 0.1 mM pyridoxal phosphate, and 2 mM dithiotreitol in 50 mM sodium phosphate buffer pH 7.4. Blanks additionally contained 5 mM semicarbazide hydrochloride (to destroy the cofactor, pyridoxal phosphate). The samples were preincubated at 37 °C for 15 min (Thermostat 5320, Eppendorf Gerätebau, Hamburg/FRG). 1 µCi of purified[4] L-[2,3-^{3}H]-ornithine (specific activity: 15–30 Ci/mmol, New England Nuclear, Dreieich/FRG) was added in 20 µl of 50 mM sodium phosphate buffer pH 7.4 (final specific activity: 20 µCi/µmole ornithine). The reaction was terminated by addition of 0.5 ml 10 mM semicarbazide hydrochloride in 0.4 M ammonium acetate buffer pH 6.5 (2 °C). In experiments where ion exchange separation was tested homogenate, blank medium, stopping mixture, 1 µCi L-[2,3-^{3}H]ornithine, and 0.1 µCi [1,4-^{14}C]putrescine (specific activity: 80–120 mCi/mmol, New England Nuclear) were mixed and used without incubation at 37 °C.

A slurry of 30 g Amberlite CG-50 ion exchange resin (H^+-form, 100–200 mesh, Sigma Chemie GmbH, München/FRG) was prepared for use by 4 washings each with 400 ml 0.2 M ammonium acetate pH 6.5, 1 mg/ml EDTA[4] and filled into glass-wool-plugged pasteur pipettes to give columns of 3 cm height. Each column was washed with 2 ml of 0.2 M ammonium acetate buffer pH 6.5 (without EDTA). The complete enzyme assay mixture was applied to a column. To remove unreacted [^{3}H]ornithine the resin was washed with 12 ml 0.2 M ammonium acetate buffer pH 6.5 (degassed by suction) followed by 2 ml 0.2 M acetic acid. [^{3}H]Putrescine was eluted with 3 ml 8 M formic acid directly into scintillation vials.

Each eluate was mixed with 15 ml of a scintillation cocktail (Aquasol-II, New England Nuclear) and counted in a β-scintillation spectrometer, ^{3}H-counting efficiency 29.5%. Using freshly purified [^{3}H]ornithine blank counting rates were below 500 cpm, i.e., 0.1% of the added ^{3}H-label. The rates increased

Table 1. Influence of pyridoxal phosphate concentrations on enzyme activity

Ornithine [mM]	0.1	0.1	2	2
Pyridoxal phosphate [mM]	0.1	2	2	0.1
Putrescine formed* [nmoles/h/mg protein]	0.96 ± 0.04	0.26 ± 0.02	2.08 ± 0.08	1.86 ± 0.08

* Mean ± SD of 3 experiments. Assay conditions: 45,000 xg supernatant, 2.1 mg protein per ml. 2 mM dithiothreitol, 50 mM sodium phosphate buffer pH 7.4. Incubation time 4 h.

Table 2. Ornithine decarboxylase activity in postnatal rat brain

Postnatal age (days)	2	4	8	16
Brain weight (mg)	307 ± 6	499 ± 19	778 ± 42	1241 ± 45
Putrescine formed* (nmoles/h/mg protein)	1.16 ± 0.11	0.73 ± 0.07	0.37 ± 0.02	0.033 ± 0.004

* Mean ± SD, double estimations from 3 brains. Assay conditions: 10,000 xg supernatants, 3.1–3.6 mg per ml. 0.1 mM ornithine, 0.1 mM pyridoxal phosphate, 2 mM dithiothreitol, 50 mM sodium phosphate buffer pH 7.4. Incubation time 3–4 h.

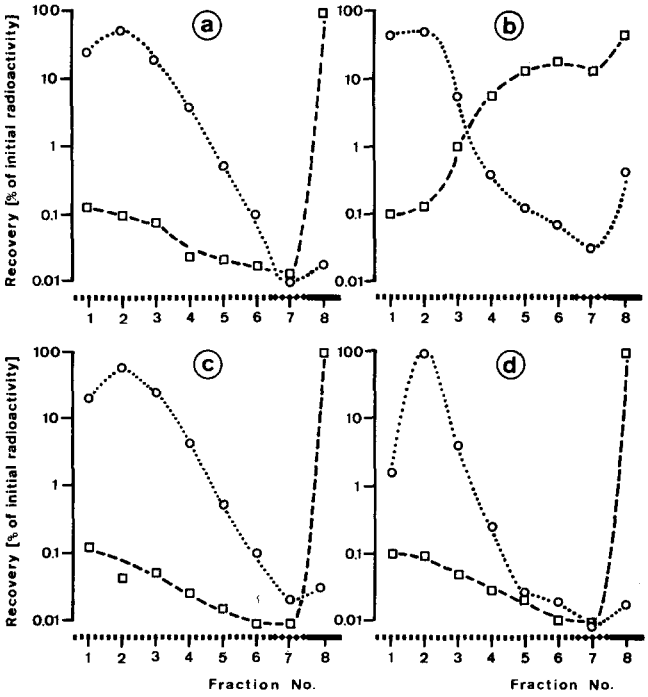

Figure 1. Ion exchange separation of [^{3}H]ornithine and [^{14}C]putrescine. *a*. Separation with 0.2 M ammonium acetate buffer pH 6.5 and Amberlite CG-50 ion exchange resin 100–200 mesh; *b* as with *a*, but with buffer pH 4.5; *c* as with *a*, but in the presence of 0.2 M unlabeled putrescine; *d* as with *a*, but with Bio-Rex 70 ion exchange resin, 200–400 mesh. ○·····○ = [^{3}H]ornithine, □——□ = [^{14}C]putrescine. Fractionation: ▪▪▪▪▪ = 0.2 M ammonium acetate buffer, ▪▪▪▪▪▪▪▪▪▪▪▪▪▪ = 0.2 M acetic acid, ▬▬ = 8 M formic acid. Fractions 1, 8: 3 ml, fractions 2–7: 2 ml.

when purified [^{3}H]ornithine was stored for weeks in a refrigerator.

For the determination of Michaelis constants duplicate aliquots of 45,000 xg supernatants of 4 brains (1.7–2.1 mg protein per ml) were incubated for 4 h at 37 °C with 100, 33, or 30 µM ornithine, in the presence of 100 µM pyridoxal phosphate, or with 10, 3.3, or 2 µM pyridoxal phosphate, in the presence of 100 µM ornithine. Each test tube contained 1 µCi [^{3}H]ornithine. After subtraction of blank values radioactivity data for the 20 and 33 µM ornithine concentrations were calculated to an apparent specific activity of 20 µCi/µmole ornithine, as used in all other assays. All radioactivity data were corrected for elution and scintillation counting efficiencies and subjected to evaluation of the Michaelis constants with the Lineweaver-Burk method[5] using linear regression analysis. The coeffients of correlation, r^2, were greater than 0.99 in any of the regression calculations.

Protein was determined according to Lowry et al.[6] with a bovine serum albumin standard. Biochemicals were purchased from Sigma, buffer salts (analytical grade) from E. Merck AG, Darmstadt/FRG. Buffer solutions were made up with water from a Milli-Q® water purification system (Millipore, Bedford/USA), conductivity < 0.1 µS.

Results and discussion. Maderdrut and Oppenheim[4] recommended a 0.2 M ammonium acetate buffer pH 4.5 for the separation of ornithine and putrescine. Most probably this was a printer's error, as with these conditions ornithine and putrescine were eluted together (fig. 1b). For the purification of [^{3}H]ornithine Maderdrut and Oppenheim[4] had used the same buffer pH 6.5, and indeed with this condition the separation worked reliably (fig. 1a). Addition of 0.2 mM unlabeled putrescine did not influence the separation (fig. 1c). Using Bio-Rex 70 ion exchange resin (200–400 mesh, Bio-Rad, München/FRG), as originally recommended[4], resulted in a somewhat better separation (fig. 1d), but the separation took several hours to complete, instead of less than 30 min with the Amberlite CG-50 resin.

Washing the column with 12 ml 0.2 M ammonium acetate buffer pH 6.5 at room temperature caused bubble formation in the ion exchange resin, resulting in a low elution velocity and deterioration of separation. This was precluded by degassing the buffer with a water pump prior to use. In the original paper[4] putrescine was eluted from the columns with 4 ml 1 M acetic acid. The usual type of liquid scintillation vial of about 20 ml was too small as to hold this liquid volume plus an appropriate volume of scintillation cocktail. 3 ml 8 M formic acid efficiently removed putrescine from the ion exchange resin (fig. 1) and were readily miscible with 15 ml of the scintillation cocktail. The mean recovery of [^{14}C]putrescine after 8 M formic acid elution was 94%. The appearance of ^{14}C-radioactivity in the previous washing steps was attributed to impurities of the crude [^{14}C]putrescine preparation, as in experiments where purified [^{14}]putrescine[4] was used, no corresponding peak was found.

Some characteristics of ornithine decarboxylase were checked with the present method. Each assay was performed fourfold. With blank counting rates of 0.1% of the added ^{3}H-label the detection limit of [^{14}C]putrescine was less than 50 pmoles per assay. Parallel estimations usually differed 5% from one another, at most 15%. Enzyme activity estimations differed less than 10% from one another when aliquots from one tissue sample were assayed on different days. At 37 °C the enzyme reaction proceeded linearly for 12 h with a 45,000 xg supernatant, and for 8 h with a 10,000 xg supernatant (fig. 2). The deviations seen in figure 2 did not originate from the assay, but rather from individual variations of enzyme activities of the investigated four brains. After 8–12 h the yellow color of the pyridoxal phosphate solution disappeared. This was probably due to degradation of the cofactor by microsomal contaminants, as the effect was much more pronounced with the 10,000 xg supernatant (fig. 2).

Michaelis constants of the enzyme were determined with respect to the substrate, ornithine, and the cofactor, pyridoxal phosphate. The Michaelis constant found for ornithine was 83 ± 4 µM. This value exceeded others reported for rat brain (41 or 33 µM[7], and 29 µM[8], respectively). K_m for pyridoxal phosphate was 4.2 ± 0.2 µM, corresponding to a value of 4.7 µM reported for rat liver[9]. No evidence was found for the existence of two different Michaelis constants for pyridoxal phosphate, as had been reported elsewhere[9, 10].

Addition to the assay medium of 2 mM pyridoxal phosphate caused a 73% inhibition of putrescine formation (table 1). This was attributed to a reduction of the concentration of free substrate caused by an increase of Schiff-base formation which

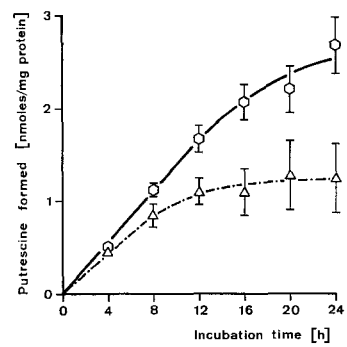

Figure 2. Formation of putrescine with incubation time. Each point represents a mean ± SD from 4 14-day-old brains (double estimations). Incubation temperature: 37 °C. Blank cpm subtracted. ○——○ = 45,000 xg supernatant, △—·—·△ = 10,000 xg supernatant. Protein concentrations of the supernatants: 2.5 mg/ml (45,000 xg) and 3.1 mg/ml (10,000 xg), respectively.

occurs spontaneously with pyridoxal phosphate and ornithine[10]. Ornithine decarboxylase does not accept this Schiff-base as a substrate[10], and preincubation of the assays is required to equilibrate enzyme, free substrate, free cofactor, and Schiff-base. The seeming inhibition of the enzyme by 2 mM pyridoxal phosphate was overcome by concomitant use of 2 mM ornithine (Table 1). The Schiff-base did not interfere with the ion-exchange procedure, as it was destroyed by semicarbazide in the stopping mixture.

Formation of a Schiff-base from ornithine and pyridoxal phosphate will disturb any evaluation of the kinetics of ornithine decarboxylase, as it decreases the concentrations of free substrate and cofactor, respectively, depending on the ratio of the respective concentrations. In the 1/V-1/[S]-diagram a substrate concentration which is in fact smaller than calculated from the weighings must result in a Michaelis constant which is greater than the true value. This effect may be responsible for differences im K_m-values reported for ornithine (e.g., 30–40 µM in the brains of young rats[7,8], 20–460 µM in neonatal mouse brain[11], 60 and 350 µM, respectively, in rat liver[8,9]) as well as for pyridoxal phosphate (e.g., 4.7 µM in rat liver[7], and 10 µM in mouse fibroblasts[10]).

The present method allows the routine performance of up to 100 complete enzyme assays per day. It is especially suitable for the detection of changes of ornithine decarboxylase activity with developmental age or following experimental treatment. As an example, table 2 shows activities of ornithine decarboxylase in the brains of young Wistar rats at different postnatal days. These values are in good accordance with values obtained with the CO_2-trapping method[12].

Acknowledgments. Thanks to Ms Ilona Schlösser for excellent assistance. This work was supported by the Bundesministerium für Forschung und Technologie of the Federal Republic of Germany.

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1 Morris, D.R., and Pardee, A.B., Biochem. biophys. Res. Comm. *20* (1965) 697.
2 Clark, J.L., Analyt. Biochem. *74* (1976) 329.
3 Djurhuus, R., Analyt. Biochem. *113* (1981) 352.
4 Maderdrut, J.L., and Oppenheim, R.W., Neuroscience *3* (1978) 587.
5 Lineweaver, H., and Burk, D., J. Am. chem. Soc. *56* (1934) 658.
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
7 Morris, G., Lau, C., and Slotkin, T., Eur. J. Pharmac. *88* (1983) 177.
8 Butler, S.R., and Schanberg, S.M., Life Sci. *18* (1976) 759.
9 Kitani, T., and Fujisawa, H., J. biol. Chem. *258* (1983) 235.
10 Clark, J.L., and Fuller, J.L., Eur. J. Biochem. *67* (1976) 303.
11 Laitinen, S.I., Laitinen, P.H., Hietala, O.A., Pajunen, A.E.I., and Piha, R.S., Neurochem. Res. *7* (1982) 1477.
12 Anderson, T.R., and Schanberg, S.M., J. Neurochem. *19* (1972) 1471.

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The enhanced induction of metallothionein by zinc, its half-life in the marine fish *Pleuronectes platessa*, and the influence of stress factors on metallothionein levels

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Summary. Intraperitoneal injection of zinc raised levels of a hepatic metallothionein-like species. Assuming that this species was metallothionein (MT) then levels were raised from approximately 20 µg/g to 300 µg/g in 7 days, and levels thereafter remained high for the next 4 weeks. The half-lives of the protein in liver and kidney from starved fish, measured using in vivo incorporation of ^{35}S cysteine at 11 °C, were approximately 27 days and 32 days respectively. The following agents failed to stimulate synthesis of MT in plaice: stress (due to catching), endotoxin, dexamethasone, cortisol and turpentine.
Key words. Metallothionein induction; zinc metallothionein half-life; plaice; stress; fish.

Metallothionein (MT) is a low molecular weight, heat stable, metal binding protein that has been isolated from a variety of vertebrate and invertebrate species[1]. It appears to be ubiquitous in vertebrate tissues and in mammals it is readily inducible by a variety of agents, including the metals copper, cadmium and zinc to which it binds[1,2]. Intraperitoneal (i.p.) injection of cadmium induces synthesis of MT in plaice liver. This MT has been isolated and characterized[3] and its partial amino acid sequence determined[4]: it is similar to mammalian MT. Metallothionein-like proteins have been demonstrated in a number of fish species as a result of metal exposure, and measurement of MT levels in fish has been suggested as a method of monitoring the degree of aquatic metal pollution[5]. Roch et al.[6] have found good correlation between hepatic MT levels in rainbow trout, caught downstream from a copper mine, and the copper level in the water. Seawater-adapted eels have been shown to respond to very high levels of mercury in the water (0.4 µg/g) by synthesizing MT[7]. Mercury was not, however, associated with MT in the estuarine fish, killifish, exposed for 4 weeks to 0.02 µg/g mercury[8]. In the case of freshwater trout, while water-borne zinc does induce synthesis of hepatic MT, cadmium alone does not[9,10]. In other freshwater species cadmium does induce synthesis of MT[11].

In mammals, a variety of non-metal agents, mostly associated with stress (including environmental stress, injury, bacterial infection, liver damage and glucocorticoid hormones) are also known to induce synthesis of MT. In order to investigate the possibility that stress could stimulate MT synthesis in fish we have investigated the effect of stress due to catching and the action of the glucocorticoid hormone cortisol and its synthetic analogue dexamethasone. In order to mimic bacterial infection and injury we have examined the effect of endotoxin and turpentine injection. For MT levels to be a useful parameter to study in fish, we need to know not only which factors are capable of stimulating synthesis of MT but also the half-life of the MT so formed. This paper reports the results of such studies.

Materials and methods. Animals. Mature plaice (200–400 g), seine-netted off the Aberdeenshire coast, were maintained unfed in a circulating seawater aquarium at 11 °C.

Induction experiments and sampling of tissue. Zinc (as sulphate) in 1.1% saline was injected i.p. as 5 successive, daily doses of 2