Subcellular Localization of Ornithine Decarboxylase in Liver of Control and Growth-Hormone-Treated Rats

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1. Ornithine-2-oxo acid aminotransferase activity was inhibited by amino-oxyacetate (10^{-5} M). This permitted the measurement of ornithine decarboxylase in the presence of mitochondria by using the $^{14}\text{CO}_2$ -trapping technique. 2. Subcellular fractionation of rat liver by differential centrifugation, followed by the assay of ornithine decarboxylase in the presence of amino oxyacetate and of marker enzymes for each fraction, demonstrated that ornithine decarboxylase was located in the cytosol. 3. The greatly increased ornithine decarboxylase activity observed after growth-hormone administration was also found to be localized in the cytosol. 4. The K_m of ornithine decarboxylase from rat liver for ornithine was $28\text{ }\mu\text{M}$. Administration of growth hormone 4 h before death did not affect the apparent affinity of ornithine decarboxylase for ornithine.

A dramatic increase in the specific activity of ornithine decarboxylase (EC 4.1.1.17) is one of the earliest effects of a growth stimulus on rat liver (Fausto, 1971). This enzyme catalyses the first (thought to be the rate-limiting) step in the synthesis of polyamines (Raina *et al.*, 1966). The intracellular localization of ornithine decarboxylase in rat liver is not well established, although several workers, including Janne *et al.* (1968), showed it to be present in the 100000g supernatant. Knowledge of the localization of ornithine decarboxylase could be of value both in determining the mechanism of its activation and in postulating roles for its eventual products, the polyamines.

Ornithine decarboxylase activity may be rapidly and sensitively measured by following the release of $^{14}\text{CO}_2$ from [1- ^{14}C]ornithine (Pegg & Williams-Ashman, 1968), but in some circumstances this carbon could also be removed by the sequential action of the mitochondrial enzymes ornithine-2-oxo acid aminotransferase (EC 2.6.1.13), 1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12), either glutamate dehydrogenase or aspartate aminotransferase and 2-oxoglutarate dehydrogenase. To assay ornithine decarboxylase in the presence of mitochondria, it is necessary to ensure that this latter pathway is not active.

In the present study we report on the use of various inhibitors to block this pathway and their effect on ornithine decarboxylase activity. We also describe experiments on the subcellular localization of ornithine decarboxylase in rat liver, made possible by the use of one of these inhibitors, namely amino-oxyacetate. Subcellular localization was similarly investigated after injection *in vivo* of growth hormone 4 h before death, a well-known stimulus to induce ornithine decarboxylase activity (Janne *et al.*, 1968). A preliminary report of this work has already appeared (Brosnan & Murphy, 1975).

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Materials and Methods

Animals

Male rats of the Sprague-Dawley strain, weighing approx. 90–100g, were obtained from the Animal Unit, Faculty of Medicine, Memorial University of Newfoundland.

Growth hormone was dissolved in 0.15M-NaCl (approx. pH 8–9) to give a concentration of 2mg/ml. This solution was injected subcutaneously at a final dose of 2mg of growth hormone/100g body wt. Control rats received a proportional subcutaneous injection of saline. Animals were killed 4 h after injection. To avoid diurnal variation in response, injections were always carried out between 9.00 and 10.00h.

Chemicals

Growth hormone (NIH-GH-B16 Bovine) was a gift of the Endocrinology Study Section, NIAMD, Bethesda, MD, U.S.A. L-[1- ^{14}C]Ornithine (7.7mCi/mmol) and NCS (Nuclear-Chicago scintillant) were purchased from Amersham-Searle Corp., Oakville, Ont., Canada. 2-Oxo[1- ^{14}C]glutaric acid (53mCi/mmol), DL-[1- ^{14}C]glutamic acid (53mCi/mmol),

[1,4-¹⁴C]putrescine (15mCi/mmol), DL-[5-¹⁴C]ornithine (8mCi/mmol) and Omnifluor were obtained from New England Nuclear, Dorval, Que., Canada. Diphenylamine (Analar grade) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

All other chemicals were of the highest analytical grade available and were obtained from either Sigma Chemical Co., St. Louis, MO, U.S.A. or Fisher Scientific, Montreal, Que., Canada.

Centre wells and rubber stoppers used in ¹⁴CO₂ trapping were purchased from Kontes Glass Company, Vineland, NJ, U.S.A.

Tissue homogenization and isolation of subcellular fractions

Rats were killed by cervical dislocation. Livers were quickly removed and placed in approximately ten vol. of isolation medium (0.33M-sucrose, 0.005M-MgCl₂, 0.01M-Tris, pH7.3). The liver was chopped with scissors, then homogenized in a smooth-glass Potter-Elvehjem homogenizer at approximately 500rev./min by five to six strokes of a motor driven loose-fitting Teflon pestle (clearance 0.3mm). After filtration through two layers of cheesecloth, the homogenate was fractionated by differential centrifugation into a nuclear fraction (N), a mitochondrial fraction (M), a lysosomal fraction (L), a microsomal fraction (P), and a soluble fraction (S). The method proposed by de Duve *et al.* (1955) and modified by Sedgwick and Hubscher (1965) was closely followed. All operations were carried out at 0–4°C.

Enzyme assays

Succinate- and NADPH-cytochrome *c* reductase activities were assayed as described by Sottocasa *et al.* (1967); β -glucuronidase as described by Gianetto and deDuve (1955); lactate dehydrogenase as described by Morrison *et al.* (1966); ornithine-2-oxo acid aminotransferase as described by Pegg *et al.* (1970).

Assay for ornithine decarboxylase

Ornithine decarboxylase activity was measured by following the release of ¹⁴CO₂ from L-[1-¹⁴C]-ornithine, by using a modification of the method of Pegg & Williams-Ashman (1968). Reaction mixtures contained 0.1 μ mol of pyridoxal phosphate, approx. 4mg of liver protein, 7.5 μ mol of Tris (pH7.3), 0.15 μ mol of EDTA, 0.1 μ mol of L-ornithine (1 μ Ci/ μ mol) in a final volume of 2.0ml. When inhibitors were used, mixtures were incubated at 37°C for 10min before the reaction was initiated by addition of labelled substrate. The reactions were carried out in 25ml Erlenmeyer flasks equipped with tight-fitting rubber stoppers; each flask carried a polypropylene centre well, in which was placed 0.2ml of NCS and a small piece of filter paper. After 60min of incubation, the reaction was terminated by addition of

0.2ml of 2.5M-H₂SO₄, injected through the rubber stopper. Shaking was then continued for 60min to collect ¹⁴CO₂. Centre wells were quickly placed in toluene containing 4g of Omnifluor/litre, and radioactivity was measured by using a Beckman LS-233 liquid-scintillation counter. Corrections for quenching were made by using the channels-ratio method. Mixtures containing acid at zero time, or those containing no enzyme, were used as blanks.

In one series of experiments, ornithine decarboxylase activity was assayed by following the production of [¹⁴C]putrescine from DL-[5-¹⁴C]ornithine. The incubation was carried out as above in polyethylene tubes and was stopped by addition of 0.1 vol. of 60% (w/v) perchloric acid. After centrifugation to remove precipitated protein, the supernatant was adjusted to pH2 and was applied to a column (41 mm $\times$ 12 mm) of Beckman PA-35 resin in a Beckman 121 amino acid analyser. Putrescine was eluted from the column at 55°C by using sodium citrate buffer (pH5.42; 2.35M-Na⁺) at a flow rate of 90ml/h. Fractions (11.0 ml) were collected and their radioactivity measured by adding a portion (2.5ml) to Aquasol and counting in a Beckman LS-233 liquid-scintillation spectrophotometer. [1,4-¹⁴C]Putrescine was used as standard.

In the experiments to determine the apparent *K_m* of ornithine decarboxylase for ornithine, the assay conditions were identical with those described above, but the final ornithine concentration was varied from 0.043 to 0.578mM. The soluble fraction was used for these assays.

For all enzyme assays, linear progress curves were observed, and enzyme activity was linear with protein concentration.

DNA and protein determination

DNA was extracted into hot HClO₄, as described by Schneider (1945), and the concentration was determined with diphenylamine reagent (Burton, 1956) with calf thymus DNA as standard. Protein was determined by the biuret method (Gornall *et al.*, 1949) after solubilization of membranous material with deoxycholate (Jacobs *et al.*, 1956). Bovine serum albumin was used as standard.

Results

Modifications of the ornithine decarboxylase assay

Since ornithine decarboxylase catalyses the formation of 1 mol of CO₂ and 1 mol of putrescine from 1 mol of ornithine, it should be possible to assay this enzyme by measuring either product. In practice this enzyme usually is measured by the production of ¹⁴CO₂ from [1-¹⁴C]ornithine, as this measurement is simple, reproducible and extremely sensitive (Pegg & Williams-Ashman, 1968). Alternatively the formation of [¹⁴C]putrescine from [5-¹⁴C]ornithine can be assessed after separation on an amino acid analyser

(see the Materials and Methods section). This method requires far more material for accurate measurement and is undesirable as a routine procedure. It is, however, essential to show that a 1:1 stoichiometry between CO_2 and putrescine formation does exist before the CO_2 -trapping technique can be used as a routine (Pegg & Williams-Ashman, 1968).

A trial fractionation indicated that ornithine decarboxylase activity followed both the cytoplasmic marker, lactate dehydrogenase, and the mitochondrial marker, succinate-cytochrome *c* reductase, if the $^{14}\text{CO}_2$ -trapping method was used. In the cytosol, a stoichiometric relationship was observed between $^{14}\text{CO}_2$ and [^{14}C]putrescine produced ($\text{CO}_2/\text{putrescine} = 1.0$); the $^{14}\text{CO}_2$ -trapping method appeared to be a true measure of ornithine decarboxylase activity. However, in the mitochondrial fraction, formation of putrescine was not equivalent to the CO_2 released ($\text{CO}_2/\text{putrescine} = 43.8:1$).

In hepatic mitochondria, ornithine could be converted into citrulline by ornithine transcarbamylase or to glutamic γ -semialdehyde by L-ornithine-2-oxo acid amino transferase (EC 2.6.1.13). With ornithine transcarbamylase the product citrulline is transported out into the cytoplasm (Gamble & Lehninger, 1973), and by this pathway there would be no release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine. In the case of ornithine-2-oxo acid aminotransferase, the product glutamic γ -semialdehyde can be metabolized to glutamate by the action of 1-pyrroline-5-carboxylate dehydrogenase (Johnson & Strecker, 1962). Glutamate could then be metabolized to 2-oxoglutarate by either glutamate dehydrogenase or aspartate aminotransferase. L-[1- ^{14}C]Ornithine may thus be converted into 2-oxo[1- ^{14}C]glutarate, and the ^{14}C then released oxidized to $^{14}\text{CO}_2$.

To determine what percentage of the ornithine was metabolized via the glutamate-2-oxoglutarate pathway, a number of inhibitors were used. The inhibitor rotenone was used to inhibit any NAD-dependent steps (Ernster *et al.*, 1963). Arsenite was used to inhibit 2-oxoglutarate dehydrogenase (Fluharty & Sanadi, 1960). Amino-oxyacetate was reported by

Rognstad & Clark (1974) to inhibit aspartate aminotransferase in lower concentration (0.2 mM) than is required to inhibit most other aminotransferases and was used initially to inhibit this enzyme. These results are shown in Table 1.

Rotenone inhibited $^{14}\text{CO}_2$ production by mitochondria by approx. 87–95% when L-[1- ^{14}C]ornithine or DL-[1- ^{14}C]glutamate was used and by approx. 75% with 2-oxo[1- ^{14}C]glutarate as substrate. On the other hand, rotenone was without effect on cytoplasmic $^{14}\text{CO}_2$ production. These results alone would seem to indicate that at least 90% of the $^{14}\text{CO}_2$ evolved from L-[1- ^{14}C]ornithine by mitochondria was not removed by ornithine decarboxylase, but rather by the proposed pathway via glutamate.

When arsenite was added to a mitochondrial assay mixture containing either 2-oxo[1- ^{14}C]glutarate or DL-[1- ^{14}C]glutamate, an inhibition of $^{14}\text{CO}_2$ release of approx. 90% was observed; with L-[1- ^{14}C]ornithine, however, an inhibition of approx. 50% was observed. There was no effect of arsenite on cytoplasmic ornithine decarboxylase activity. These results indicate that in mitochondrial assays, only about one-half the CO_2 removed from ornithine was through the action of 2-oxoglutarate dehydrogenase and that the other half was removed by some other unidentified pathway. The additional action of rotenone in this case could be to decrease the supply of 2-oxoglutarate required by ornithine-2-oxo acid aminotransferase.

In the presence of amino-oxyacetate, $^{14}\text{CO}_2$ liberation from DL-[1- ^{14}C]glutamate was inhibited by approx. 27%. With L-[1- ^{14}C]ornithine as substrate, 0.2 mM-amino-oxyacetate almost completely inhibited $^{14}\text{CO}_2$ evolution.

These results indicate that some enzyme, involved in converting ornithine into glutamate, is much more sensitive to amino-oxyacetate than is aspartate aminotransferase. Since amino-oxyacetate is known to inhibit pyridoxal phosphate-requiring enzymes, especially aminotransferases, it appeared likely that the sensitive enzyme was ornithine aminotransferase.

Fig. 1 shows that 0.01 mM-amino-oxyacetate

Table 1. Effects of inhibitors on mitochondrial $^{14}\text{CO}_2$ evolution by using labelled ornithine, glutamate and 2-oxoglutarate as substrates

Incubation conditions were those described for ornithine decarboxylase assay, except that the radioactive substrate was varied as indicated. Assays were preincubated for 10 min in the presence of inhibitor before the addition of substrate. To assess non-enzymic release of $^{14}\text{CO}_2$, acid was added to selected vials, either with or without inhibitor, before initiation of the reaction with substrate. These values were low, and were subtracted from all experimental results.

1- ^{14}C -labelled substrate (1 $\mu\text{Ci}/\mu\text{mol}$)	Inhibitor ...	Inhibition (%)		
		Rotenone (0.1 mM)	Arsenite (1.0 mM)	Amino-oxyacetate (0.2 mM)
L-Ornithine (0.05 mM)		87.2	44.3	97.9
DL-Glutamate (0.125 mM)		94.7	88.5	27.3
2-Oxoglutarate (0.125 mM)		75.5	89.2	12.1

depressed mitochondrial ornithine-2-oxo acid aminotransferase activity by 75%, and 0.2 mM gave complete inhibition. Mitochondrial $^{14}\text{CO}_2$ evolution was almost completely inhibited at 0.01 mM-amino-oxyacetate (99%), whereas the cytosol $^{14}\text{CO}_2$ evolution was unaffected at 0.01 mM.

Therefore it appeared that the use of 0.01 mM-

Fig. 1. Effect of amino-oxyacetate on rat liver ornithine decarboxylase activity (cytosol or mitochondria) and on mitochondrial ornithine transaminase activity

Assays were preincubated for 10 min in the presence of amino-oxyacetate. Reactions were initiated by the addition of ornithine, as described in the Materials and Methods section. ●, Ornithine decarboxylation, rat liver cytosol; ○, ornithine decarboxylation, rat liver mitochondria; △, ornithine transaminase, rat liver mitochondria.

amino-oxyacetate in all ornithine decarboxylase assays would prevent the artifacts due to the oxidative metabolism of ornithine via ornithine-2-oxo acid aminotransferase, without seriously affecting the measurement of ornithine decarboxylase activity.

Subcellular localization of ornithine decarboxylase in livers of control and growth-hormone-treated rats

In fractionation studies the recoveries of enzymes, DNA and protein generally ranged from 80 to 110%, with the exception of ornithine decarboxylase, which gave a recovery of approx. 100–200% (Table 2). The reason for such a discrepancy remains unclear. It may be that there is an ornithine decarboxylase inhibitor in the crude homogenate which is not present in the cytosol. A deviation from linearity was sometimes observed with increasing volume of homogenate, which would lend credence to this interpretation.

The distribution of the marker enzymes succinate-cytochrome *c* reductase (mitochondria), β -glucuronidase (lysosomes), NADPH-cytochrome *c* reductase (microsomal fraction) and lactate dehydrogenase (cytosol) and of DNA (nuclei) was similar to that found by other investigators (de Duve *et al.*, 1955; Kalra & Brosnan, 1973).

Table 2 shows that the subcellular distribution of ornithine decarboxylase in liver from control rats was similar to that of the cytosolic marker enzyme, lactate dehydrogenase. This would indicate that ornithine decarboxylase is largely in the cytosol of rat liver cells.

Table 2. Specific activities and recovery of ornithine decarboxylase, marker enzymes, DNA and protein from subcellular fractions of livers of control rats

Specific activities of marker enzymes are given as nmol of product formed/min per mg of protein. Specific activity of ornithine decarboxylase is given as d.p.m./60 min per mg of protein (2000 d.p.m.=1 nmol). Ornithine decarboxylase assays were in the presence of 0.01 mM-amino oxyacetate. DNA is expressed as $\mu\text{g}/\text{mg}$ of protein and protein as mg/g wet wt. of liver. All values are presented as means $\pm$ S.D. Abbreviations: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, cytosolic fraction. Numbers in parentheses refer to the percentage of total recovered activity in each fraction. ND, not detectable.

Marker	No. of expts.	Recovery (%)	Specific activity				
			N	M	L	P	S
Protein	3	101.4 $\pm$ 3.1	35.3 $\pm$ 2.7	42.0 $\pm$ 5.5	14.8 $\pm$ 7.9	5.6 $\pm$ 1.1	67.8 $\pm$ 10.1
DNA	4	90.2 $\pm$ 19.4	21.0 $\pm$ 3.6 (78.6)	2.1 $\pm$ 0.5 (9.3)	1.8 $\pm$ 1.3 (2.8)	1.7 $\pm$ 1.7 (0.7)	1.2 $\pm$ 1.1 (8.6)
Succinate-cytochrome <i>c</i> reductase	4	84.7 $\pm$ 12.9	0.012 $\pm$ 0.005 (20.5)	0.039 $\pm$ 0.014 (79.5)	ND (0)	ND (0)	ND (0)
β -Glucuronidase	3	92.4 $\pm$ 23.5	1.19 $\pm$ 0.49 (16.5)	2.72 $\pm$ 0.28 (45.0)	3.52 $\pm$ 0.19 (20.5)	0.29 $\pm$ 0.50 (0.6)	0.65 $\pm$ 0.71 (17.4)
NADPH-cytochrome <i>c</i> reductase	4	73.2 $\pm$ 14.9	0.014 $\pm$ 0.007 (38.0)	0.017 $\pm$ 0.010 (13.8)	0.041 $\pm$ 0.027 (28.4)	0.057 $\pm$ 0.033 (19.7)	ND (0)
Lactate dehydrogenase	4	97.1 $\pm$ 18.9	0.03 $\pm$ 0.03 (5.3)	0.03 $\pm$ 0.03 (2.1)	0.03 $\pm$ 0.04 (1.5)	0.47 $\pm$ 0.16 (2.7)	2.47 $\pm$ 0.39 (88.4)
Ornithine decarboxylase	4	151.0 $\pm$ 52.2	156 $\pm$ 182 (16.0)	171 $\pm$ 110 (20.9)	20 $\pm$ 34 (0.9)	54 $\pm$ 76 (0.9)	312 $\pm$ 170 (61.4)

Table 3. Specific activities and recovery of ornithine decarboxylase, marker enzyme, DNA and protein from subcellular fractions of livers of growth-hormone-treated rats

Specific activities of marker enzymes are given as nmol of product formed/min per mg of protein. Specific activity of ornithine decarboxylase is given as d.p.m./60 min/mg of protein (2000 d.p.m. = 1 nmol). Ornithine decarboxylase assays were in the presence of 0.01 mM-amino-oxyacetate. DNA is expressed as $\mu\text{g}/\text{mg}$ of protein and protein as mg/g wet wt. of liver. Values are as means $\pm$ s.d. Abbreviations: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, cytosolic fraction. Numbers in parentheses refer to the percentage of total recovered activity in each fraction. ND, not detectable.

Marker	No. of expts.	Recovery (%)	Specific activity				
			N	M	L	P	S
Protein	4	97.4 $\pm$ 10.6	36.5 $\pm$ 17.1	35.3 $\pm$ 7.7	14.4 $\pm$ 7.5	10.6 $\pm$ 4.6	66.3 $\pm$ 13.9
DNA	4	90.6 $\pm$ 12.6	16.0 $\pm$ 2.9 (65.5)	2.6 $\pm$ 0.9 (8.9)	3.2 $\pm$ 1.2 (5.9)	2.7 $\pm$ 0.4 (3.3)	2.1 $\pm$ 1.0 (16.4)
Succinate-cytochrome c reductase	4	96.2 $\pm$ 18.3	0.010 $\pm$ 0.005 (7.5)	0.047 $\pm$ 0.023 (92.5)	ND* (0)	ND (0)	ND (0)
β -Glucuronidase	3	113.1 $\pm$ 15.2	0.70 $\pm$ 0.10 (7.5)	4.69 $\pm$ 2.03 (48.8)	6.13 $\pm$ 1.29 (26.0)	1.58 $\pm$ 1.76 (4.9)	0.65 $\pm$ 0.67 (12.7)
NADPH-cytochrome c reductase	4	78.9 $\pm$ 21.0	0.008 $\pm$ 0.002 (10.2)	0.015 $\pm$ 0.003 (34.7)	0.016 $\pm$ 0.011 (26.9)	0.036 $\pm$ 0.007 (28.2)	ND (0)
Lactate dehydrogenase	4	93.3 $\pm$ 16.1	0.19 $\pm$ 0.006 (8.0)	0.20 $\pm$ 0.18 (3.7)	0.04 $\pm$ 0.05 (1.1)	0.32 $\pm$ 0.16 (1.3)	1.93 $\pm$ 0.47 (85.8)
Ornithine decarboxylase	4	190.5 $\pm$ 52.5	98 $\pm$ 86 (2.1)	150 $\pm$ 164 (3.1)	170 $\pm$ 132 (1.4)	198 $\pm$ 135 (1.2)	2405 $\pm$ 858 (92.2)

Fig. 2. Double-reciprocal plot of the velocity of $^{14}\text{CO}_2$ release against L-ornithine concentration

Soluble fraction (approx. 7 mg of protein) was incubated with various concentrations of L-[1- ^{14}C]ornithine ($1 \mu\text{Ci}/\mu\text{mol}$) under the conditions described in the Materials and Methods section. The endogenous concentration of ornithine was $36 \mu\text{M}$ for control supernatant and $35 \mu\text{M}$ for supernatant from growth-hormone-treated rats. Curves were fitted by least-squares analysis. Mixtures containing acid at zero time were used to correct for non-enzymic CO_2 formation at each concentration of ornithine used. Δ , Cytosol from liver of control rat; $\circ$, cytosol from liver of growth-hormone-treated rat.

Table 3 shows the subcellular distribution of the marker enzymes, DNA and ornithine decarboxylase in liver from growth-hormone-treated rats. The

striking increase in ornithine decarboxylase activity was entirely due to increased activity in the cytosol fraction.

Tables 2 and 3 list mean specific activities for the enzymes used in the present study, from control and growth-hormone-treated rats respectively. At 4 h after growth-hormone injection, the specific activity of these marker enzymes remained unchanged. Growth hormone did, however, markedly increase the specific activity of soluble ornithine decarboxylase relative to that in controls.

K_m of ornithine decarboxylase for ornithine

The increased activity of ornithine decarboxylase after growth-hormone injection could be due to increased production of the enzyme, to production of a more active isoenzyme of ornithine decarboxylase, or to modification of the existing enzyme. The latter two possibilities might be expected to yield enzymes with kinetic characteristics different from those seen in the enzyme from control rats. Therefore with the cytosol fraction, the K_m for ornithine was determined in control and growth-hormone-treated rats. Fig. 2 shows that the extracts from livers of control and growth-hormone-treated rats displayed an identical apparent K_m (0.028 mM) for ornithine.

Discussion

The present study was initiated to determine the subcellular localization of hepatic ornithine decarboxylase. It became evident that the generally used radioactive assay for ornithine decarboxylase was subject to error, owing to interference from competing

enzymes present in the mitochondrial fraction. The present experiments indicated that this pathway in liver was approx. 3–30-fold more active than ornithine decarboxylase itself, when measured under standard assay conditions for ornithine decarboxylase. The initial enzyme in this pathway appears to be ornithine-2-oxo acid aminotransferase. It is evident that in any assay of ornithine decarboxylase by the CO_2 -trapping technique with any preparation that includes mitochondria, the results will be a measure of both ornithine decarboxylase activity and the activity of this second pathway.

It is also possible that severe damage to mitochondria could result in the release of these enzymes, all of which are thought to be soluble, into the cytosol fraction. Inclusion of this second pathway as 'ornithine decarboxylase activity' would lead to misinterpretation of the response of ornithine decarboxylase to various stimuli. There is no reason to believe that these two pathways should respond to stimuli in a similar fashion; in fact, we showed that injection of growth hormone into rats does not affect the activity of ornithine-2-oxo acid aminotransferase (Murphy & Brosnan, 1975), whereas the activity of ornithine decarboxylase in these same animals is markedly stimulated. Thus it is extremely important that ornithine-2-oxo acid aminotransferase either be removed completely or inhibited before ornithine decarboxylase is measured by using the $^{14}\text{CO}_2$ -trapping technique. Other workers have been aware of this particular difficulty (see, e.g., Janne & Williams-Ashman, 1971), but hitherto no satisfactory solution had been proposed.

We showed that it is possible to inhibit this pathway completely by the use of amino-oxyacetate at 0.01 mM, a concentration of inhibitor which consistently showed no effect on the activity of ornithine decarboxylase.

It is noteworthy that with 0.01 mM-amino-oxyacetate, mitochondrial ornithine-2-oxo-acid aminotransferase is inhibited by about 75%, whereas the mitochondrial $^{14}\text{CO}_2$ evolution is inhibited by 99%. This discrepancy could be for one of several reasons. The amino-oxyacetate apparently inhibited $^{14}\text{CO}_2$ evolution by blocking aspartate aminotransferase as well as ornithine-2-oxo acid aminotransferase (Table 1, column 3). The difference in conditions between ornithine-2-oxo acid aminotransferase and $^{14}\text{CO}_2$ evolution assays might also be responsible for the difference in response of the two assays to amino-oxyacetate. The ornithine-2-oxo acid aminotransferase assay medium is iso-osmotic, whereas the $^{14}\text{CO}_2$ evolution-assay medium is hypo-osmotic. The concentrations of substrates were also much higher in the ornithine-2-oxo acid aminotransferase assay than in the decarboxylation assay.

Previous studies on the localization of ornithine decarboxylase using rat liver (Ono *et al.*, 1972) and

rat prostate (Pegg and Williams-Ashman, 1968) involved preparation of highly purified mitochondrial, nuclear, microsomal and cytosolic fractions; other tissue components were discarded. The purity and intactness of organelles were not monitored by the use of marker enzymes. No attempts were made to recover 100% of the homogenate enzyme activity and thus to detect possible inactivation or loss of enzyme during preparation of fractions, as suggested by de Duve (1967). In each of the aforementioned studies, ornithine decarboxylase activity, as measured by the CO_2 -trapping technique, appeared only in the cytosolic fraction. Such studies would not rule out the presence of the enzyme in discarded cell components or the loss or inactivation of ornithine decarboxylase during preparation of organelles.

By including amino-oxyacetate in the incubation medium to inhibit ornithine aminotransferase, we were able to carry out a total subcellular fractionation on rat liver. These results would indicate that in this tissue ornithine decarboxylase is in fact localized largely in the cytosol.

Since we have shown that ornithine decarboxylase is a cytoplasmic enzyme, it would appear that putrescine is synthesized in this cellular compartment, and it will be necessary to take this finding into account when considering the further regulation of polyamine biosynthesis and the possible effects of polyamines on nucleic acid metabolism (Kremzner, 1970).

Weber *et al.* (1972) and Kremzner (1970) have independently suggested that ornithine-2-oxo acid aminotransferase activity could regulate the activity of ornithine decarboxylase. However, we have shown that ornithine decarboxylase is located in the cytosol, whereas ornithine-2-oxo acid aminotransferase is known to be a mitochondrial enzyme (Peraino & Pitot, 1963). If there is a mechanism by which the aminotransferase can exert control over decarboxylase activity by regulating cytosolic ornithine concentrations, it must also include the process(es) governing mitochondrial ornithine transport.

It is known that the subcellular locus of a number of enzymes may alter during dietary manipulation (e.g., hexokinase in intestine; Jones & Mayer, 1973) or after various stimuli (e.g., tyrosine aminotransferase; Sapico *et al.*, 1974). The present studies, however, indicate that the increased ornithine decarboxylase activity is located exclusively in the cytosol of rat liver after animals have been injected with growth hormone. Thus growth hormone does not cause appearance of new ornithine decarboxylase activity in a second compartment or translocation of it to another organelle.

We have also shown that there is no change in the apparent K_m of the enzyme for ornithine after growth-hormone injection. Assays were carried out in the presence of excess pyridoxal phosphate, so that changes in the affinity of the enzyme for this cofactor

would not be reflected in the activity of the enzyme. These data would indicate that there is actually an increase in the concentration or activity ($V_{\max}$) of ornithine decarboxylase under these conditions.

Since growth hormone is thought to interact with the plasma membrane without actually entering the cell (Lesniak *et al.*, 1974), it is unlikely that this hormone would be able to interact directly with ornithine decarboxylase to modify its activity. Thus it appears that there must be some step(s) intermediate between growth-hormone interaction with the cell and ornithine decarboxylase activation and/or synthesis.

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