APPARENT INDUCTION, KINETIC AND PHYSICAL PROPERTIES OF THE MULTIPLE SPECIES OF ORNITHINE DECARBOXYLASE, FORMS A AND B, IN THE KIDNEY AND LIVER OF HORMONE-TREATED RAT.

by

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ABSTRACT

Ornithine decarboxylase occurs in the kidney and liver of control and hormone treated rats as two ionic forms, designated ODC A and B in order of elution by DEAE-Sepharose chromatography. These studies were designed to better characterize the multiple forms of ODC and to investigate the functions and possible origins of ODC A and B in growth hormone, prolactin and/or dexamethasone stimulated tissue. The two species of ODC existed in separate and distinct proportions in control rat kidney and liver. Within the kidney and liver of growth hormone, prolactin and dexamethasone stimulated rats, the proportions of ODC A and B were altered, reflecting the increased half-life of ODC B. In growth hormone-stimulated animals, form B had an increased half-life of 25 minutes in the kidney and 60 minutes in the liver. Hormone stimulated rats were also given either LiCl, actinomycin D or putrescine, which decreased total ODC activity. The ODC A:B ratio was altered in some instances, but only within the kidney. In these cases, the enhanced proportion and most likely the stability of the B form of ODC was inhibited or removed, as in the response of ODC to LiCl in the stimulated kidney. Within the liver, there was no alteration of the A:B ratio. Possibly, the liver uses different signalling or regulatory mechanisms. ODC B did not have any kinetic advantage over A in either the kidney or liver of growth hormone-treated rats, as K_m^{orn} and V_{max} values did not differ significantly

between forms A and B in either case. The charge separation of ODC A and B from the kidney and liver was shown to be dependent on the state of phosphorylation based on the evidence that prior treatment of supernatant containing ODC with alkaline phosphatase resulted in the elution of active ODC A only by DEAE chromatography. Finally, ODC A and B did not differ in molecular weight (~48kD) as seen on an SDS-PAGE gel. In growth hormone-treated rats, ODC showed complex responses in terms of activity or kinetics. Not only did the ratio of liver ODC A and B not respond to lithium, actinomycin or putrescine treatment, but there was evidence of multiple species of ODC within forms A and B having different kinetic and half-life properties.

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ABBREVIATIONS

ADOMET	S-adenosyl-L-methionine
ADOMETDC	S-adenosyl-L-methionine decarboxylase
AlkPase	alkaline phosphatase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
DEAE	diethylaminoethyl
DEX	dexamethasone
DFMO	alpha-difluormethylornithine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
GH	growth hormone
Hepes	[4-(2-hydroxyethyl0]-piperazine ethane sulfonic acid
IP	intraperitoneally
K_m^{orn}	substrate binding constant for ornithine
KCL	potassium chloride
kD	kilodaltons
KH_2PO_4	potassium phosphate, monobasic

l	liters
LiCl	lithium chloride
mCi	millicurie
mg	milligram
ml	milliliter
mM	millimolar
mmol	millimole
mRNA	messenger ribonucleic acid
MTA	5'-methylthioadenosine
NaCl	sodium chloride
NaN_3	sodium azide
NBT	nitro blue tetrazolium
Na_2HPO_4	sodium phosphate, dibasic
nmol	nanomole
ODC	ornithine decarboxylase
ORN	ornithine
PAGE	polyacrylamide gel electrophoresis
PLP	pyridoxal-5'-phosphate
PMSF	phenylmethylsulfonylfluoride
PO	polyamine oxidase
Poly A	polyadenylated
pmol	picomole
PRL	prolactin
PUT	putrescine

rRNA	ribosomal ribonucleic acid
SAT	spermine/spermidine-5'-acetyl transferase
SDS	sodium dodecyl sulphate
SPD	spermidine
SPM	spermine
TCA	trichloroacetic acid
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
TPA	12-O-tetradecanoylphorbol 13-acetate
tRNA	transfer ribonucleic acid
uCi	microcuries
ug	microgram
ul	microliter
uM	micromolar

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"That which does not kill you, makes you stronger"

Nietchze

To Mom and Jen,

for making this possible.

INTRODUCTION

GENERAL INTRODUCTION

Ornithine Decarboxylase (L-ornithine carboxyl-lyase, E.C. 4.1.1.17, ODC) is the initial and rate limiting enzyme in the polyamine biosynthetic pathway (1-4). The products of the pathway, putrescine (PUT), spermidine (SPD), and spermine (SPM) are normal constituents of prokaryotic and eukaryotic cells. At physiological pH, PUT, SPD and SPM are protonated and possess 2, 3, and 4 positive charges respectively and thus are known as the organic cations of the cell. Polyamines have a variety of physiological and biochemical effects and have been shown to play a role in membrane function, proliferation and differentiation (5, 7). By virtue of their charge, they can complex with nucleic acids to provide conformational stability. Spermine has been shown to bind to the DNA helix, spanning the major or minor groove and stabilizing it (8). There has also been evidence of PUT, SPD and/or SPM binding to and stabilizing the stem-loop structures in rRNA and mRNA; further, tRNA conformation is stabilized through binding of polyamines to specific sites (9). Additionally, they can influence the conformation of structural proteins as well as the activity and location of enzymes. Spermine and spermidine were shown to induce the

polymerization of spectrin and dramatically decreased the lateral diffusions of transmembrane glycoproteins (10). Polyamines can also affect the activity of certain enzymes involved in phosphorylation and dephosphorylation, such as protein kinase C, casein kinase II and alkaline phosphatase. Finally, by association with phospholipids, polyamines can affect the cellular plasma membrane and influence the action of enzymes in the endoplasmic reticulum (11). Polyamines affect the activity of membrane bound cholinesterase and are shown to decrease the hydrolytic activity of phospholipases A_2 and C on mitochondrial membranes (10). Submillimolar concentrations of polyamines can stabilize spheroplasts and mitochondria against osmotic shock and induce the aggregation of subcellular organelles (11). It has been well documented that polyamines are essential for cell growth (12-14). Specifically, putrescine has been shown to be a growth factor for both prokaryotes and eukaryotes and has been demonstrated to be conjugated to discrete nuclear proteins intracellularly (15-18). Spermidine has been shown to increase the rate of chain elogation of DNA and RNA, increase protein synthesis and to be a specific translation factor for fidelity of protein synthesis (19-23). Finally, spermine increases the efficiency of acylation of tRNA, a process required in protein synthesis (24, 25).

The physiological functions of cells vary widely in response to stimuli; however, in any stimulated cell, a large and rapid increase in the activity of ODC, which is then followed by an increase of the polyamines, is almost always part of the initial response. This has been observed in tissues responding to stimuli as varied as growth factors, viral infections, mitogens

and foreign toxic substances (26-30). The increase in ODC activity results mainly from an increase in the amount of ODC protein and in the half life of the induced ODC. The amount of the ODC protein is increased primarily by increased transcription of the gene, along with increased translation of the mRNA (4, 7, 31). The relative contributions of the increases in translation and transcription seem to be stimulus specific. Additionally, in some systems, induced ODC protein has been found to be more stable (32, 33).

It is now well documented that ODC is present in mammalian tissue, in two ionic forms which are separable by ion exchange chromatography. These two forms have been observed in rat liver, kidney, heart, and thymus, mouse kidney and HTC cells under varied stimuli, including steroids and heavy metals (34-40). The two forms are named ODC A and ODC B in order of elution from DEAE Sepharose column. Evidence suggests that these ionic forms are not artifactual (41). There has been much speculation as to the origin of these two species. In theory, since ODC has been shown to be a multigene family (42-45), these two forms could be separate gene products with the charge difference of the proteins attributable to differing amino acid content. Conversely, the gene transcripts may not differ and instead, differences between the two species could depend on a post-translational modification of the protein. While the regulation of ODC has been extensively investigated at the molecular level, the connection between the regulation of ODC and the form in which it occurs in mammalian tissues has not yet been defined. The physiological significance of ODC A and B and their origin are major interests in the field of

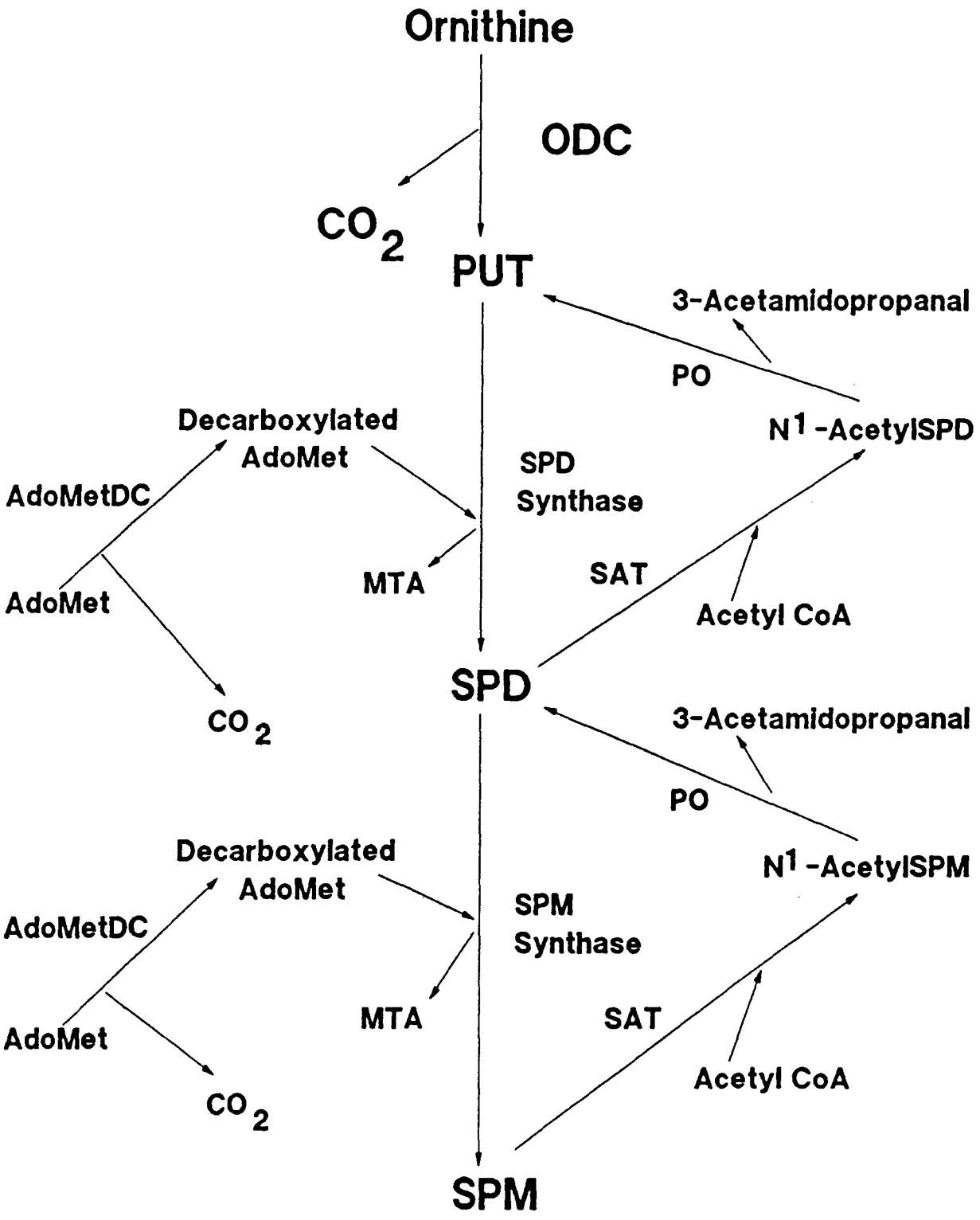
polyamines. If their occurrence is biologically important, it might be expected that stimuli which affect total ODC activity would also affect the proportions and/or properties of ODC A and B. Properties of ODC directly involved with its stability or kinetic function would most likely be associated with changes in cell activity. Additionally, the physical properties of the enzymes which cause the interconversion of the two species of ODC could also be related to a specific stimulus. Thus, changes in post-translational modifications, such as phosphorylation, or in size of the ODC monomer could provide the basis for the charge separation of ODC A and B.

PATHWAY OF POLYAMINE BIOSYNTHESIS

ODC, the initial enzyme in the polyamine biosynthetic pathway (see figure 1), has the shortest half life, in the range of 10-20 mins (46), of any of the enzymes involved in the polyamine biosynthesis; indeed, it has the shortest half life recorded for any known mammalian enzyme. ODC is also the most highly inducible enzyme involved in polyamine biosynthesis and as such the activity of this enzyme mainly determines the activity of the pathway. ODC is thus the rate limiting enzyme of this pathway (46). Decarboxylation of the amino acid ornithine by ODC results in the formation of putrescine, a diamine. Aminopropyl groups, donated by decarboxylated S-adenosyl-L-methionine (ADOMET), are subsequently added to the diamine,

by spermidine and spermine synthase, to give spermidine and spermine. The decarboxylation of ADOMET is catalyzed by ADOMET decarboxylase. This reaction is rate limiting in the synthesis of spermidine and spermine. The changes in ADOMET decarboxylase activity that occur after stimulus are usually similar to, but less than, those of ODC. Although the half life of S-adenosyl-L-methionine is longer than that of ODC, it is still much shorter than that of the spermidine and spermine synthases. These synthases exhibit considerably higher activities and longer half lives, so it is unlikely that they are involved in the control of polyamine synthesis (47). The reactions forming spermidine and spermine are effectively irreversible but spermidine and spermine can be converted back to putrescine by the polyamine acetylation/oxidation pathway which involves the two enzymes spermidine/spermine-N1-acetyltransferase and polyamine oxidase. The activity of the polyamine oxidase appears not to change significantly in response to growth stimulus, although the conversion of spermine to spermidine and spermidine to putrescine appears to be enhanced in tissues undergoing rapid proliferation (47).

Figure 1: The Polyamine Metabolic Pathway:
ODC, ornithine decarboxylase; PUT, putrescine; SPD, spermidine; SPM, spermine; PO, polyamine oxidase; SAT, spermidine/spermine-N^1-acetyltransferase; ADOMET, S-adenosyl-L-methionine; ADOMETDC, S-adenosyl-L-methionine decarboxylase; MTA, 5'-methylthioadenosine


Ornithine
ODC
CO_2
PUT
3-Acetamidopropanal
PO
N^1-AcetylSPD
Decarboxylated
AdoMet
AdoMetDC
AdoMet
CO_2
SPD
Synthase
MTA
SAT
Acetyl CoA
SPD
3-Acetamidopropanal
PO
N^1-AcetylSPM
Decarboxylated
AdoMet
AdoMetDC
AdoMet
CO_2
SPM
Synthase
MTA
SAT
Acetyl CoA
SPM

CELLULAR REGULATION OF ODC

The complexity of the regulation of ODC would appear to be related to the physiological importance of the polyamines. Besides being present as only 0.00014% of soluble cellular protein in thioacetamide stimulated liver (48), ODC also has the shortest documented half life (10-20 min) (46). As ODC is tied intimately with cell growth, its activity can change rapidly in response to a wide range of stimuli which affect cell growth, such as tumor promoting agents, viral transformations, hormones and mitogens (26-30). ODC is rapidly induced, beginning within 2-3 hours of treatment in rat tissues, and increases in ODC activity of 5-500 fold have been reported (49-51). In stimulated systems, studies with a radiolabelled suicide substrate inhibitor of ODC, known as DFMO (difluoromethylornithine), have shown that the increased ODC activity is mainly due to concomitant increases in the amount of ODC protein (52-54). Also seen in androgen stimulated mice and growth hormone stimulated rats, subsequent treatment with cycloheximide, an inhibitor of protein synthesis, prevented ODC induction (55, 56). Therefore, the accumulation of ODC protein seems to depend on an increased rate of synthesis. The increased rate of ODC synthesis can result from stimulation of the transcription of the gene and/or stimulation of translation of mRNA and this is dependent on the stimulus used. Increased gene

transcription has been observed in androgen stimulated BHK cells, without concomitant increase in mRNA levels (57). On the other hand, in rat hepatoma cells stimulated with TPA, increases in ODC activity could be blocked by actinomycin D, an inhibitor of mRNA synthesis (58). Also, the stability and the efficiency of translation of the ODC mRNA may be increased, as can be seen in serum stimulated mouse S49 cells and in the kidneys of testosterone stimulated mice (94). In addition to the increase in protein concentration, the increased ODC activity also depends on the increase in the stability of the ODC protein, as shown by an increase in half life. This prolonged stability has been shown in many systems, including rat kidney stimulated with dexamethasone and mouse kidney stimulated with androgen (32, 36, 37, 59, 60).

Because of its important role in cell growth, ODC also has a network of negative regulatory factors which affect it. Besides the inherent control of its extremely short half life, there are other negative effectors present at almost every level of ODC synthesis. At the primary level, transcription of the gene itself can be suppressed. Additionally, it has been shown that there are binding sites for regulatory factors on the ODC mRNA. The most important of the regulatory factors are most likely the polyamines themselves. Normal polyamine concentrations vary between types of cells and tissues, but have been observed in the micromolar to millimolar range. At micromolar concentrations, the polyamines are able to depress ODC synthesis by selectively inhibiting the translation of the ODC mRNA (61, 62).

Also, the addition of polyamines to HTC cells was shown to induce a noncompetitive protein inhibitor of ODC, which was termed antizyme. This protein binds to ODC, directly inhibiting its activity (63). In the complex, the ODC molecule seems more susceptible to degradation. Antizyme to ODC has now been induced in a variety of cells, including fibroblasts, nerve and hepatic cells, as well as in thyroid and plant cells (63). Another possible way to regulate ODC is through phosphorylation of the enzyme. A polyamine dependent protein kinase, identified in the slime mold Physarum polycephalum, was capable of the phosphorylation and subsequent inactivation of ODC (64). However, this polyamine dependent kinase has not been found in mammalian tissues.

HORMONAL INDUCTION OF ODC

ODC induction exhibits a remarkably constant pattern of expression in response to stimulation with hormones even though they interact with cells by very different mechanisms. With most hormone stimulated rat tissues, the time course of ODC induction is quite similar; once stimulated, ODC activity is rapidly induced to a maximum value approximately 4-6 hours post stimulation, whereupon the activity diminishes to control values by 8 hours (65-67). Induction of ODC is related to the amount of hormone in a dose dependent manner. One of the first such hormones to be studied extensively was growth hormone (GH). Of related

interest is the similar polypeptide, prolactin (PRL). The majority of studies indicate that new rRNA synthesis is required for significant increases in protein synthesis in response to trophic hormones, such as GH and PRL (68-70). Most of these hormones induce ODC at least in part by a cyclic AMP dependent mechanism (71). Trophic agents that elevate cyclic AMP concentration also promote an elevation in *de novo* synthesis of ODC (71). In many of these systems, the addition of cyclic AMP analogs and/or phosphodiesterase inhibitors to tissues and cells elevates ODC activity (72-76). More recent evidence indicates that the phosphatidylinositol pathway also has a role in the hormonal induction of ODC. Increases in inositol phosphates have been shown to precede the increase in ODC activity in some systems. Additionally, LiCl, primarily an inhibitor of the phophatidylinositol pathway through its uncompetitive inhibition of inositol phosphate phosphatase (108), has been shown to decrease ODC activity (92). In the majority of these animal and cell systems, ODC has been shown to be transcriptionally and translationally regulated as determined by the effects of actinomycin D and cycloheximide on its activity (72-76).

Another group of hormones having well documented effects on ODC are the steroids. As with the trophic hormones, early stimulation of ODC in the tissue is related to the steroid in a dose dependent manner (71, 77). Steroids elicit response, not by cyclic AMP or phosphatidylinositol mediated signals but by interaction with high affinity cytosolic receptors. The hormone-receptor complex translocates to the nucleus to directly affect gene transcription (78). However, the effects of the steroids can be expressed at

the transcriptional, translational or both, levels in the regulation of ODC (57, 77, 79). This is in marked contrast to the effect of the trophic hormones, which regulate ODC by both transcriptional and translational steps regardless of the system involved (80). Although the trophic hormones and the steroids regulate ODC through different avenues, in both systems ODC exists in the two ionic forms, A and B.

FORM OF ODC IN MAMMALIAN TISSUES

Regulation of ODC has been well characterized at the molecular level in terms of ODC synthesis and stability. However, the form in which ODC exists in mammalian tissue has not yet been associated with a regulatory function. In mammalian tissues, ODC exists in two forms; isozymes, with differing pI's. These two forms are named ODC A and ODC B in the order of their elution in DEAE Sepharose chromatography. Initially seen in the liver of dexamethasone-treated calf and in the liver of thioacetimide-treated or partially hepatectomized rat, multiple species of ODC have now been observed in 3T3 cells, in the kidney of testosterone stimulated mouse, in the liver of chloroform, maleate or cobalt treated rat and in the heart of isoproterenol treated rat (34, 35, 37-39, 81-83). The occurrence of two forms is not confined to stimulated tissue; they have also been observed in the heart, liver, thymus and kidney of untreated rats (34,

35, 37-39, 81-83) . Since these forms are present even with the additon of 8M urea and are eluted from Fast Protein Liquid Chromatography after little pretreatment of cellular cytosol, it is unlikely that the ionic forms are a result of nonspecific binding of ODC with other substances or nonspecific protein degradation in the tissue preparations (41). Additionally, ODC A and B isoforms from every source examined to date have had the same molecular weight and cannot be distinguished on a SDS denaturing gel (71). Animal tissues usually contain 2-3 species of ODC mRNA, but when isolated, these mRNA have only differed at their poly A sites or the 5' untranslated region and so are probably not responsible for the charge disparity of ODC A and B (84-86). Finally, the active ODC gene itself has been sequenced and appears to contain only one start sequence for transcription (86, 109, 110).

Further, there has been some evidence that the charge disparity between A and B may be due to phosphorylation. Native ODC has been isolated from Friend murine erythroleukemia cells as a phosphoprotein and appears to be predominantly phosphorylated the single serine residue, ser 303 (87, 88). Additionally, ODC A isolated from testosterone treated mouse kidney can readily be labelled by ^{32}P-ATP when incubated with casein kinase II in vitro. However, ODC B, the more acidic form, cannot accept ^{32}P label without prior alkaline phosphatase treatment (89).

CURRENT STUDY

This study was undertaken primarily to better characterize the multiple forms of ODC and to investigate the origins and possible functions of ODC A and B. The experiments reported here were designed with three major objectives;

1) to establish patterns of expression of the two forms of ODC in the liver and kidney of control rats and of animals treated with dexamethasone, prolactin or growth hormone, to see if their proportions were changed on stimulation. The effects of agents which were known to alter the level of ODC activity in tissue of hormone treated rats, eg actinomycin D, putrescine and LiCl, were also used to define any possible changes of A and B isoformsduring a decrease in ODC activity.

2) to compare some kinetic and physical properties of ODC A and B isolated from hormone stimulated tissue to see if change in proportion of A and B is associated with a change in properties of ODC.

3) and finally, to investigate the occurence of phosphorylation in the origin of the charge disparity of ODC A and B in hormone stimulated tissue.

MATERIALS AND METHODS

[1-*14*C]-L-ornithine hydrochloride was obtained from Amersham Corporation (Oakville, Ontario). DEAE Sepharose CL-6B was purchased from Pharmacia Fine Chemicals (Dorval, Quebec). Affigel 10, Bradford dye, silver stain reagents, molecular weight standards and any other electrophoreisis reagents were bought from Biorad Laboratories (Richmond, California). L-ornithine hydrochloride, pyridoxal-5'-phosphate, pyridoxamine-5'-phosphate, dithiothreitol, dexamethasone, actinomycin D, putrescine, lithium chloride and cycloheximide were obtained from Sigma chemical Company (St. Louis, Missouri). BCIP (5-bromo-4-chloro-3-indoyl phosphate), NBT (nitro blue tetrazolium), prestained high molecular weight standards, alkaline phosphatase conjugated goat anti rabbit immunoglobins and any other immunological reagents were obtained from Bethesda Research Laboratories.

TREATMENT OF ANIMALS

Immature female Wistar rats, age 4-8 weeks and adult male CD-1 mice were obtained from Animal Care Unit (U.B.C. strain) and Canadian Breeding Farm (Charles River, Quebec). Animals were kept in a 12 hr dark/light cycle room for 48 hrs prior to experiment and were given standard laboratory chow and water ad libitum. Dexamethasone, dissolved in ethanol at 2 mg/ml was administered by intraperitoneal injection as a suspension in 500ul of 0.9% (w/v) NaCl. Each rat, assuming an average body weight of 80 g, received 200 ug of the hormone. Both growth hormone (bovine or ovine) and prolactin (bovine) were dissolved in saline and adjusted to pH 8.2-8.3 with sodium bicarbonate prior to IP injection. Each animal received 500 ug of GH or PRL in a volume of 500 ul. Rats were killed 5 hrs after treatment with either dexamethasone, growth hormone or prolactin. Other hormone treated rats were additionally treated at hour 4 with either LiCl (5 umol/g in 500 ul saline), actinomycin D (2.5 mg/kg in 500 ul saline) or putrescine (125 mg/kg in 500 ul saline) and then killed at hour 5 post hormone. Testosterone propionate in saline (100 mg/kg) was administered by subcutaneous injection and the mice were killed 24 hrs later. Control animals were untreated. Rats and mice were killed by cervical dislocation following brief exposure to CO_2.

TISSUE PREPARATION

After killing, the kidney and or livers were removed immediately from the animals, quick frozen in liquid nitrogen and stored at -70°C. Prior to experiment, tissues were partly thawed and homogenized in ice-cold buffer A (3.5 ml/g tissue for kidney or 4 ml/g tissue for liver). Buffer A contained 50 mM Hepes, 3 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM PMSF at a pH of 7.3 at room temperature. Homogenization was performed using a Potter-Elvehjem homogenizer with the pestle being driven at approximately 600 rpm during the 6-8 strokes. The homogenate was kept on ice throughout this procedure. The resulting kidney homogenate was centrifuged at 20000 xg_{av} at 4°C for 20 minutes. The resulting liver homogenate was centrifuged at 100000 xg_{av} at 4°C for 35 min. After centrifugation, the supernatant was decanted and used in subsequent experiments.

ODC ASSAY

Aliquots of the supernatant fractions were used for assay of ODC. The ODC activity was measured by a slight modification of the previous method (67). The reaction mixture, in a final volume of 400 ul, contained pyridoxal phosphate (0.2 mM) and L-ornithine (0.25 mM containing 0.150 uCi L-1-^{14}C ORN (58 mCi/mmol)). All assay tubes were kept on ice until starting the reaction with the addition of enzyme. In the assay ^{14}C-CO_2 was collected in

200 ul hyamine hydroxide following acidification of the reaction mixture with 2 M citric acid. All assays were carried out in duplicate or triplicate and an average value was used in data analysis. One unit of enzyme activity was defined as 1 pmol CO_2 released per 30 min incubation at 37°C and specific activity was expressed as units of activity per mg protein.

PROTEIN DETERMINATION

Protein concentration was determined by the method of Bradford (90). A standard curve for the protein assay based on BSA was also done with every set of samples.

SEPARATION OF THE IONIC FORMS OF ODC

A settled volume of 40 mls of DEAE Sepharose CL-6B suspended in buffer B (50 mM Hepes, 3 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 150 mM NaCl, pH 8.0 at room temperature) was degassed for 30 min. After decanting the fines, the gel was packed in a Pharmacia column (1.5x28 cm) to a height of 23 cm. The column was then moved to a 4°C cold room and washed with 4 volumes of buffer. Supernatant prepared from either the kidneys or livers of treated animals was loaded directly and the column washed for at least 20x5 ml fractions with buffer B at a flow rate of approximately 35 ml/hr. The column was then eluted with a linear gradient composed of 200 mls of buffer B and 200 mls of buffer C (50 mM Hepes, 3 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 250 mM NaCl, pH 8.0 at room temperature) with an additional 170x2.5 ml fractions being collected. Aliquots of the eluent were taken from every second fraction for assays of ODC activity. If ODC A and B were needed for further experiments, enzyme activity of each ionic form was separately pooled and concentrated by ultrafiltration under a nitrogen pressure of 50 psi with a Diaflo YM10 membrane. Just prior to ultrafiltration, Brij 35 was added to the pooled enzyme to a final concentration of 0.02% in order to stabilize the enzyme. All ultrafiltration procedures were carried out at 4°C.

DETERMINATION OF ODC HALF LIFE *IN VIVO*

Cycloheximide (25 mg/kg) was administered intraperitoneally in 500 ul of saline to rats given growth hormone 5 hrs previously. At intervals of 15, 30 and 60 minutes post cycloheximide, the animals were killed and the livers and kidneys removed. Subsequently, DEAE elution profiles of the ODC in the rat liver and kidney were determined. Groups of 3-6 rats were sampled and the tissues pooled for DEAE chromatography. After chromatography, ODC A and B were pooled separately and concentrated by ultrafiltration. ODC activity was then assayed in the ODC A and B samples. This activity was expressed as a percentage value of the activity measured in similarly treated samples of ODC A and B from rats not treated with cycloheximide. These activites were correlated with the duration of cycloheximide treatment and graphed on semilog plot. Enzyme decay is exponential in nature and thus to interpret the data, a semilog graph had to be used. Any deviations from first order rate of decay on semi-log plot will be observed as a curve.

ALKALINE PHOSPHATASE TREATMENT OF TISSUE EXTRACT

The supernatant from liver and kidney of growth hormone and growth hormone + LiCl treated rats was prepared as described earlier. To the resulting supernatant, alkaline phosphatase (final concentration of 200 Units) and magnesium chloride(final concentration 1.0 mM) were added. The mixture was then incubated in a slowly shaking water bath at 37°C for 30 minutes. Subsequently, DEAE profiles of the ODC in the samples was determined. Separation or inhibition of the alkaline phosphatase from the supernatant prior to chromatography was not necessary as it eluted from the column within the first five fractions collected i.e. void volume.

ENZYME PURIFICATION

ODC A and B from the livers and kidneys of rats treated with growth hormone for 5 hours were partially purified prior to kinetic studies. ODC was purified by two main steps; DEAE Sepharose CL-6B chromatography and pyridoxamine-5'-phosphate Affigel chromatography.

Step 1-DEAE Sepharose CL-6B chromatography

DEAE chromatography was performed as described earlier. The tissues from 10 rats were pooled for ODC purification. Both ODC A and ODC B were pooled individually from the column and concentrated to a volume of approximately 10 ml for Affigel chromatography.

Step 2-pyridoxamine-5'-phosphate Affigel chromatography

The affinity gel was prepared by adding 110mg pyridoxamine-5'-phosphate hydrochloride to 25 ml of Affigel 10 in 0.10 M sodium phosphate buffer, pH 7.0. The coupling reaction was allowed to proceed for approximately 24 hr at $4^{o}C$ with rotational mixing. The unreacted groups on the agarose were then coupled with 1 M ethanolamine for an hour and the gel washed extensively on a buchner funnel with 1 M NaCl in 0.01 M sodium phosphate buffer, pH 7.0. The gel was then degassed for 30 minutes and packed into a column of 1.5 cm diameter to a height of 15 cm. The column was washed with 5 volumes of buffer D (50 mM tris-HCl, 3 mM DTT, 0.1 mM EDTA, 10 mM NaCl, 0.1 mM PMSF, 0.02% Brij 35, pH 7.3) at a flow rate of 30 ml/hour. ODC A or ODC B from previous DEAE chromatography was loaded directly onto the column and washed with buffer D for 30x5 ml fractions. The ODC activity was then eluted from the column with buffer E (50 mM Tris-HCl, 3 mM DTT, 0.1 mM EDTA, 10 mM NaCl, 0.1 mM PMSF,

0.02% Brij 35, 50 uM PLP, pH 7.3) at the same flow rate and an additional 80x5 ml fractions were collected. Aliquots were taken from every second fraction for the determination of ODC activity. All Affigel chromatography was done at 4°C. The ODC activity was pooled and further concentrated to a volume of approximately 3 mls by ultrafiltration as stated earlier, but without any further addition of Brij 35.

The buffer of the resulting partially purified ODC was exchanged for buffer K (50 mM Hepes, 3 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 0.02% Brij 35, 50 mM NaCl pH 7.3) by elution through a Biorad ECONO-PAC 10DG desalting column which had been washed with buffer K. The resulting volume of the partially purified ODC was approximately 10 mls.

DETERMINATION OF K_m^{orn} AND V_{max} FOR KIDNEY ODC B, AND LIVER ODC A AND B

Aliquots of 100ul from the resulting partially purified ODC were examined for ODC activity in the presence of 5, 10, 25, 50, 75, 100, 250 and 500 uM ornithine. These assays were done in duplicate and at two different salt concentrations (50 mM and 150 mM NaCl) in order to observe any kinetic differences due to possible changes in subunit structure due to affects of NaCl on binding of substrate or possible dimerization of ODC monomers. All other parameters for the ODC assay were as stated previously. The experimental values for K_m^{orn} and V_{max} were determined by Eadie-Hofstee plot (111).

IMMUNOPRECIPITATION OF ODC

To further concentrate ODC for gel electrophoresis, samples of kidney ODC A and B and liver ODC A and B from the desalting column eluent were precipitated using an anti-ODC antibody that had been previously prepared. This polyclonal antisera had been raised in a rabbit host using purified ODC

from the kidneys of testosterone stimulated mice as the antigen. Aliquots of 200 ul of ODC (semipurified by DEAE Sepharose chromatography) were combined with 100ul of a 1/5 dilution of rabbit anti ODC serum in an Eppendorf tube.. This was gently shaken for 30 minutes at 4°C. A 450 ul aliquot of 1:2 suspension of protein A Sepharose CL-4B in buffer A was added and the mixture was shaken for another 30 minutes at 4°C. The suspension was washed with 1.0 ml of buffer A and spun briefly in an Eppendorf centrifuge to collect the protein A Sepharose CL-4B immunocomplex. This wash was repeated 2-3 times. The protein A Sepharose Cl-4B was eluted by the addition of 500ul of buffer S (0.58% glacial acetic acid, 0.15 M NaCl) and the suspension was spun once again to collect the supernatant. The supernatant was treated with 250 ul TCA(30%) and 100 ul 0.1% BSA and spun to collect the resulting precipitate. The precipitate was washed once with 500 ul of ethanol and dissolved in PAGE sample buffer.

ELECTROPHORESIS OF ODC

The molecular weights of kidney ODC B, liver ODC A and B were estimated by electrophoresis using a discontinuous polyacrylamide gel (PAGE). The method used was that of Laemmli (91) with some modifications. The sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol and 0.01% bromophenol blue) was not made with any beta-mercaptoethanol, as any reducing agent in the sample buffer seemed to interfere with later transfer of

the proteins to nitrocellulose. All gels were run using the BioRad MINI-PROTEAN II DUAL SLAB CELL system. All gels were done in duplicate so that protein could be detected by both antibody and protein staining methods.

IMMUNOBLOTTING OF THE GEL

After the gel had been developed, it was immunoblotted onto nitrocellulose using the BioRad MINI TRANS-BLOT ELECTROPHORETIC TRANSFER CELL. The method was as stated in the BIORAD TRANS-BLOT instructions. The transfer buffer was 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Transfers were done at 150 mA constant current for 1 hr. The nitrocellulose was then incubated with 5% bovine serum albumin in KBS buffer (137 mM NaCl, 1.5 mM KH_2PO_4, 7.2 mM Na_2HPO_4, 0.02% NaN_3, 2.7 mM KCl, 0.05% Tween 20) while gently shaking for 1 hour at room temperature or overnight at 4^oC. The blot was then washed 3 times with KBS and further incubated with 25 mls of a 1/500 dilution of rabbit ODC antibody in KBS, with gentle shaking for 1 hour. The blot was again washed 3-5 times with KBS and finally incubated with 1/3000 dilution of alkaline phosphatase conjugated to anti-rabbit antibody in KBS for an additional hour with gentle shaking. The blot was washed a final 3-5 times with KBS and 10mls of alkaline phosphatase substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) with NBT and BCIP substrate were added as per BioRad

IMMUNO-BLOT kit instructions. The blot was left in the substrate containing buffer until color development occurred. The reaction was terminated by extensive washing with distilled water.

STAINING OF GEL

After electrophoresis, the gel was fixed and stained for protein using the BioRad SILVER STAIN kit. The stained gel was placed between two acetate sheets which had previously been thoroughly wetted with distilled water and then air-dried.

RESULTS

RELATIVE PROPORTIONS OF ODC ACTIVITY IN THE MULTIPLE FORMS OF ODC DURING HORMONAL INDUCTION

Previously, it has been demonstrated that multiple forms of ODC, which are separable by DEAE Sepharose chromatography, exist in mammalian tissue. In each case, A is designated as the form eluted earlier in the gradient, and B is the more acidic. Thus, these experiments were first directed towards establishing the patterns of appearance of ODC A and B in tissues of control and hormone treated rats. The data presented in figures 2 and 3 demonstrate that ODC A and B occur in distinct, specific proportions in rat liver and kidney; and further, these proportions are changed when the rat is treated with different hormones. In each case, with the treatment of hormone, the activity of ODC rapidly increases to a maximum of 8X control for rat kidney and 15X control for rat liver (see table 1). The specific activities observed are in agreement with previous studies (65, 66, 67, 71). ODC A and B are both induced in response to hormone treatment, but not equally. The change in proportion of ODC A and B varied with hormone treatment and tissue. In figure 2, the ratio of ODC A:ODC B in control rat

kidney is seen to be 50:50. However, in the kidney of rat treated with GH, PRL or DEX for 5 hrs, the pattern has been shifted to an ODC A:ODC B of approximately 40:60. The proportions in liver are somewhat different (fig 3). In control rat liver, the ODC A:ODC B ratio is 70:30, notably different from control kidney. Additionally, the response of the ODC A:ODC B ratio to hormonal stimulation differs in the liver. In rat treated for 5 hrs with DEX or PRL, the ODC A:ODC B ratio is shifted to 60:40. In the liver of rats stimulated with GH, the ODC A:ODC B ratio is affected to a greater degree with a shift to 50:50. Even though the ODC A:ODC B ratios differed between kidney and liver and to some extent, hormone treatment, in each case where ODC activity was induced, ODC B activity was increased to a greater degree than ODC A activity. Each of the represented DEAE Sepharose profiles was repeated at least 3 times, with a minimum column efficiency of 75%. The only exception was that of the control kidney and liver which had the lower column efficiencies of 65 and 60%. As ODC is so unstable in the unstimulated tissue, attempts to obtain higher column efficiencies were unfeasible.

Figure 2: DEAE Sepharose Chromatography of ODC Activity in Kidney from Normal and Hormone-Treated Animals: Kidney extracts, prepared from 3-5 a) normal, b) growth hormone-treated, c) prolactin-treated and d) dexamethasone-treated animals were applied to DEAE Sepharose CL-6B columns and the different forms of ODC separated as described in Materials and Methods. The hormone treatment used was also as described in Materials and Methods.

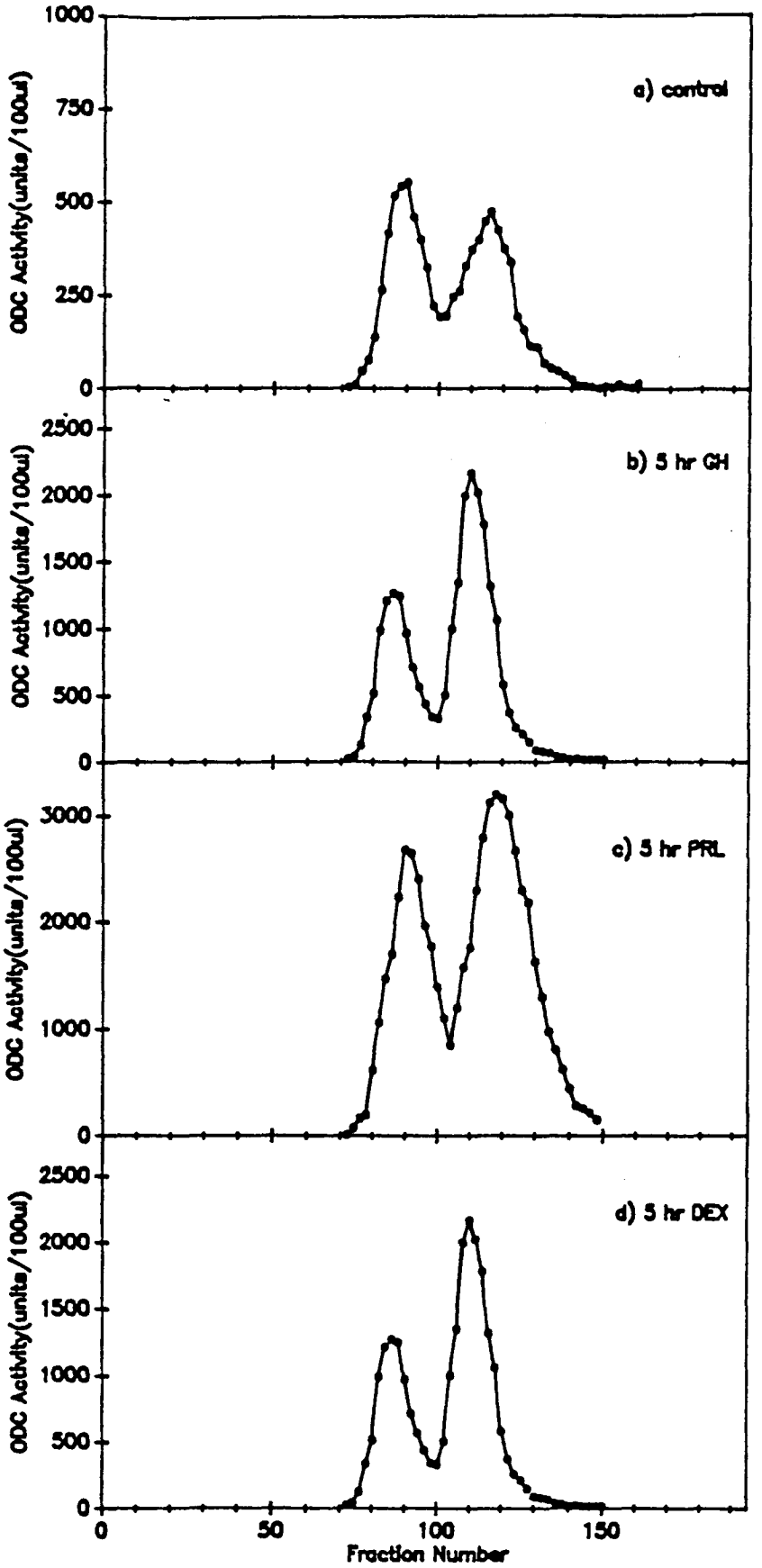
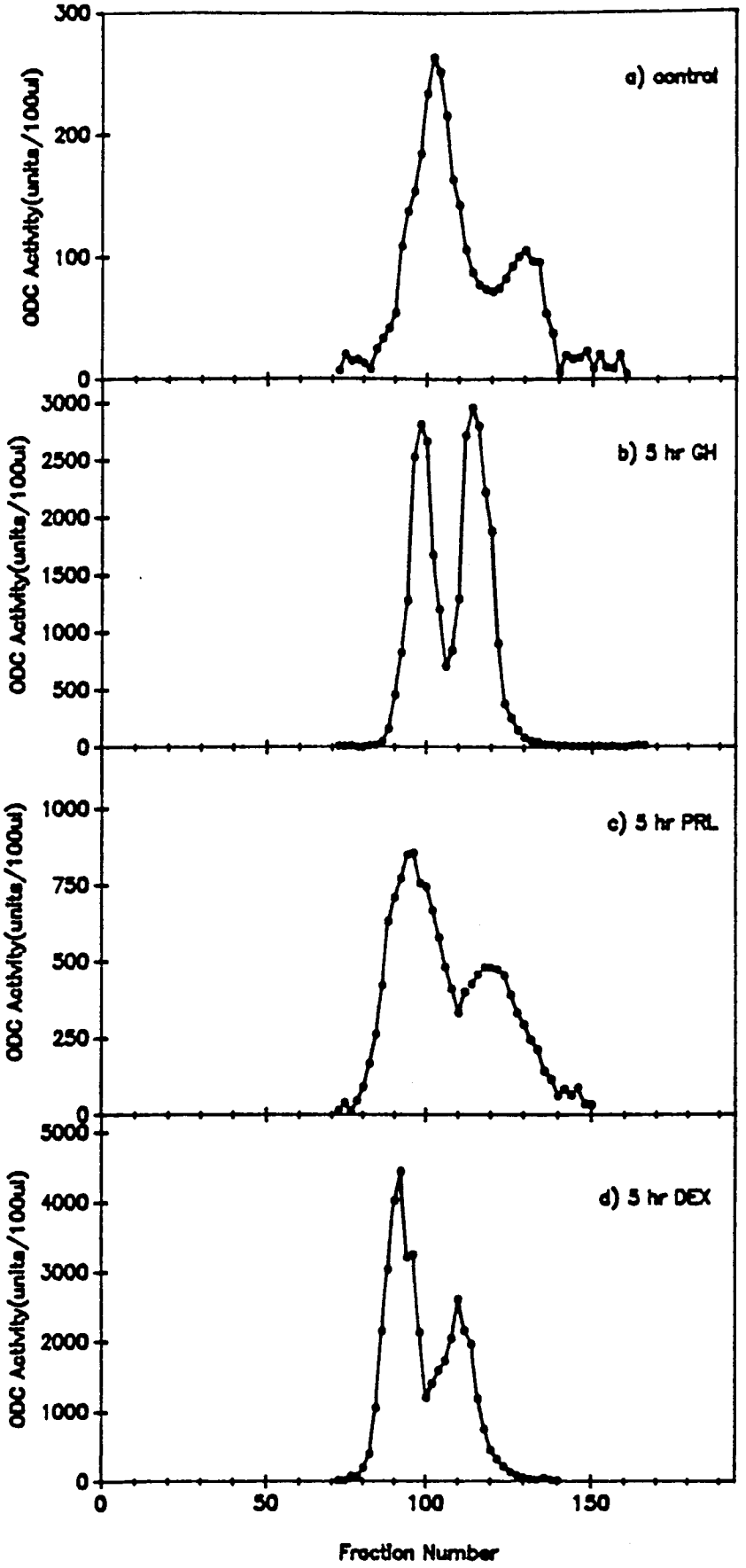

a) control
b) 5 hr GH
c) 5 hr PRL
d) 5 hr DEX
ODC Activity(units/100ul)
Fraction Number
0
250
500
750
1000
1500
2000
2500
3000
50
100
150

Figure 3: DEAE Sepharose Chromatography of ODC Activity in Liver from Normal and Hormone-Treated Rats: Liver extracts, prepared from 3-5 a) normal, b) growth hormone-treated, c) prolactin-treated and d) dexamethasone-treated animals were applied to DEAE Sepharose CL-6B columns and the different forms of ODC separated as described in Materials and Methods. The hormone treatment used was also as described in Materials and Methods.


a) control
b) 5 hr GH
c) 5 hr PRL
d) 5 hr DEX
ODC Activity(units/100ul)
Fraction Number
0
50
100
150
300
200
100
3000
2500
2000
1500
1000
500
1000
750
500
250
5000
4000
3000
2000
1000

RELATIVE PROPORTIONS OF THE IONIC FORMS OF ODC DURING A DECREASE IN THE TOTAL ACTIVITY OF ODC

Since the patterns (ratios) of ODC A:B isoforms were established in the control and hormone stimulated tissues, other agents which are known to affect ODC activity were also injected in vivo to observe their effects on the distribution of isoforms A and B. Three agents which appear to act by differing mechanisms were chosen: actinomycin, an inhibitor of RNA synthesis; LiCl, an inhibitor of the phosphatidylinositol and other signal transduction pathways; and PUT, a natural negative feedback inhibitor of ODC which acts through stimulation of antizyme and repression of translation of ODC mRNA. Each of these agents were given 1 hour before killing the rats treated 4 hrs earlier with either GH, PRL or DEX. Previous experiments have shown the effect of these agents on ODC activity under these conditions (92, 71) and the results illustrating the effects of these agents in the present study are seen in table 1.

LiCl had the same overall effect in opposing the effects of each of the three hormones on kidney ODC. The specific activity of ODC was decreased to approximately half of the value observed in hormone stimulated tissue and the A:B ratio reverted to the ratio observed in control kidney (50:50). Actinomycin D also decreased the ODC specific activity, which had previously been increased by each of the hormones, by at least 40%, but the effect of actinomycin D on the ratio of ODC A:B appears to vary with the hormone

used. Actinomycin D, caused the A:B ratio in ODC from the kidneys of PRL treated rat to revert to the 50:50 in control rats, but did not change the 40:60 ratio in the kidneys of both GH and DEX treated animals. PUT affected the total ODC activity in the kidneys of hormone stimulated rat in the same overall manner as LiCl, with the concomitant 50% decrease in ODC activity. Treatment with PUT also resulted in an ODC A:B ratio of 50:50 in kidneys from rat treated with GH. The effect of PUT on the distribution of ODC from the kidneys of DEX treated rats however was not definitive.

In its response to agents which inhibit ODC, the liver again differed from the kidney. As seen in table 1, none of the inhibiting agents seemed to change the original stimulated profiles for ODC A and B from the livers of DEX, GH or PRL treated rats. ODC from the livers of both DEX and PRL stimulated rats kept to the A:B ratio of 60:40 regardless of LiCl, ACT D or PUT addition. ODC from GH stimulated rat liver also kept to its unique A:B ratio of 50:50 regardless of agent. As with the kidney however, these agents all decreased the ODC specific activity. Thus, the agents appeared to equally affect the activity of ODC A and B in liver.

To further test the effects of hormones on A:B ratio, two hormones of differing method of action, DEX and GH, were injected at the same time into the rats. The resulting A:B profile for kidney was 40:60, similar to that of single dose hormone stimulated kidney (see table 1). However, the profile of ODC from livers of GH+DEX treated rats was 50:50, similar to ODC from GH

stimulated rat liver, and not similar to ODC from DEX stimulated liver (see table 1), as with the ODC specific activity.

Table 1: Specific Activity of ODC and the Proportions of the Multiple Ionic Forms in the Kidney and Liver of Normal Rats and Rats Treated with Growth Hormone, Prolactin or Dexamethasone.

KIDNEY			LIVER		
Treatment Of Animals	Specific(units) Activity	A:B Ratio	Treatment Of Animals	Specific(units) Activity	A:B Ratio
CONTROL	1000	50:50	CONTROL	100	70:30
5hGH	8000	40:60	5hGH	1500	50:50
5hPRL	7000	40:60	5hPRL	900	60:40
5hDEX	8000	40:60	5hDEX	500	60:40
5hGH + 1hLiCl	3000	50:50	5hGH + 1hLiCl	800	50:50
5hPRL + 1hLiCl	4000	50:50	5hPRL + 1hLiCl	400	60:40
5hDEX + 1hLiCl	4000	50:50	5hDEX + 1hLiCl	300	60:40
5hGH + 1hAct.D	6000	40:60	5hGH + 1hAct.D	900	50:50
5hPRL + 1hAct.D	4000	50:50	5hPRL + 1hAct.D	550	60:40
5hDEX + 1hAct.D	5500	40:60	5hDEX + 1hAct.D	300	60:40
5hGH + 1hPUT	5000	50:50	5hGH + 1hPUT	600	50:50
5hDEX + 1hPUT	5000	45:55	5hDEX + 1hPUT	300	60:40
5hDEX + GH	8800	40:60	5hDEX + GH	1500	50:50

HALF LIVES OF LIVER AND KIDNEY ODC A AND B *IN VIVO*

To assess the possible significance of the two forms *in vivo*, the half lives for both liver and kidney ODC A and B were determined. The half life of ODC was determined by measuring the activity of enzyme following the administration of cycloheximide. Rats were treated with GH for 5 hrs and then with cycloheximide for 15, 30 or 60 mins. DEAE Sepharose profiles were then determined for each tissue at each time period. From figures 9 and 10, it is shown that both kidney ODC A and ODC B activities decay in a linear fashion, indicating a species with a single half life. The half lives for kidney A and kidney B were found to be 7 mins and 25 mins respectively. However, the decay of both liver ODC A and ODC B is curvilinear and indicates the presence of two components with different half lives. The half lives for liver A appear to be 10 min and approximately 25 mins while liver B is more disparate with half lives of 10 mins and approx 60 mins.

Figure 4: Effect of Cycloheximide on the Two Major Ionic Species of ODC in Kidneys of Growth Hormone-Treated Rats.

Rats were given 500 ug GH in 0.9% NaCl. After 5 hrs, when induced ODC activity had reached a peak, the animals were injected with 25 mg/kg cycloheximide. The two ionic species of the enzyme were separated by DEAE-Sepharose CL-6B column chromatography as described under Materials and Methods. Samples were prepared at 15 min, 30 min and 60 min after protein synthesis was blocked. Results are shown for form A (●-●) and form B (o-o). Each point represents ODC assays on 5 animals.

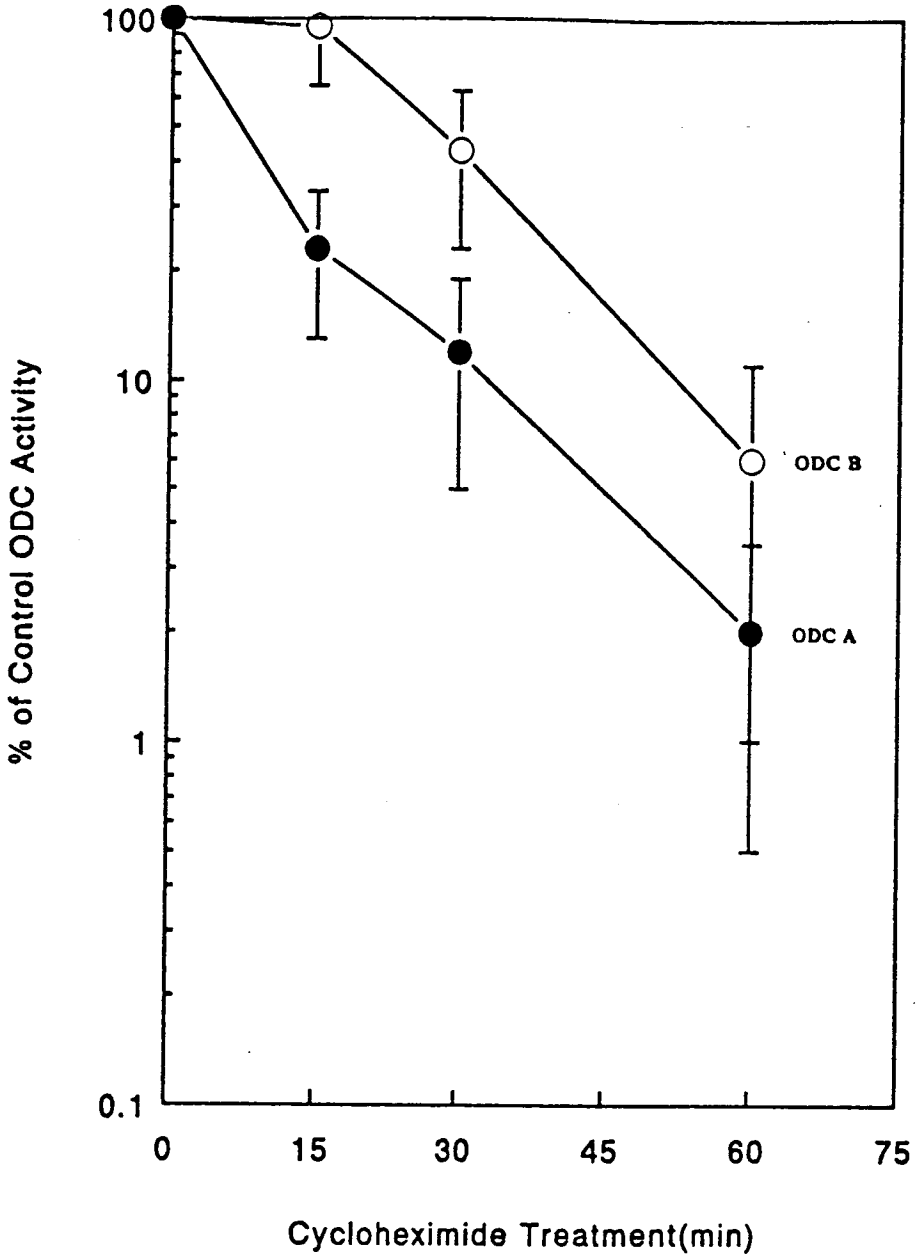
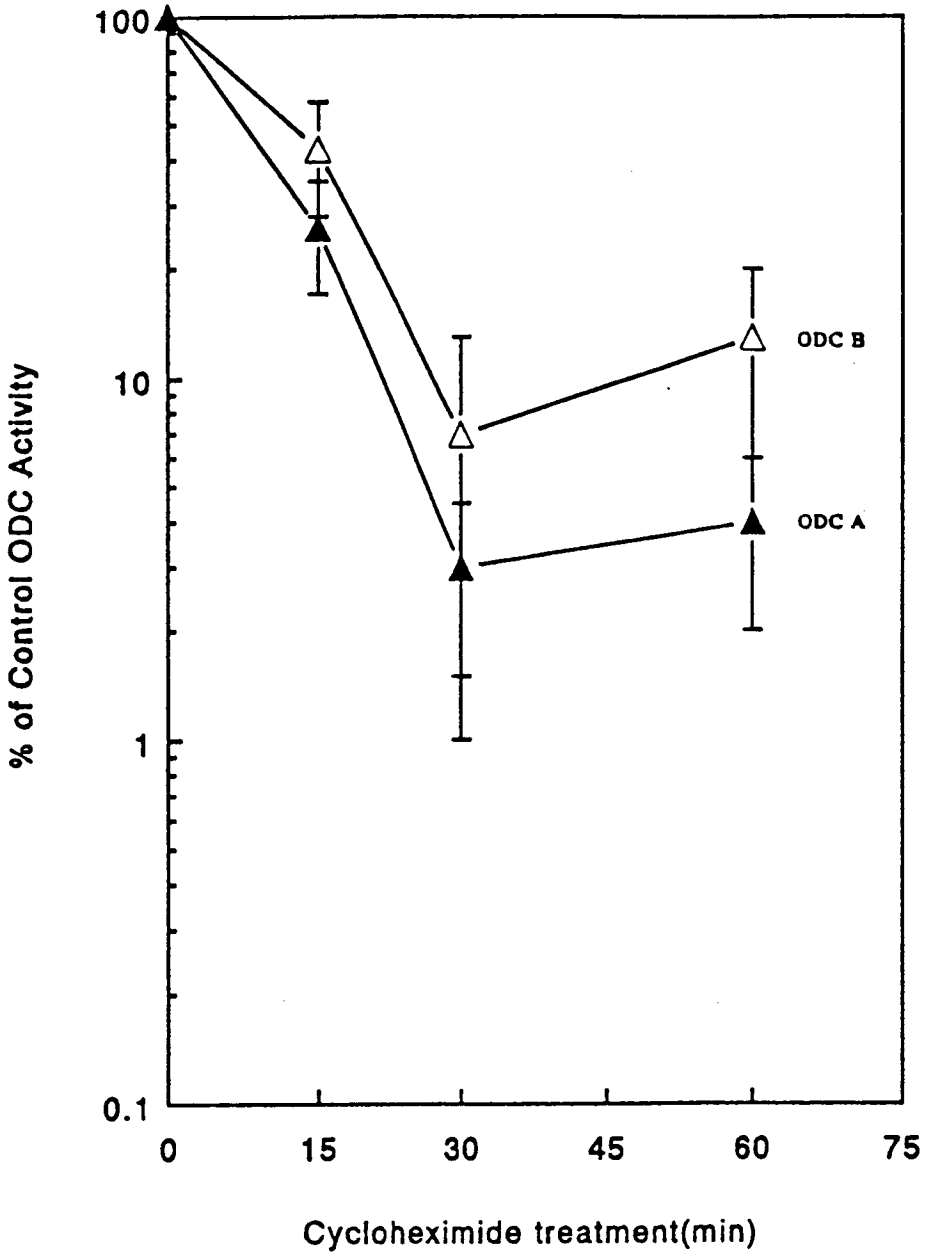

100
10
1
0.1
% of Control ODC Activity
0
15
30
45
60
75
Cycloheximide Treatment(min)
ODC B
ODC A

Figure 5: Effect of Cycloheximide on the Two Major Ionic Species of ODC in Livers of Growth Hormone-Treated Rats.

Rats were given 500 ug GH in 0.9% NaCl. After 5 hrs, when induced ODC activity had reached a peak, the animals were injected with 25 mg/kg cycloheximide. The two ionic species of the enzyme were separated by DEAE-Sepharose CL-6B column chromatography as described under Materials and Methods. Samples were prepared at 15 min, 30 min and 60 min after protein synthesis was blocked. Results are shown for form A (●-●) and form B (o-o). Each point represents ODC assays on 5 animals.


100
10
1
0.1
% of Control ODC Activity
0
15
30
45
60
75
Cycloheximide treatment(min)
ODC B
ODC A

KINETIC PROPERTIES OF KIDNEY ODC A AND B FROM THE LIVER OF GH STIMULATED RAT

To see if the two forms differ in other properties, the similarities and differences between ODC A and B at the kinetic level were investigated. ODC A and B from the liver and kidney of GH stimulated rat were separately purified by the procedures outlined in materials and methods. The two main chromatographic procedures included DEAE-Sepharose Cl-6B ion exchange and pyridoxamine phosphate affinity. ODC A and B from the liver and kidney of GH stimulated rat were examined because the responses of ODC activity to hormone induction and inhibition differed between these tissues. Ornithine concentration was varied from 5-500 uM and kinetic studies were also done in the presence of 50 or 150 mM NaCl. Table 2 shows the results of the determination of the enzyme kinetic parameters for kidney B, liver A and liver B. Figures 6-8 show the Eadie-Hofstee plots for kidney B, liver A and liver B.

Figure 6: Eadie-Hofstee Graphical Analysis of Ornithine Kinetics for B Form ODC Purified from Kidneys of Growth Hormone-Treated Rats.

ODC was purified by DEAE Sepharose and Pyridoxamine Affinity Chromatography from rat kidneys 5 hr after injection of hormone. Assays were in triplicate and contained 0.2 mM pyridoxal-5'-phosphate with varied ornithine concentrations over the range of 5-500 uM, and in the presence of 50 mM (o-o) and 150 mM (●-●) NaCl. For full details, see Materials and Methods

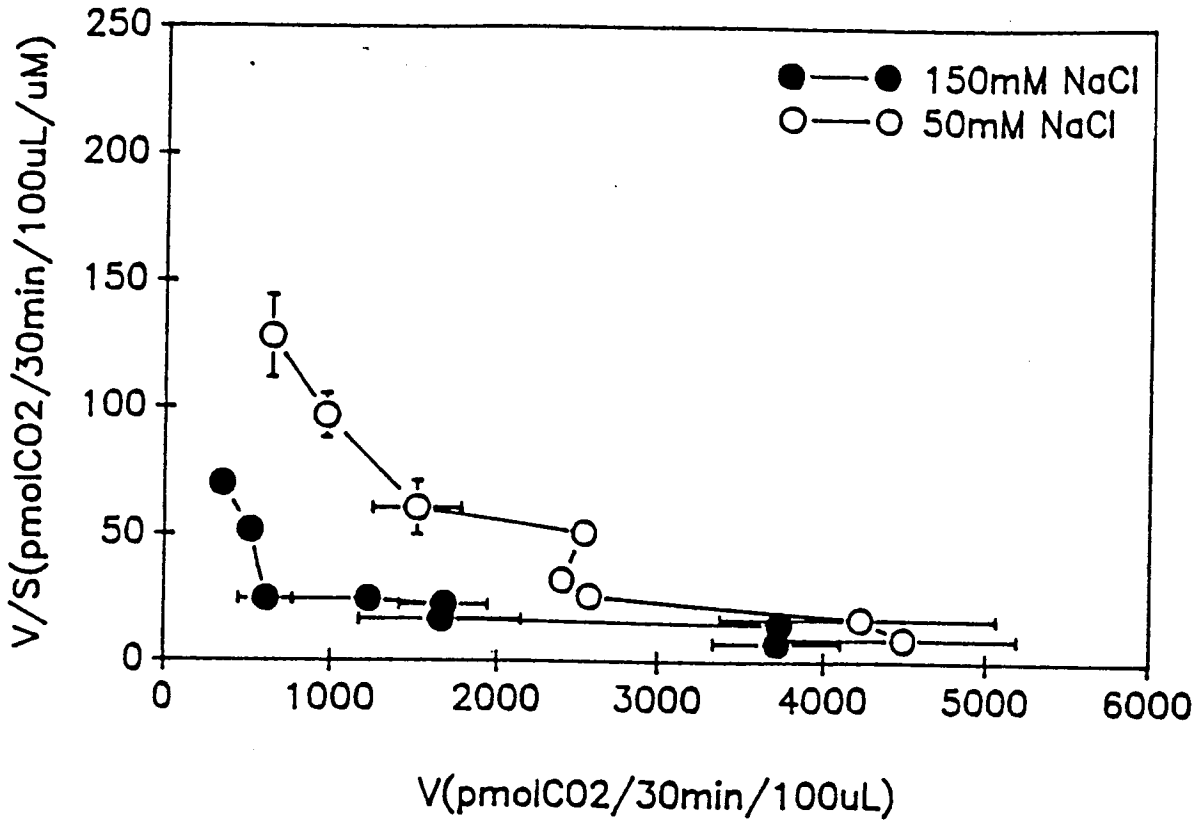

250
200
150
100
50
0
0
1000
2000
3000
4000
5000
6000
V/S(pmolCO2/30min/100uL/uM)
V(pmolCO2/30min/100uL)
150mM NaCl
50mM NaCl

Figure 7: Eadie-Hofstee Graphical Analysis of Ornithine Kinetics for A Form ODC Purified from Livers of Growth Hormone-Treated Rats.

ODC was purified by DEAE Sepharose and Pyridoxamine Affinity Chromatography from rat livers 5 hr after injection of hormone. Assays were in triplicate and contained 0.2 mM pyridoxal-5'-phosphate with varied ornithine concentrations over the range of 5-500 uM, and in the presence of 50 mM (o-o) and 150 mM (●-●) NaCl. For full details, see Materials and Methods

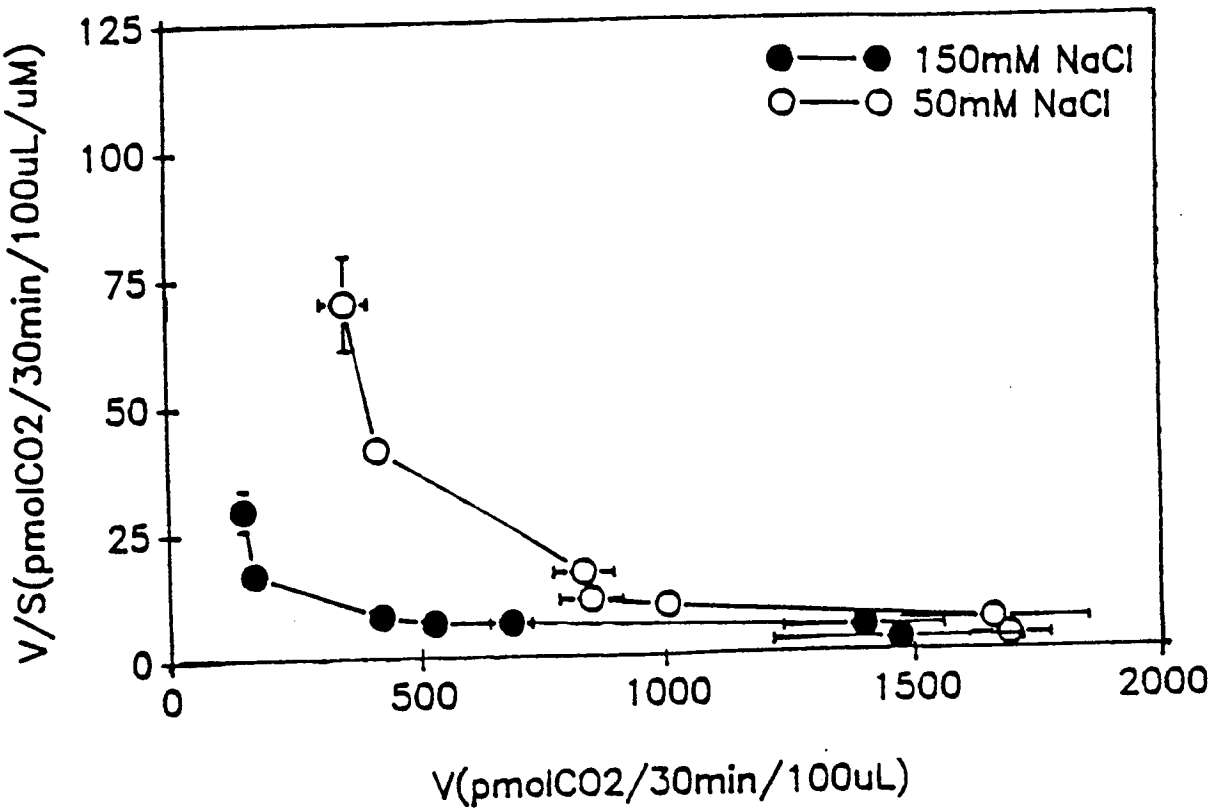
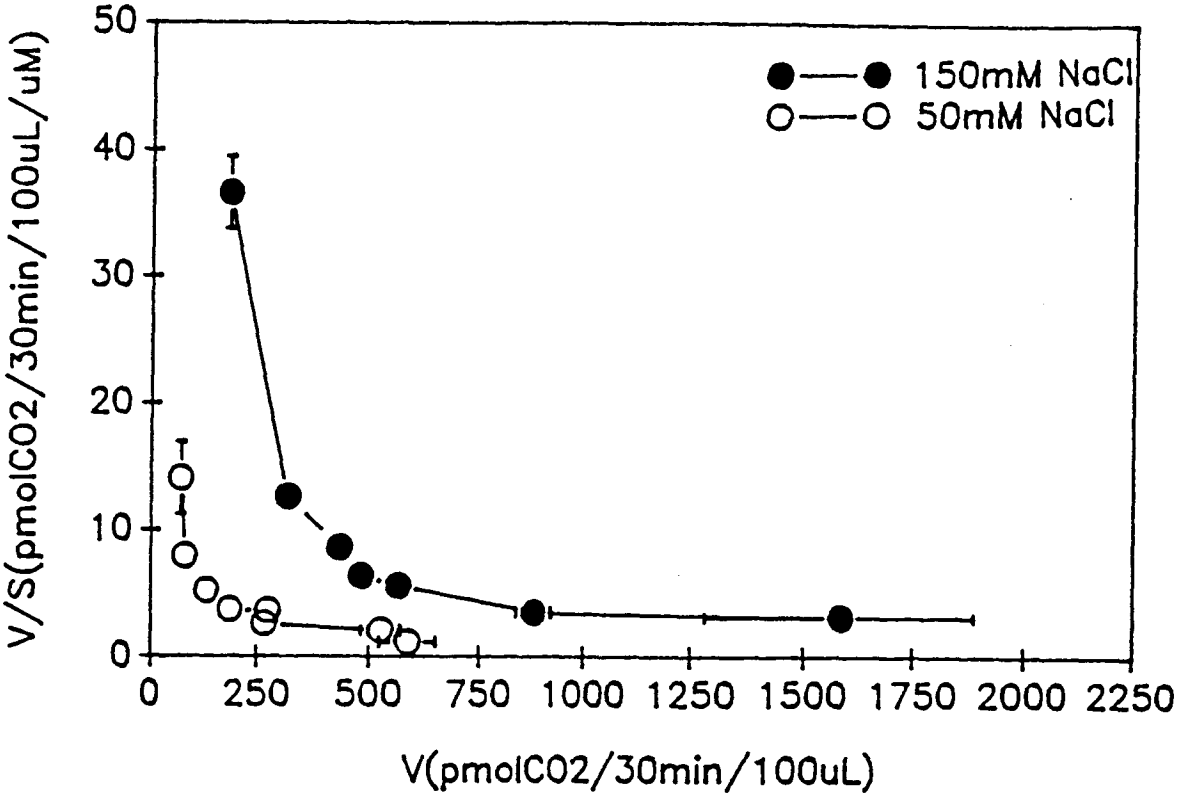

125
100
75
50
25
0
0
500
1000
1500
2000
V/S(pmolCO2/30min/100uL/uM)
V(pmolCO2/30min/100uL)
150mM NaCl
50mM NaCl

Figure 8: Eadie-Hofstee Graphical Analysis of Ornithine Kinetics for B Form ODC Purified from Livers of Growth Hormone-Treated Rats.

ODC was purified by DEAE Sepharose and Pyridoxamine Affinity Chromatography from rat kidneys 5 hr after injection of hormone. Assays were in triplicate and contained 0.2 mM pyridoxal-5'-phosphate with varied ornithine concentrations over the range of 5-500 uM, and in the presence of 50 mM (o-o) and 150 mM (●-●) NaCl. For full details, see Materials and Methods


150mM NaCl
50mM NaCl
V/S(pmolCO2/30min/100uL/uM)
V(pmolCO2/30min/100uL)
0
10
20
30
40
50
0
250
500
750
1000
1250
1500
1750
2000
2250

From figures 7 and 8, it can be seen that ODC does not respond in a first order manner to increases in substrate. The Eadie-Hofstee plot of kidney ODC B is probably first order in nature (figure 6) as a least squares analysis of these points puts a straight line within the error bars of this data. The subsequent plots for liver ODC A and B are not. From figures 7 and 8, it appears that A and B from liver have two different V_{max} and K_m^{orn} values; signifying that there may be two species with different kinetic properties within A and B. An increase in ionic concentration seems to accentuate this property, for in each case the plots became more curved at the higher salt concentrations. Previous studies have shown that higher salt concentrations will increase the apparent value of the K_m^{orn} (93). Ionic concentration affected both the K_m and V_{max} in each case. See table 2 for actual K_m^{orn} and V_{max} values. From table 2, it seems as though kidney B possesses the highest V_{max} at either salt concentration. The lowest V_{max} values were those corresponding to liver B. Within liver A and liver B there were two different kinetic components of ODC, consisting of one species with a low K_m value and a low V_{max} and another species with a high K_m value and a high V_{max}.

Table 2: Kinetic Properties of Purified Multiple Ionic Forms of ODC from Kidney and Liver of Growth Hormone-Treated Rats.

Tissue	[NaCl] (mM)	Korn (uM)		Vmax(units/ 100ul)	
		A	B	A	B
5h GH KIDNEY	50	---	37	---	4350
	150	---	87	---	4200
5h GH LIVER	50	9 167	6 136	940 2500	485 2400
	150	14 224	9 320	500 2250	150 750

ROLE OF PHOSPHORYLATION IN CHARGE SEPARATION OF A AND B

As stated earlier, phosphorylation may play a role in the charge heterogeneity of ODC A and B. To further test if phosphorylation is involved in the charge separation of A and B, tissue supernatant from 24 hr testosterone treated mouse kidney was treated with alkaline phosphatase for 1 hr before loading onto a DEAE Sepharose column. The resulting profile showed that all of the ODC activity eluted in the region corresponding to ODC A (figure 4). Supernatants from the kidneys and livers from GH stimulated Wistar rats were treated with alkaline phosphatase for 30 min with the same results (figure 4 and 5). Additionally, supernatants were prepared from the livers and kidneys of rats treated with GH and LiCl and also subject to alkaline phosphatase treatment. However, phosphatase treatment of liver and kidney supernatant again gave the identical results of the seeming conversion of all ODC to ODC A. A supernatant from the liver treated with GH+LiCl, was incubated without alkaline phosphatase, with a resulting profile of 77:27, thus indicating that any seeming conversion from ODC B to A is not solely due to unspecific breakdown.

All the DEAE Sepharose column profiles of phosphatase treated tissue had efficiencies of 75% or higher. ODC activity was decreased about 40% by the incubation, regardless of phosphatase treatment. However, the decrease in ODC activity in form B is not wholly accounted for by this nonspecific decrease.

Figure 9: Effect of Alkaline Phosphatase on the Two Major Ionic Species of ODC from Kidneys of Growth Hormone- or Growth Hormone plus LiCL-Treated Animals.

The rats were treated as described in Materials and Methods. Supernatants from the kidneys were incubated with alkaline phosphatase (200 Units) for 30 min at 37°C and were applied to DEAE-Sepharose Cl-6B columns. Chromatography was run as described in Materials and Methods.

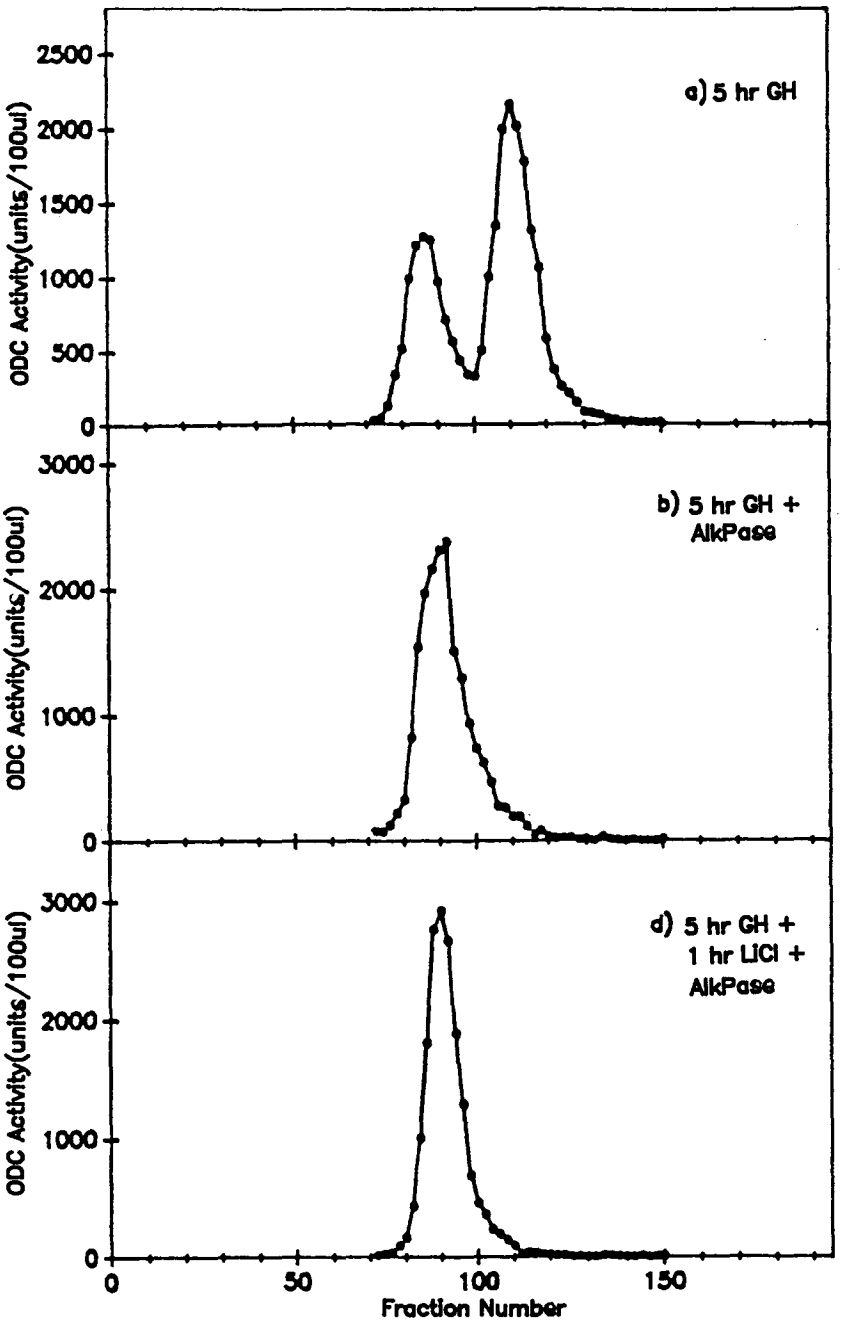
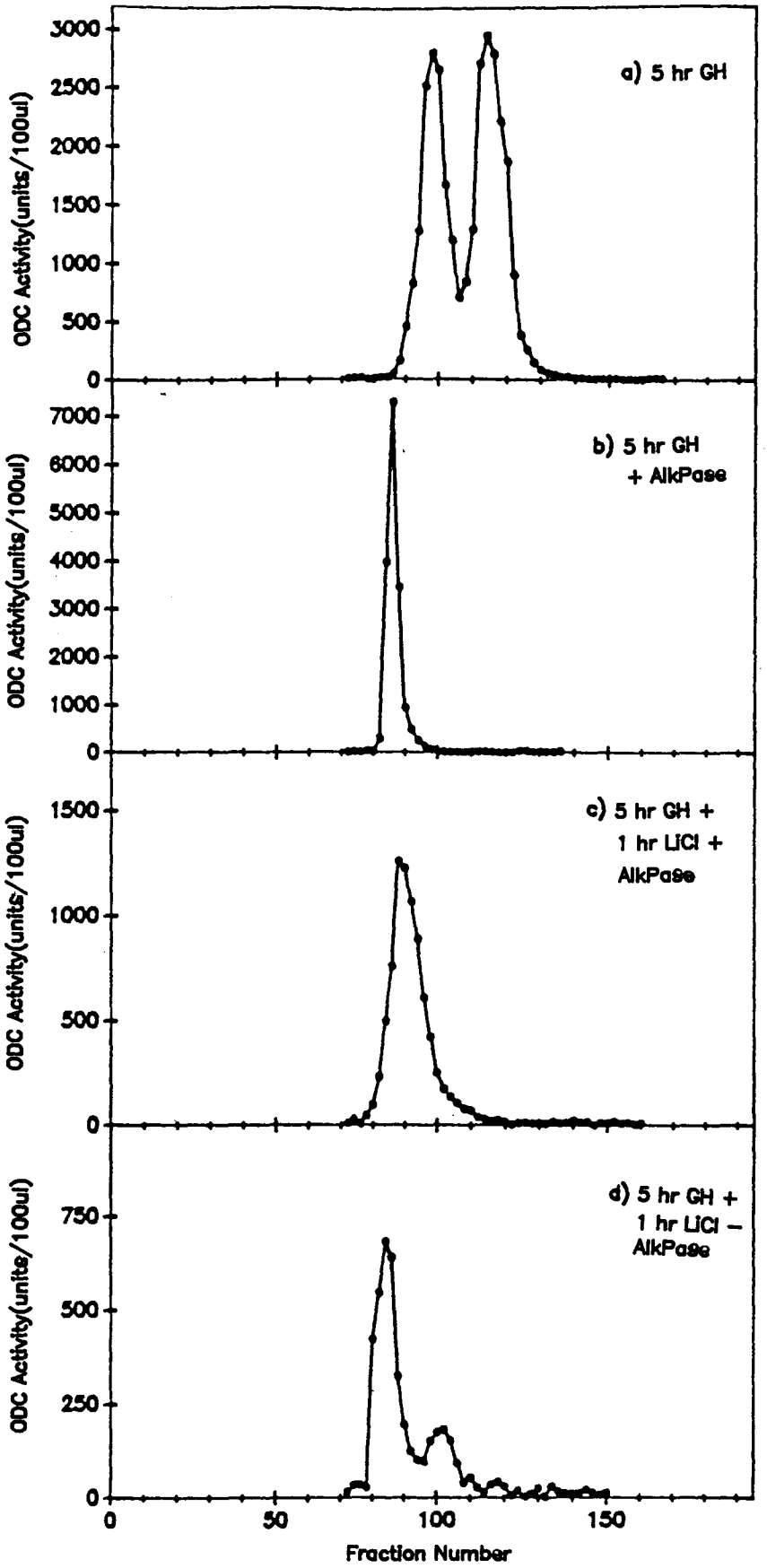

a) 5 hr GH
b) 5 hr GH + AlkPase
d) 5 hr GH + 1 hr LiCl + AlkPase
ODC Activity(units/100ul)
Fraction Number
0
500
1000
1500
2000
2500
3000
50
100
150

Figure 10: Effect of Alkaline Phosphatase on the Two Major Ionic Species of ODC from Livers of Growth Hormone- or Growth Hormone plus LiCl-Treated Rats.

The rats were treated as described in Materials and Methods. Supernatants from the livers were incubated with alkaline phosphatase (200 Units) for 30 min at 37°C and were applied to DEAE-Sepharose Cl-6B columns. An additional GH + LiCl Supernatant was incubated without alkaline phosphatase prior to chromatography as a control. Chromatography was run as described in Materials and Methods.


a) 5 hr GH
b) 5 hr GH
+ AlkPase
c) 5 hr GH +
1 hr LiCl +
AlkPase
d) 5 hr GH +
1 hr LiCl –
AlkPase
ODC Activity(units/100ul)
Fraction Number
0
50
100
150
3000
2500
2000
1500
1000
500
7000
6000
5000
4000
3000
2000
1000
750
250

PHYSICAL PROPERTIES OF ODC A AND B FROM THE LIVER AND KIDNEY OF GH TREATED RATS

Partially purified ODC A and B from liver and kidney of growth hormone stimulated rats were run on a SDS denaturing PAGE and detected via western blotting. As seen in figure 11, there appears to be no difference in the apparent molecular weight of kidney A and B and liver A and B, which is seen as approximately 48 kD. The corresponding protein gel is not shown as the BSA added to the ODC supernatants prior to immunoprecipitation for stabilization, overshadowed the ODC on the protein stained gel.

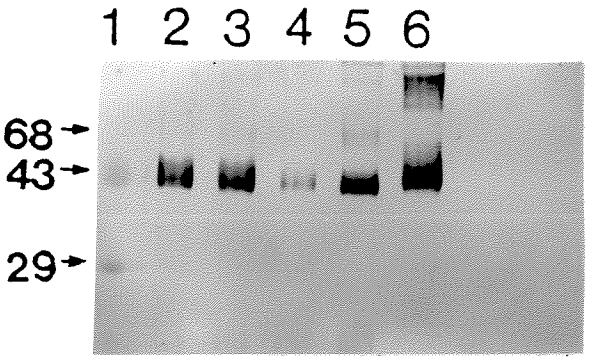


Figure 11: Electrophoresis of the Multiple Species of ODC on SDS-PAGE Gels.

The positions of the standards are shown by arrows. The results shown are forms A and B of enzyme purified from kidney and livers of GH treated rats as described in Materials and Methods. The standard purified ODC was from the kidneys of 24 hr testosterone treated mice. Aliquots were run on a discontinuous SDS gel and then electrophoretically transferred to nitrocellulose. Blots were reacted against the anti-ODC antibody as described in the materials and methods. The contents in the lanes are as follows: 1) prestained high molecular weight standards 2) ODC A from GH kidney 3) ODC B from GH kidney 4) ODC A from GH liver 5) ODC B from GH liver 6) ODC standard.

DISCUSSION

The physiological role of the multiple species of ODC observed in mammalian tissue is thus far undefined. Stimuli which affect the functioning of the cell also affect the activity of ODC. If the multiple species of ODC are biologically significant, alterations in total ODC activity might involve changes in the proportions, or changes in the physical and/or kinetic properties of these forms.

The results reported here suggest that multiple species of ODC in mammalian tissue are physiologically important. Hormonal stimulation of the ODC activity in rat kidney resulted in increases in activity in both A and B, and an increase in the proportion of form B. The ratio of ODC A and B changed with hormone stimulation to a value of 40:60 with each of the hormones studied. This increase in the proportion of form B reflects the increase in half life of that species, as seen in the cycloheximide studies (figure 4). This finding, in hormone stimulated rats is similar to findings in other stimulated systems such as serum stimulated HTC cells, and kidneys of testosterone stimulated mouse and liver of the cobalt stimulated rat (34). When rats were first treated with hormone for 4 hrs and then subsequently treated with agents which decreased the total specific activity of ODC, the

proportions of ODC A and B again changed. In the kidney of hormone stimulated animals, LiCl was able to change the ratio of ODC A and B to the control value of 50:50, indicating that the half life advantage of hormone stimulated ODC B is removed in the prescence of lithium. This has been corroborated with half-life studies (Richards, J. F. R., unpublished data). Actinomycin D did not have the uniformity of effect in the kidney that LiCl showed. While ODC activity was decreased within each of the hormonally treated groups, actinomycin D caused reversion of the A:B ratio to 50:50 in the PRL stimulated rat only. In both the GH and DEX stimulated animal treated with actinomycin D, there was no change in A:B ratio from their previous hormone stimulated values. As actinomycin D inhibits RNA generation, these results may indicate that PRL requires transcription to induce ODC activity whereas GH and DEX can induce ODC activity through both transcription and translational pathways. Putrescine had limited effects on the proportions of forms A and B. In spite of the decrease in ODC activity, the ratio of A:B in the kidney reverted back to the control value of 50:50 only in GH stimulated rats.

As actinomycin D and putrescine had differing effects on hormone stimulated tissue, this would corroborate the existence of stimulus specific regulatory processes regarding ODC, as seen in other stimulated systems (94-96), such as the kidneys of the testosterone stimulated mouse and the regenerating rat liver.

The kinetic properties of ODC from the kidney of growth hormone stimulated rat were investigated to see if form B might have an advantage over form A in function as well as stability. From the results, this is not the case. The kinetic values observed for ODC B from the kidney of GH stimulated rat were well within values previously reported for those corresponding to ODC A (38, 39, 81, 97). Thus, ODC B does not have kinetic advantage over ODC A in the kidneys of GH stimulated animals, and kinetic properties alone cannot be the basis for the biological relevance of the two ionic forms. This is further substantiated by the kinetic studies of ODC A and B from HTC cells and the kidneys of testosterone stimulated mice (96, 36).

In the livers of hormone stimulated animals, increases in the total specific activity of ODC also resulted in the preferential increase of form B relative to form A. The proportions of A:B seen in the liver differed from those seen in the kidney, both for unstimulated and stimulated tissues. Also, GH was able to stimulate the increase in activity of form B to a greater extent in the liver than the other hormones. As within the kidneys of hormone stimulated animals, ODC B had a longer half-life than form A. However, the activity of form B was proportionally less in the liver than in the kidney and experimentally it was found that total ODC activity from the liver was less stable than that from the kidney. Similar A:B ratios as well as increases in half-life of form B have also been observed in the livers of chloroform and cobalt stimulated rats (34, 37). Interestingly, there was

evidence of multiple half life species within both ODC A and B in the liver of the growth hormone-stimulated rat which has been undocumented as yet.

With the use of the inhibiting agents, the total specific activity of ODC was decreased in all three hormone stimulated systems. However, neither LiCl, actinomycin D nor putrescine changed the A:B ratio from the hormone stimulated values. This is perhaps indicative of differing regulatory mechanisms involved with ODC between the liver and kidney, the pathways in the liver relying less on regulation of ODC by half-life.

The kinetic properties of forms A and B from the liver of growth hormone-stimulated rats were also examined. The results show evidence of multiple kinetic species. These two kinetic species consist of a low K_m^{orn}, low V_{max} species and a high K_m^{orn}, high V_{max} species. These species, seen in both ODC A and B, are unusual but do not seem to give kinetic advantage to one ionic form over the other. These results could also imply dimerization of A and B and subsequent cooperativity within the dimers. However, the elevated salt conditions and extreme enzyme dilution that is required in the conventional DEAE chromatography which was used to separate A and B contraindicate the formation of dimers (41, 113). There is some possibility that the dimers, A:A and B:B, could form within the assay conditions of the kinetic experiments; however,as the ODC assays for the kinetic experiments were done under the same conditions for ODC purified from both kidney and liver and anomalous results were not observed for ODC from the kidney, it is

unlikely that the results observed for the ODC purified from the liver are due to dimerization.

The multiple half life species observed in both form A and B might be associated with these kinetic species. There would seem to exist additional multiple functional forms of ODC within the liver of growth hormone stimulated animals. The liver from growth hormone stimulated rats appears to have many distinguishing features when compared to those from the kidney. The effects of growth hormone in mammalian tissue are partially regulated by its intermediaries, the somatomedins. It has been documented that there are at least 3 types of somatomedins; A, B, and C. The kidney has a preponderance of receptors for somatomedin C whereas the majority of receptors on the liver recognize form A (98). Possibly this could account for the tissue specific responses of ODC to growth hormone.

The origin of the charge separation of ODC A and B is still uncertain and under investigation. Of the possible mechanisms to explain this charge difference, the post-translational modification, phosphorylation, has been the more fully investigated. First evidence of the phosphorylation of ODC was through the work of Donato et al which used polyclonal and monoclonal antibodies to distinguish subpopulations of phosphorylated ODC from mammalian ODC immunoprecipitated from many species (112). Evidence of the possible phosphorylation of ODC was then investigated through the *in vitro* phosphorylation of ODC by casein kinase II from RAW 264 cells (99).

Additionally, native ODC has been isolated from Friend murine erythroleukemia cells as a phosphoprotein being predominantly phosphorylated at a serine residue (87, 88). *In vivo* evidence of phosphorylation was observed in ODC from the kidneys of testosterone stimulated mice (89). ODC A isolated from this system was shown to readily accept ^{32}P from ^{32}P-ATP when incubated with casien kinase II; however, ODC B could not accept the ^{32}P label without prior treatment with alkaline phosphatase. Further *in vivo* evidence of phosphorylation has been observed in ODC from HTC cells and mouse myeloma cells (87,100). To further confirm the role of phosphorylation in the charge disparity of A:B, a series of supernatants from the kidneys and livers of hormone-treated rats were incubated with alkaline phosphatase and then subject to DEAE chromatography. The resulting ODC had the characteristics of ODC A on a DEAE column. This indicates that the charge difference between A and B, as seen on the DEAE column, is attributable to phosphorylation. Similar results were obtained in HTC cells, liver from mouse and rat and normal and tumorigenic colonic mucosa from mouse and human (100, 101). This effect of alkaline phosphatase was also noted on crude extract from GH treated kidney and liver also treated with LiCl, once again indicating that phosphorylation is responsible for the charge difference between A and B and not some other post-translational modification such as the attachment of a small peptide tail (102). Additionally, an incubation without alkaline phosphatase was run on a column and although ODC B had been preferentially degraded or acted upon by a cellular phosphatase, the total

activity loss of ODC was greater than in the previous results and ODC eluting in the area corresponding to form B was still evident in this untreated supernatant. However, a recent report found that an alanine substitution for serine 303, the primary site of phosphorylation in ODC, did not affect the half life of the ODC enzyme *in vitro* (103); indicating that although A and B may be separated on DEAE chromatography on the basis of their phosphorylated states, the phosphorylation state is not important biologically. However, a previous study showed that ODC synthesized *in vitro* from a cDNA differed from the native enzyme in certain properties, including stability of the enzyme (104).

Finally, the physical properties of liver and kidney ODC A and B were found to be similar on an SDS-PAGE Western. Their molecular weight for the monomer was found to be 48 kD, which is low (~13%) according to the expected weight of 55kD calculated from the mRNA sequence. However, there have been several reports of ODC with Mr of 50 kD (105-107), in which case the value reported here is low by only 4%. This could be due to minor degradation or the slightly different conditions of running the SDS-PAGE Western which was used. These four samples were also run on an isoelectric focusing gel and these preliminary results indicated at least two differently charged isoforms within ODC A and B from the liver of growth hormone stimulated animals. This experiment was not repeated and thus is not

presented. However, it provides suggestive evidence of the existence of further forms of ODC.

SUMMARY

Within the kidney and liver of both hormone stimulated rat and hormone stimulated rat given other agents, the proportions of ODC A and B were altered. This reflects the change in half life of ODC B as seen in the data presented. When ODC activity is increased, so is the proportion and stability of ODC B. This is seen in both the liver and kidney of growth hormone treated animals. In colonic mucosa, (101) it has been shown that with tumorigenesis comes an increase in the proportion of ODC B, providing further evidence for the biological importance of the multiple forms of ODC. However, in some instances, only seen in the kidney, where ODC activity is decreased, the enhanced stability of B is inhibited or removed, as in the response of ODC to LiCl in the stimulated kidney (Richards, J. F. unpublished data). In the liver, there was no additional change in the hormone stimulated A:B ratio, although ODC activity was decreased. This could represent different signalling mechanisms or perhaps different regulatory mechanisms in the liver. Growth hormone stimulated rat liver seemed to be a complex system in that ODC did not show typical responses in terms of activity or kinetics. Not only did the ratio of liver ODC A and B not respond to any of the agents which decreased ODC activity, but there was

evidence of multiple species of ODC within forms A and B having different kinetic and half-life properties. Finally, the charge separation of ODC A and B from kidney and liver was shown to be dependent on the state of phosphorylation. ODC A and B did not differ in molecular weight.

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