neurites (*4*). The effect of the extract on parasympathetic and sympathetic neurons is an acceleration of the rate of neurite elongation (*5*). This response cannot be due to enhanced survival of the neurons, since the cells do not normally die within 2 hours under these conditions.

The presence of at least two distinct growth factors in the rat hippocampal formation suggests that such factors are involved in development and in the specificity of neuronal sprouting after damage or deafferentation. Such specificity might be due in part to the release or "unmasking" of growth factors specific to the denervation. Different affinities of afferent fibers for such factors could provide selectivity in sprouting responses. For example, the fact that sympathetic fibers can be diverted into the central nervous system by intracerebral injections of NGF and the observation that radioactive NGF is selectively transported by septal perikarya led to the hypothesis that NGF, or a similar factor, might be involved in sympathetic sprouting after septal lesions (*3*). This hypothesis is strengthened by our finding of an NGF-like factor in hippocampal extracts.

Since sympathetic fibers invade only brain regions whose cholinergic fibers have been removed, it is possible that central cholinergic fibers normally respond to or control the availability of an NGF-like factor. This hypothesis is supported by the observation that NGF (*10*) or hippocampal extracts (*11*) increase cholinergic activity in fetal telencephalic neurons in vitro. Whether or not the substances identified in this study are actually involved in the development of the central nervous system or in denervation-induced plasticity must be addressed in the future. The detection of such substances, however, provides a basis for designing further experiments and hypotheses.

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7. The hippocampal formation was rapidly dissected from the fresh brains of female hooded rats (150 to 200 g) and homogenized immediately or frozen for later use. A single hippocampal formation (50 mg wet weight) was homogenized in 2.5 ml of Hanks balanced salt solution and centrifuged at 100,000*g* for 30 minutes. The supernatant was saved and diluted with Hanks balanced salt solution before being added to the culture medium.
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Autoradiographic Identification of Ornithine Decarboxylase in Mouse Kidney by Means of α-[5-^{14}C]Difluoromethylornithine

Abstract. *α-Difluoromethylornithine is an enzyme-activated irreversible inhibitor of ornithine decarboxylase that forms a covalent bond with the enzyme. When α-[5-^{14}C]difluoromethylornithine was administered to androgen-treated mice, only ornithine decarboxylase became labeled. Autoradiographic examination of kidney, liver, and brain indicated much more extensive incorporation of labeled difluoromethylornithine into kidney protein than into the protein of the other tissues. Such incorporation was greatly reduced by prior treatment of the mice with cycloheximide. These results correlate with the presence of ornithine decarboxylase activity which is much higher in the kidney than in the other tissues and is lost rapidly when protein synthesis is inhibited. The binding of this drug in vivo, therefore, is useful for determining the distribution of ornithine decarboxylase. The enzyme was predominantly located in the proximal tubule cells of the kidney in androgen-treated mice.*

Ornithine decarboxylase is the first enzyme in the polyamine biosynthetic pathway and is known to be highly inducible in response to a wide variety of trophic stimuli including hormones, mitogens, drugs, and tissue damage (*1*). The importance of this enzyme in cell growth has been confirmed by experiments with specific inhibitors which reduce polyamine production and inhibit cell growth (*2*). One of the most useful inhibitors is α-difluoromethylornithine (DFMO), which acts as an enzyme-activated, irreversible inhibitor (*3*). In agreement with this concept, we have observed that radioactivity becomes incorporated into the enzyme as it is inactivated by incubation with ^{14}C-labeled DFMO in vitro (*4*). Such incorporation might provide a means to selectively label the enzyme in vivo and could provide a method by which the cellular and subcellular distribution of the enzyme could be investigated. Many of the target tissues in which ornithine decarboxylase has been shown to be induced are complex mixtures of different cell types and it would be of interest to examine the relative extent to which the enzyme is affected in each one. To test whether the radioactive drug can be used in this way we have examined the enzyme in the androgen-treated mouse kidney. Ornithine decarboxylase is highly androgen responsive in the mouse, and the amount present increases substantially when castrated mice are treated with large doses of testosterone (*5*). The results indicate that [^{14}C]DFMO exclusively labels the enzyme in vivo and that the enzyme is predominantly located in the proximal tubule cells of the mouse kidney.

Androgen-treated mice were injected with [^{14}C]DFMO at a dose of 1 mg/kg and killed 60 minutes later. This treatment led to the loss of 90 percent of the ornithine decarboxylase activity in tissue extracts. When extracts from the kidneys of these mice were prepared and subjected to polyacrylamide gel electrophoresis only one labeled protein band (molecular weight 55,000) was observed. This band corresponded to the position of authentic, labeled ornithine decarboxylase (*4*). Autoradiographs of the kidney sections indicated that there was substantial radioactivity present in the area of the cortex but much less in the medulla (Fig. 1A). The labeling of the kidney was much greater and more regionally localized than that in the liver or brain (Fig. 1, C and D). These results correlate well with the ornithine decarboxylase activities in the tissues (Table 1). As an additional confirmation that the labeling did represent ornithine decarboxylase, similar autoradiographs were prepared from mice treated with cycloheximide 6 hours before they were killed. As expected from the rapid turnover of the enzyme (*1*) this treatment greatly re-

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duced the activity in all tissues (Table 1). No labeling of liver or brain was detected in autoradiographs of cycloheximide-treated mice (results not shown) and labeling in the kidney was much reduced, although there was still a clear difference between the cortex and medulla (Fig. 1B).

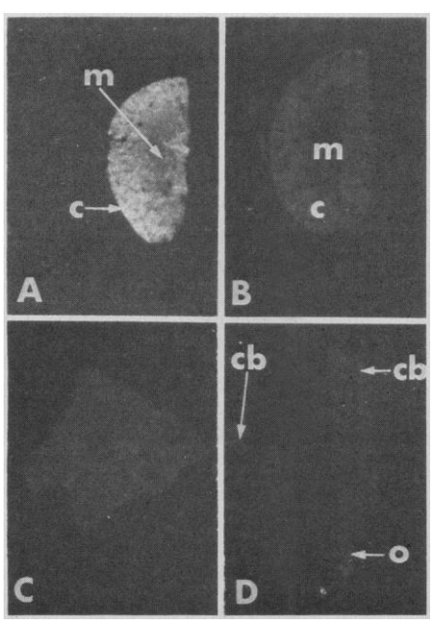
The distribution of ornithine decarboxylase was studied in greater detail by histological autoradiography (Fig. 2). A large number of grains occurred over the cells of the proximal convoluted tubules, with much lower amounts corresponding to the distal convoluted tubules and renal corpuscles (Fig. 2A). The labeling was much less intense over the structures in the medulla (Fig. 2B). The distribution of labeled protein shown in Fig. 2 could be attributed to the selective uptake of the drug into certain cells resulting in a higher degree of inactivation, and hence labeling, of the enzyme. This is unlikely, however, since DFMO is taken up at the same rate by passive diffusion in a number of cell types (*6*). Furthermore, Table 1 shows that 90 percent of the total enzyme activity is inhibited by the dose of the drug used in this labeling study.

To confirm the results of our autoradiographic studies, we attempted to dissect mouse kidneys into the cortex and medulla and measure activities in homogenates of these tissues. Although cross-contamination was probably substantial because of imprecision in dissection, extracts from the cortex were two to three times more active than those from the medulla. These results are consistent with the autoradiographic observations. Treatment of the mice with 1 mg of DFMO per kilogram of body weight reduced the activities in both fractions by approximately 90 percent.

The method we have described should prove useful in determining the distribution of ornithine decarboxylase, and is limited only by the very low amounts of this protein in mammalian cells (*4*). Even in the induced mouse kidney the enzyme is only about one part in 10,000 of the cytosolic protein (*7*). As shown in Table 1, the amount in the kidney is three orders of magnitude greater than that in other tissues such as liver and brain. The [^{14}C]DFMO used in the present experiments, therefore, does not have a high enough specific activity for detailed studies in such tissues but it should prove a relatively easy task to synthesize the drug containing tritium at a much higher specific activity. That the labeling by DFMO is highly specific is demonstrated both by the presence of only one labeled band in tissue extracts analyzed by polyacrylamide gel electrophoresis under denaturing conditions and by the

Table 1. Ornithine decarboxylase activities in mouse tissue extracts. Mice were injected intraperitoneally with testosterone propionate (100 mg/kg) in sesame oil (4 mg/ml) 3 days before they were killed. Cycloheximide (20 mg/kg) and DFMO (1 mg/kg) were injected intraperitoneally 6 hours and 1 hour before the mice were killed, respectively. Ornithine decarboxylase activity was determined as described (*4*) with L-[1-^{14}C]ornithine (New England Nuclear) being used as substrate. All assays were carried out under conditions in which the activity was proportional to the amount of protein added and to the time of incubation. Results were expressed as units of enzyme per milligram of protein or per gram, wet weight, of tissue. One unit catalyzed the release of 1 nmole of CO_2 per 30 minutes at 37°C. Protein was determined by the method of Bradford (*13*). Activity of the enzyme in brain and liver was reduced to undetectable levels by cycloheximide treatment. At a dose of 1 mg/kg, [5-^{14}C]DFMO produced exactly the same inhibition of ornithine decarboxylase activity (90 percent) as the unlabeled drug.

Tissue	Units of ornithine decarboxylase activity	
	Per milligram of protein	Per gram wet weight
Brain	0.009	0.15
Liver	0.015	1.05
Kidney	175.0	4900.0
Kidney (cycloheximide treated)	9.0	257.0
Kidney (DFMO treated)	18.0	492.0



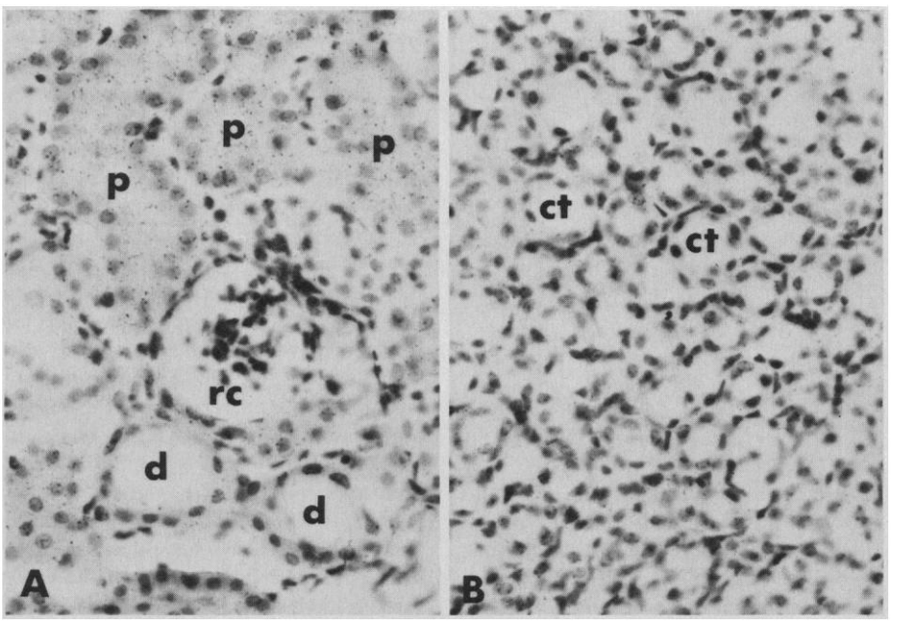


Fig. 1 (left). Autoradiographs of organs from mice treated with [^{14}C]DFMO as described in Table 1. Tissues were perfused with 10 percent neutral buffered Formalin, embedded in polyester wax and sectioned at 10 μm. Autoradiographic sections were incubated for 4 weeks with DuPont x-ray film. The autoradiographs shown were printed directly from the x-ray film (×2.5). (A) Androgen-treated mouse kidney with the cortex (*c*) and medulla (*m*) indicated. (B) Cycloheximide-treated mouse kidney. (C) Liver. (D) Brain, showing a midsagittal section with cerebellum (*cb*) and olfactory bulb (*o*) marked. Fig. 2 (right). Histological autoradiographs from the androgen-induced kidney of mice treated with [^{14}C]DFMO. Tissues were treated as in Fig. 1 and the sections were incubated for 6 weeks at 4°C in Kodak NTB-2 emulsion. (A) A section of cortex showing extensive labeling of the proximal convoluted tubules (*p*) and much less activity over the renal corpuscle (*rc*) and distal convoluted tubules (*d*). (B) A section of medulla with only sparse labeling over the collecting tubules (*ct*) (×345).

decrease in labeling when tissues of mice treated with cycloheximide were used. Recently, an attempt was made to determine the distribution of ornithine decarboxylase by the use of conjugates of DFMO with rhodamine or biotin (*8*). This method depends on the specific binding of such derivatives to ornithine decarboxylase. This was not demonstrated unequivocally and appears unlikely since ornithine decarboxylase has little affinity for substrate analogs with additions to the amino groups (*9*). The method also depends on the maintenance of ornithine decarboxylase activity in sections fixed with glutaraldehyde and formaldehyde or on the passage of the DFMO conjugates across the cell membrane. The present use of labeled DFMO avoids these difficulties.

The histological autoradiographs obtained in the present study suggest that ornithine decarboxylase in the proximal tubule cells occurs predominantly in the cytoplasm. This is in agreement with many studies in which enzyme activities were assayed in extracts from various subcellular fractions, but there have been a few reports of a nuclear location for ornithine decarboxylase [see (*10*)]. Our results do not rule out the possibility that a small proportion of the enzyme is present in organelles such as the nucleus. Studies with [^{3}H]DFMO might help to resolve this question.

The induction of a number of proteins in the mouse kidney in response to androgens is well established and it is known that androgen administration results in hypertrophy of the proximal tubule cells and an increase in the content of microsomal and lysosomal β-glucuronidase (*11*). The present results indicate that the induced renal ornithine decarboxylase, a cytoplasmic enzyme, is also present in the proximal tubules. These results strengthen the association between the activity of this enzyme and cellular hypertrophy. Androgens produce significant increases in RNA synthesis in the mouse kidney, and a number of messenger RNA's that are specifically induced by androgens have been isolated although not all of the gene products are identified (*11*). The mouse kidney should, therefore, provide a valuable system in which to study the induction of ornithine decarboxylase.

Our results contrast with a report that rat kidney ornithine decarboxylase is located predominantly in the medullary region as determined by dissection of the kidney and measurement of the activity in extracts prepared from homogenates of the cortex and medulla (*12*). Ornithine decarboxylase is not androgen dependent in the rat kidney and rat kidneys do not show the marked hypertrophy of the proximal tubules observed in the mouse.

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Muscular Contraction: Kinetics of Crossbridge Attachment Studied by High-Frequency Stiffness Measurements

Abstract. *Instantaneous stiffness of frog skeletal muscle, an indication of the proportion of attached crossbridges, was determined during the tetanus rise and after a step length change imposed during the tetanus plateau. During the onset of contraction as well as after a step, the ratio of stiffness to force differed from that determined during the tetanus plateau. The data after a step are predicted by the Huxley-Simmons model of muscular contraction, but the results during the rise suggest that a long-lived state may exist between crossbridge attachment and force generation.*

It is widely accepted that the contractile force of a striated muscle fiber develops because of interactions between actin and myosin filaments in each half-sarcomere. These interactions also produce an increase in fiber stiffness, which is thought to be directly proportional to the number of attachments (crossbridges) between actin and myosin filaments (*1, 2*). To estimate the number of crossbridges attached during the rise of tetanic force and during the phase of quick recovery from a step length change imposed on the tetanus plateau, we determined the moment-to-moment relation between stiffness and force. Our data indicate that the ratio of stiffness to force is higher during the quick recovery phase than during the plateau, which is predicted by the theory Huxley and Simmons (*1*) proposed to account for the molecular mechanism of contraction. However, we observed that the rise in stiffness preceded the development of force during the onset of contraction. This may be due to the existence of a long-lived crossbridge state between attachment and force generation.

To determine stiffness we applied small sinusoidal length changes (amplitude, 0.02 to 0.05 percent of fiber length; frequency range, 1 to 9 kHz) to one end of a fiber and recorded the corresponding changes in force at the other end. Stiffness was calculated as a ratio between the force and length sinusoids. In order to compare our data with some of the data in the literature (*3*), we also used step length changes to determine stiffness. The forces recorded at the end of step length changes of various amplitudes were plotted against the sizes of the length changes. We thus obtained "T_1 curves" (*1*), which allowed us to estimate the amount of compliance in the end attachments of our preparations by comparing our results to those obtained by Ford *et al.* (*3*), who used a servo system to control the length of a fiber segment between two markers. The in-

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