

# DOPAMINE METABOLISM FOLLOWING IRREVERSIBLE INACTIVATION OF AROMATIC AMINO ACID DECARBOXYLASE IN RETINA<sup>1</sup>

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## Abstract

The effects of an intravitreal injection of  $\alpha$ -fluoromethyl-dopa, an irreversible mechanism-based inactivator of aromatic L-amino acid decarboxylase, on the retinal dopamine content of light-adapted chicks and rabbits have been examined. A single administration of 10 nmol of  $\alpha$ -fluoromethyl-dopa totally inactivates aromatic L-amino acid decarboxylase within 2 hr *in vivo* in rabbits. By 4 to 6 hr, the level of dihydroxyphenylalanine increased 7-fold and the levels of dopamine and dihydroxyphenylacetic acid fell by 90%. With an injection of 50 nmol, similar results were observed in chicks. The levels of dihydroxyphenylacetic acid began to fall soon after injection when significant (30 to 40%) amounts of dopamine were still present. These results are discussed in relation to the use of tissue dihydroxyphenylacetic acid levels to indicate the level of dopaminergic neuronal activity or dopamine synthesis. Recovery by the retinae of both species was shown by the return of aromatic L-amino acid decarboxylase activity and the resynthesis of dopamine.

A considerable amount of information about the functions of neurotransmitters in the peripheral and central nervous systems has been obtained by studying the effects of drugs which interfere with their synthesis or degradation. Interpretation of these results can be complicated by drug actions other than those desirable for the particular purpose in mind. For example, *p*-chlorophenylalanine (PCPA)<sup>3</sup> is used to block tryptophan hydroxylase *in vivo* and the resultant decline in tissue 5-hydroxytryptamine (5-HT) is used to give a measure of the rate of turnover (Kore and Weissman, 1966). However, PCPA also will interfere with catecholamine biosynthesis (Sanders-Bush et al., 1974). Similarly, brocresine (NSD 1055) is used to inhibit aromatic L-amino acid decarboxylase (L-AAAD) to study the rate of synthesis

and turnover of the biogenic amine neurotransmitters, but it also inhibits histamine biosynthesis (Moles and Sheperd, 1973).

Mechanism-based irreversible inactivators largely overcome the problem of specificity (Rando, 1974). These inactivators are substrates for the target enzyme, but they also contain latent reactive groupings within their structure. It is not until the enzyme metabolizes the inactivator that the reactive grouping is unmasked and the enzyme is inactivated. (*S*)- $\alpha$ -Fluoromethyl-dopa ( $\alpha$ -FMD) is an inhibitor of this type which inactivates L-AAAD (Kollonitsch et al., 1978).

Clearly, inhibition of L-AAAD by  $\alpha$ -FMD will block the formation of all catecholamines and indoleamines since this enzyme is common to all of their biosynthetic pathways (McGeer and McGeer, 1973). This complication is less important in tissues where only one of these amines is present as a neurotransmitter.

Of the biogenic amines that appear to act as neurotransmitters in the brain, only for dopamine is there strong evidence for a role in the vertebrate retina. With retinae from various species, it has been shown that dopamine is present in appreciable quantities, is synthesized by the intact retina, and is released by a  $Ca^{2+}$ -dependent mechanism (see Ehinger, 1976; Starr, 1977 for reviews). In addition, dopamine and some of the drugs which affect its actions have effects on the activity of some retinal neurons (Ames and Pollen, 1969). In the rabbit and chick retinae, histochemical methods have localized dopamine to a defined subset of amacrine cells (Ehinger, 1967; Haggendal and Malmfors, 1965). Norepi-

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<sup>3</sup> The abbreviations used are: L-AAAD, aromatic L-amino acid decarboxylase; DOPA, dihydroxyphenylalanine; DOPAC, dihydroxyphenylacetic acid; EDTA, ethylenediaminetetra-acetate;  $\alpha$ -FMD,  $\alpha$ -fluoromethyl-dopa; 5-HT, 5-hydroxytryptamine; MAO, monoamine oxidase; PCPA, *p*-chlorophenylalanine; Tris, tris(hydroxymethyl)aminomethane.

nephrite has not been found in significant amounts in the retinae of those species so far studied (Haggendal and Malmfors, 1965; Nichols et al., 1967). There is, however, some evidence to suggest a neurotransmitter function for 5-HT in chick and bovine retina (Suzuki et al., 1977; Thomas and Redburn, 1979) but not in rabbit (Floren and Hansson, 1980).

The retina is an unusually favorable tissue for investigations of neurotransmitter function and metabolism. It is a relatively simple structure and its morphology has been much studied by both light and electron microscopy. Because it is located at the periphery, it can be removed with little damage for incubation *in vitro* or selectively exposed to test compounds *in vivo* by their injection into the eye. Finally, the retina can be activated by natural stimuli and thus provides both a means of identifying the effects of neurotransmitter release in physiological experiments and of controlling the demand for neurotransmitters in biochemical ones. We therefore sought to characterize the effects of  $\alpha$ -FMD on retinal L-AAAD and dopamine. We report that this compound rapidly inactivates L-AAAD when intravitreally administered to rabbits and chicks and demonstrate the resultant dramatic effect on the retinal content of dopamine, dihydroxyphenylalanine (DOPA), and dihydroxyphenylacetic acid (DOPAC).

### Materials and Methods

Young adult New Zealand white rabbits (2 to 3 kg) were obtained from Margarets Home Farm, Attleboro, MA and 12-day-old white Leghorn chicks were obtained from Spafas, Inc., Norwich, CT. All animals were exposed to normal fluorescent room lighting for at least 16 hr before and throughout any experiment. Animals were injected intraocularly under ether anesthesia with 10  $\mu$ l of drug solution dissolved in 0.9% (w/v) saline which had been sterilized by membrane filtration (0.45- $\mu$ m pores). Rabbits were killed by cervical dislocation and chicks by decapitation. The eyes were enucleated, the cornea was cut away with a razor blade, and the vitreous body was removed. The eyecup was cut into two portions through the optic nerve and the half-retinae were teased out under a dissecting microscope. The tissue was stored at  $-70^{\circ}\text{C}$  in preweighed 1.5-ml microcentrifuge tubes until required for assay.

Dopamine, its metabolites, and  $\alpha$ -FMD were assayed by a combination of high pressure liquid chromatography and electrochemical detection after isolation of catechols on alumina (Mefford et al., 1980). Briefly, half-retinae were sonicated in cold 0.1 M perchloric acid (100  $\mu$ l for chick and 200  $\mu$ l for rabbit) containing 0.4 mM sodium bisulfite, 0.1 mM disodium EDTA, and 3 ng of dihydroxybenzylamine as the internal standard and centrifuged at  $27,000 \times g$  for 30 min. To 100  $\mu$ l of the supernatant, 10 mg of acid-washed alumina and 1 ml of 0.5 M Tris-HCl, pH 8.6, were added and the 1.5-ml microcentrifuge tubes were shaken mechanically by inversion for 10 min. The tubes were centrifuged, the supernatant was aspirated off, and the alumina was washed with two 1-ml aliquots of distilled water. Catechols were eluted by agitation of the alumina with 100  $\mu$ l of 0.1 N HCl for 10 min and then centrifuged. Usually 30  $\mu$ l of the eluate was injected onto

the liquid chromatograph. The mixture was resolved by reverse phase ion pair chromatography on a RP-18 guard column (3  $\times$  0.46 cm, Brownlee) and  $\mu$ Bondapak C<sub>18</sub> (30  $\times$  0.4 cm, Waters) analytical column through which was pumped 50 mM sodium phosphate, pH 3.5, containing 0.1 gm/liter of sodium heptane sulfonic acid, 8% (v/v) methanol, and 0.1 mM disodium EDTA at 1 ml/min. Catechols in the eluate were assayed electrochemically with a TL-3 flow cell packed with carbon paste (silicone grease base) and connected to an LC-3 controller (Bioanalytical Systems). The working electrode was operated at 0.60 V. Authentic samples of DOPA,  $\alpha$ -FMD, dopamine, and DOPAC were carried through the extraction procedure with the internal standard added. Tissue levels of a compound were computed by the internal standard-peak height ratio method and expressed as nanograms per gm of tissue, wet weight. The limits of sensitivity as given by a peak height of 5 times base line noise were 30 to 50 pg per injection for DOPAC and 20 to 25 pg for other catechols. The efficiency of extraction was about 60% for the internal standard, 37% for DOPA and  $\alpha$ -FMD, 40% for dopamine, and 25% for DOPAC.

L-AAAD activity in retinal homogenates was assayed by measuring the release of  $^{14}\text{C}$  from L-[1- $^{14}\text{C}$ ]DOPA. Half-retinae of either species were sonicated in 200  $\mu$ l of cold 5 mM sodium phosphate, at pH 7, containing 10 mM 2-mercaptoethanol and centrifuged at  $27,000 \times g$  for 30 min. Aliquots of the supernatant were dispensed into 6  $\times$  50 mm glass test tubes on ice and made up to 50  $\mu$ l with homogenization buffer. To this was added 50  $\mu$ l of a solution containing 0.1 M sodium phosphate, pH 7.0, 56  $\mu$ M pyridoxal 5-phosphate, 20 mM 2-mercaptoethanol, 1 mM L-DOPA, and 2  $\mu$ Ci/ml of DL-[1- $^{14}\text{C}$ ]DOPA. The tubes were placed on 20-ml glass scintillation vials containing 600  $\mu$ l of 1 N NaOH and the vials then were closed with rubber septum caps. The vials were incubated for 1 hr at  $37^{\circ}\text{C}$  and then 100  $\mu$ l of 10% trichloroacetic acid was injected into the assay mixture through the septum. The vials were incubated further for 1 hr at  $37^{\circ}\text{C}$  and allowed to cool to room temperature. Then 500  $\mu$ l of the NaOH was removed for the determination of absorbed  $^{14}\text{C}$  content by liquid scintillation spectrometry after addition of 5 ml of Aquasol (New England Nuclear). To ensure good emulsion formation with the Aquasol, the alkali was partly neutralized by addition of 50  $\mu$ l of 3 N HCl. Under these conditions, the assay was linear with time up to 1 hr and with protein up to 500  $\mu$ g. Enzyme activity was expressed as nanomoles of  $^{14}\text{C}$  formed per mg of protein per hr after allowing for the fact that 50% of the radioactivity in the substrate was in the D isomer and therefore was not available to L-AAAD.

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

### Results

Preliminary experiments indicate that an injection of 10 to 15 nmol of  $\alpha$ -FMD into a rabbit eye of about 1.5 ml volume was the minimum dose to inhibit L-AAAD in retina maximally within 4 hr. Assuming uniform dispersal throughout the posterior eye chamber, this gives a concentration of about  $7 \times 10^{-6}$  M, which agrees well with the concentration,  $10^{-5}$  M, required to give maximal in-

hibition in retinal homogenates (Fig. 1). The short term effects of an injection of 10 nmol of  $\alpha$ -FMD are shown in detail in Figures 2 and 3. Retinal levels of  $\alpha$ -FMD did not reach a maximum value until 4 hr after injection and thereafter fell exponentially with a half-time of about 4 hr. Within 2 hr, L-AAAD activity had been reduced to blank values and, at 24 hr, there was no measurable recovery of the enzyme (Fig. 2). Dopamine levels were reduced by 70% within 2 hr and by 95% at 8 hr (Fig. 3). The level of dopamine appeared to begin to recover at 24 hr, but the value at this time point is not significantly different from the values at the preceding two time points. Calculated from the data for 2 and 4 hr after  $\alpha$ -FMD, the half-life of dopamine in this experiment was 41 min. Levels of the dopamine metabolite DOPAC were depleted in a similar manner after  $\alpha$ -FMD treatment (Fig. 3). On the other hand, the retinal content of DOPA, which is the normal substrate for L-AAAD, underwent an apparently linear increase that plateaued 4 hr after the  $\alpha$ -FMD injection at about 7 times the control values. In long term experiments, it was found that it took up to 16 days for L-AAAD levels to return to normal after total inhibition by  $\alpha$ -FMD. Assuming that L-AAAD is degraded by a first order process and synthesized by a zero order mechanism, this enzyme has a half-life of about 4 days in the retina. In these experiments, dopamine levels began to approach control values after 48 hr, when the L-AAAD activity had reached only 15 to 20% of control.

That the levels of the dopamine returned to normal after exposure of the retina to  $\alpha$ -FMD is evidence that the cells containing dopamine were not destroyed by the experimental treatment. To look for possible morphological changes, we injected  $\alpha$ -FMD into rabbit eyes three times at intervals of 3 days. One week after the last

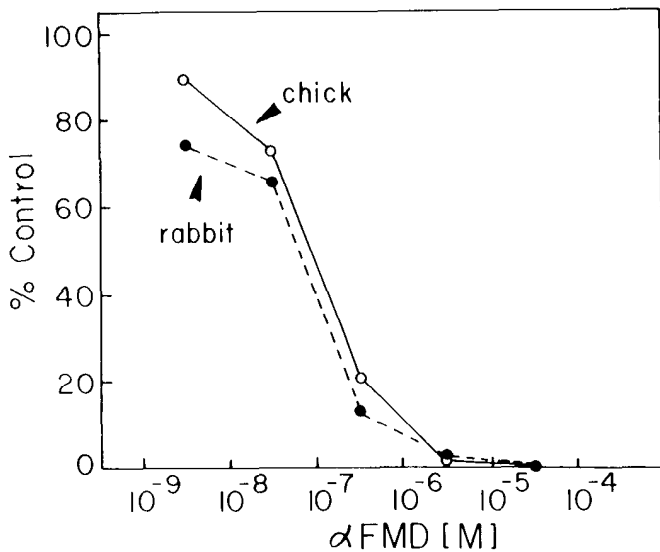


Figure 1. The effect of increasing concentration of  $\alpha$ -FMD on the L-AAAD activity of rabbit and chick retinal homogenates. Aliquots of homogenate were incubated for 30 min at 37°C with the indicated concentrations of  $\alpha$ -FMD before adding L-DOPA (final concentration, 0.5 mM) and assaying for residual L-AAAD activity. The results are expressed as the percentage of the enzyme activity observed when incubated in the absence of the inhibitor.

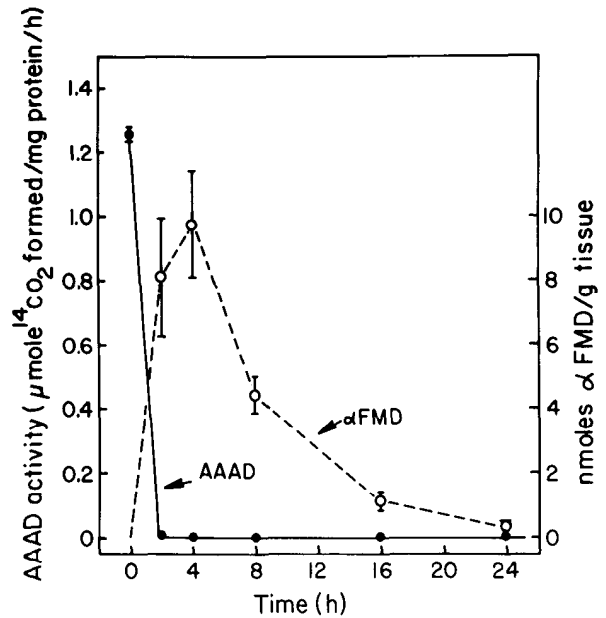


Figure 2. The retinal content of  $\alpha$ -FMD after a single intravitreal injection of 10 nmol and the effects on L-AAAD activity in young adult rabbits. Each point is the mean ( $\pm$ SEM) of data from four retinae. The data for L-AAAD activity for 2 to 24 hr after the injection was not distinguishable from the blank value. Animals were light-adapted for 24 hr before the start of the experiment.

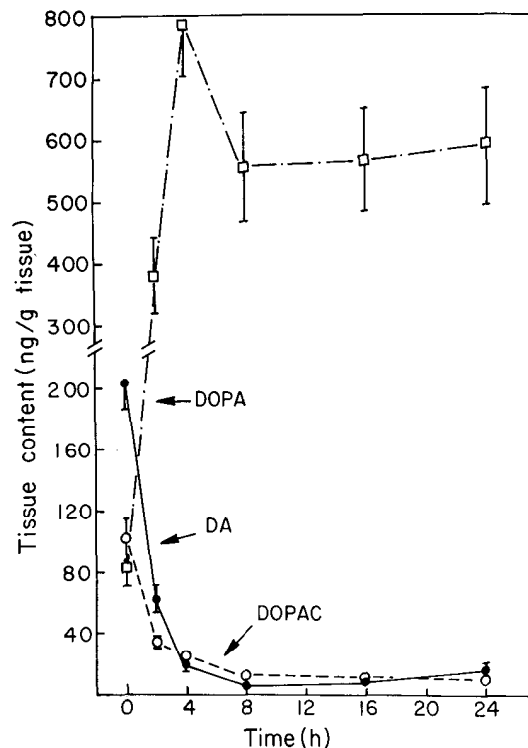


Figure 3. The effect of a 10-nmol injection of  $\alpha$ -FMD (intravitreally) on the retinal content of dopamine (DA), DOPA, and DOPAC in young adult rabbits. Each point is the mean ( $\pm$ SEM) of data from the same four retinae from which Figure 1 was derived.

injection, the retinae were removed, fixed in glutaraldehyde, dehydrated in graded ethanols, and embedded in glycol methacrylate resin. The structure of the treated retinae was indistinguishable, at the level of light microscopy, from that of untreated controls.

In the chick, an intravitreal injection of 50 nmol of  $\alpha$ -FMD was the minimum dose that would inhibit retinal L-AAAD totally within 2 hr. The  $\alpha$ -FMD was eliminated more rapidly than that from rabbit retina, with a half-life of about 1 hr. Figure 4 shows the short term effects of this dose on dopamine content in chick retina and the activity of L-AAAD. Retinal dopamine levels were reduced by 85% 2 hr after this treatment; within 4 hr, the levels were at the limits of sensitivity of the assay (i.e., about 300 pg/retina). The approximate half-time for this loss of dopamine was 1 hr. The recovery of dopamine synthesis by chick retina was studied after two injections of 50 nmol of  $\alpha$ -FMD as shown in Figure 5. Chick retinal L-AAAD was beginning to reappear 12 hr after the last injection and then steadily increased in activity up to 35% of untreated values 36 hr later. From these data, a half-life of about 3 days was calculated for L-AAAD in chick retina. At 12 to 16 hr, L-AAAD activity, dopamine, and DOPAC again could be detected. By 24 hr, dopamine and DOPAC levels had reached 50% of control values when only 10% of L-AAAD had recovered, and at this time, L-DOPA had fallen from 10- to 2-fold of untreated levels. The retinal dopamine content had reached untreated values after 48 hr. The levels of DOPAC paralleled the increase in retinal dopamine up to 36 hr and then appeared to reach a steady value at about 60% of control.

### Discussion

Our results show that, after inhibition of synthesis with  $\alpha$ -FMD, retinal dopamine levels can be reduced by greater than 90% within 4 hr in light-adapted chicks and rabbits. In chicks, the dopamine level was reduced from

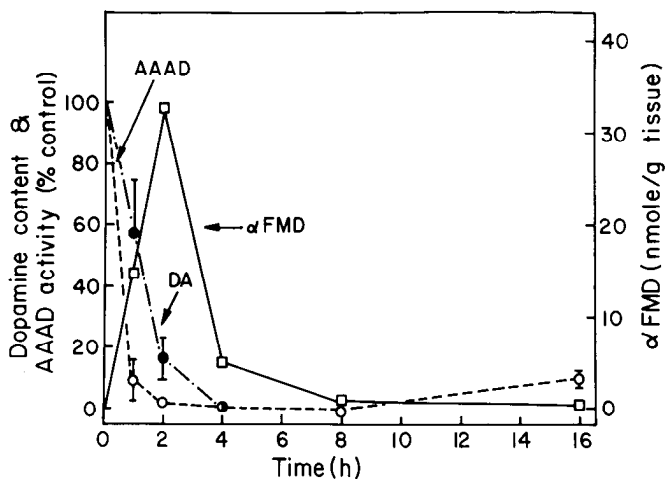


Figure 4. The effect of a 50-nmol injection of  $\alpha$ -FMD (intravitreally) on the retinal content of  $\alpha$ -FMD and dopamine (DA) and on L-AAAD activity in 12-day-old chicks. Each point is the mean of data from four animals except that for  $\alpha$ -FMD which is the mean of data from two animals. The animals were light-adapted for 24 hr before the experiment.

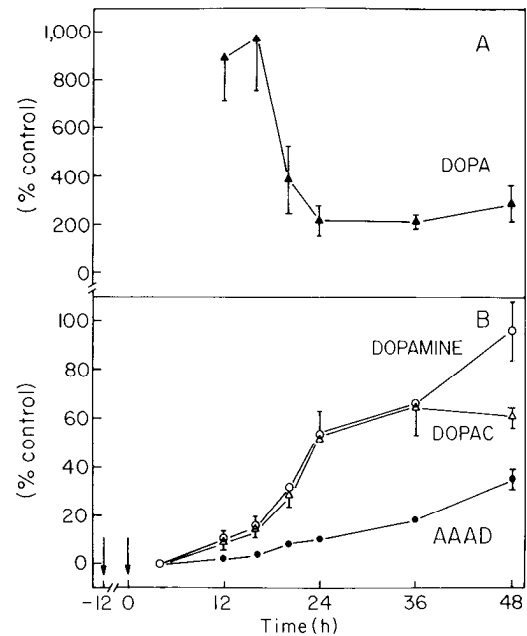


Figure 5. The recovery of L-AAAD activity and dopamine synthesis after  $\alpha$ -FMD. Light-adapted 12-day-old chicks received intravitreal injections of 50 nmol of  $\alpha$ -FMD 12 hr before and at the beginning of the experiment. Each point is the mean ( $\pm$ SEM) of data from four animals expressed as a percentage of the result for the untreated contralateral eye. Animals were kept in normal room light throughout the experiment. In panel A, the data for DOPA are presented. In panel B, the data for dopamine, DOPAC, and AAAD recovery are presented.

control levels of 100 to 120 ng/gm of tissue to the limits of sensitivity of the assay (about 2 to 3 ng/gm of tissue). Rabbit retinae contain about 200 ng of dopamine/gm of tissue. After treatment with  $\alpha$ -FMD, this level fell to 10% of control values (20 ng/gm of tissue) after 4 hr and to about 5% (5 to 10 ng/gm of tissue) after 8 and 16 hr. The small amount of dopamine may represent synthesis by uninhibited L-AAAD beyond the limits of the *in vitro* assay. The recovery of dopaminergic function *in vivo* after treatment with  $\alpha$ -FMD should depend upon the rate of *de novo* synthesis of L-AAAD protein. In chick, recovery of dopamine synthesis as indicated by the reduction of the elevated DOPA levels as well as increased dopamine content was apparent within 16 hr of the treatment with  $\alpha$ -FMD when only a small proportion of L-AAAD activity had returned. This confirms that, *in vivo*, this enzyme is not the rate-limiting step in the synthesis of dopamine (Iuvone et al., 1978). In the experiments with rabbits, dopamine levels had not recovered 24 hr after  $\alpha$ -FMD, but recovery was observed at later times. The slower recovery of dopamine in rabbit retina compared to that in chick retina may result from several factors. The rate of elimination of  $\alpha$ -FMD from the retina, and therefore probably from the intravitreal injection site, was about 4 times faster in chicks than in rabbits. This may be due to the smaller volume of the eye in 12-day-old chicks ( $\sim 0.5$  ml) compared to that in young adult rabbits ( $\approx 1.5$  ml). This would lengthen the time during which sufficient  $\alpha$ -FMD would be present in the retina to inhibit newly synthesized L-AAAD in rab-

bits. Secondly, the rate of synthesis of L-AAAD was slightly faster in chicks and, in the early phase of recovery, this would speed transmitter recovery.

The observation that dopamine synthesis recovers in both species along with L-AAAD activity suggests that the depletion of dopamine after  $\alpha$ -FMD treatment is the result of a specific lesion (i.e., inhibition of L-AAAD) and is not due to some cytotoxic effect. This is supported further by the unchanged retinal morphology observed in the light microscope when rabbits were repeatedly injected intravitreally with  $\alpha$ -FMD.

Metabolism of dopamine in nervous tissues can lead to the formation of two acidic metabolites. DOPAC is formed by the action of monoamine oxidase (MAO) on dopamine while homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) is produced by the combined action of MAO and catechol O-methyltransferase (Sharma, 1973). It has been suggested that, in the corpus striatum, DOPAC and homovanillic acid reflect intraneuronal and extraneuronal metabolism of dopamine, respectively (Roffler-Tarlov et al., 1971). The tissue levels of these metabolites have been used, therefore, as indices of the amount of dopaminergic neuronal activity (Roffler-Tarlov et al., 1971; Walters and Roth, 1972; Westerink and Korf, 1976; Beart and Gundlach, 1980). The basis of this approach is the assumption that the cytoplasmic level of dopamine in the neuron is low except when large amounts of dopamine are being recaptured after periods of high activity. The level of cytoplasmic dopamine then would reflect the rate of dopamine release, and since MAO can act only on the cytoplasmic dopamine, the level of DOPAC would be related directly to the neuron's activity.

We observed that, after treatment with  $\alpha$ -FMD, the retina's content of DOPAC fell (and rose again) in parallel with its content of dopamine: 2 hr after treatment with  $\alpha$ -FMD, dopamine stores had been depleted by 75% and retinal DOPAC levels were reduced by about 70%. What does this mean for the relation between DOPAC and activity? Classically, it has been thought that the packaging of neurotransmitter into vesicles provides a mechanism by which neurotransmitter release can remain constant in the face of moderate changes in the presynaptic content of neurotransmitter (Potter, 1970). This has been supported by experiments in which tissue amine levels were reduced by reserpine; it appears that severe depletion (>80%) of the transmitter is necessary to impair neuronal transmission (Muscholl and Vogt, 1958; Carlsson, 1966; Gillespie and McGrath, 1974). If this is correct, the neurotransmitter release in our experiments still should have been occurring at near normal levels when dopamine had been only partly depleted and, therefore, DOPAC levels should not have fallen.

An explanation for our finding is that there are, in fact, two sources of the intracellular dopamine on which MAO acts—re-uptake after release and new synthesis. The parallel rise and fall of dopamine and DOPAC content after  $\alpha$ -FMD treatment suggests that DOPAC more accurately reflects the rate of synthesis of dopamine than the rate of its release. Since increased activity leads to increased transmitter synthesis in dopaminergic neurons (Murrin and Roth, 1976), the use of DOPAC levels as a

measure of neuronal function is thus not necessarily invalidated if our interpretation is correct. It is possible, in addition, that more than 20% of neuronal stores are necessary to maintain the normal level of neurotransmitter release in the dopaminergic neurons of the retina. An investigation of the effects of tyrosine hydroxylase inhibitors on the tissue content of DOPAC and homovanillic acid should help to resolve this problem.

In conclusion, our experiments have shown that inhibition of L-AAAD by  $\alpha$ -FMD produces a rapid and dramatic depletion of retinal dopamine and DOPAC in light-adapted rabbits and chicks. The tissues recover from the effects of  $\alpha$ -FMD when new enzyme protein is synthesized. The use of  $\alpha$ -FMD as a tool to investigate the synthesis and turnover of indoleamine neurotransmitter in chick retina is described in the accompanying paper (Parkinson and Rando, 1981).

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