do not exhibit the extensive species differences found with the serum enzyme [6].

The differences in the affinities of DFP and propoxur for the two enzymes are not surprising, since the chemical structure of these inhibitors is quite different. On the other hand, the significant variation in the contribution of affinity to inhibition suggests that attempts should be made to measure the individual inhibition constants before attributing a variation in inhibition rates to differences in affinity.

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Effect of fighting and diphenylhydantoin on the uptake of ^{3}H-*l*-norepinephrine *in vitro* in synaptosomes isolated from retired male breeding mice

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In a recent study by Hendley *et al.* [1] significant increases in maximum velocity (V_{max}) and the Michaelis constant (K_m) for norepinephrine (NE) uptake in cortical homogenates were observed after the last of a series of short, intense daily fights between male mice made previously aggressive by long-term individual caging. Further observations after acute fighting showed a reversible decrease in affinity for NE uptake but no significant increase in uptake velocity [2]. The present study was likewise designed to investigate changes in central NE kinetics in acutely fighting animals, but in this instance, the effect of the drug, diphenylhydantoin (DPH, Dilantin), on NE uptake was also assessed.

The choice of DPH as a pharmacologic agent in the present study stems from earlier work reported from our laboratory in which a significant decrease in the uptake of NE in isolated rat synaptosomes incubated in physiologic media was shown after adminstration of DPH both *in vitro* [3] and *in vivo* [4]. A DPH effect on the uptake of NE in brain slices or subcellular preparations has since been confirmed by others [5, 6].

DPH was also of interest to us, since it has been observed to prevent fighting in the present animal model* as well as in electro-shocked mice [7,8]. Furthermore, there have been reports that DPH reduces aggresive behavior and anger in humans [9, 10], although studies with a contrasting conclusion have been reported [11].

In the present experiments, white male Swiss CD-1 retired breeders (Charles River Mouse Farms) were used. Since these animals often fight spontaneously,* procedures such as prolonged solitary confinement, electric shock or pharmacological manipulations are not needed to elicit aggression. The animals were divided into two separate groups upon receipt because fighting occurred more readily between strange animals than in cage-mates. After 3 days, combat pairs were obtained by taking one animal from each group and then placing them together in a confined space where they were permitted to fight vigorously. However, as with every aggressive animal model, all combat pairs did not fight equally well. Therefore, in order to obtain uniform results, it was sometimes necessary to maintain or induce fighting by applying pressure to the backs or tails of the animals with forceps. After sustained fighting for 5 min, the animals were extremely excited and there was a marked increase in respirations and heart rate. At this point, the combatants were immediately decapitated and their brains were removed within 30 sec and placed in ice-cold buffer. Pooled control brains were obtained from similar animals that were not allowed to fight.

Whole brain synaptosomes from fighting and non-fighting mice were prepared by the method of Whittaker [12]. Synaptosomal protein (0·15 to 0·3 mg per assay [13]) was preincubated in an oxygenated Krebs–Henseleit buffer for 5 min with and without 10^{-4} M DPH at 37°. An identical set of samples was incubated at 0° to serve as blanks. Then 0·05 to 2×10^{-6} M tritiated NE (New England Nuclear Corp.; spec. act., 7·2 Ci/m-mole) was added. After 5 min of additional incubation, uptake in the experimental samples was terminated by rapidly chilling the samples to 0°. After one centrifugation and washing, the pellet was solubilized (NCS-Amersham Searle), fluor (PPO/POPOP† in toluene base) was added and the samples were counted.

The uptake of ^{3}H-NE was significantly altered by fighting and by DPH in synaptosomes isolated from white Swiss male mice retired from breeding activity. The double reciprocal plot of 1/net uptake velocity in nmoles/g of synaptosomal protein/5 min) versus 1/tritiated norepinephrine, 10^{-6} M, gives the lines as shown by a linear regression least squares analysis of the data (Figs. 1 and 2). The slope and intercept were computed and, from them, V_{max} and the K_m were calculated. Each data point represents the mean value of two experiments, except for the point at 0·084 μM NE for fighting control mice. The latter data point represents a single value. The paired experiments gave values which were within a range up to 8 per cent of their mean, except for the members of one pair which were within 17 per cent of their mean.

* S. Bogoch and M. Baldwin, personal communication

† PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-2-(4-methyl 5-phenyloxazolyl) benzene.

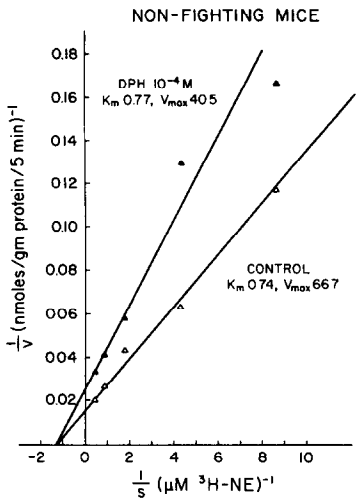

Fig. 1. Non-fighting mice. Double reciprocal plot of v (in nmoles of *l*-norepinephrine taken up per g of synaptosomal protein per 5 min) against s, the μM concentration of *l*-norepinephrine.

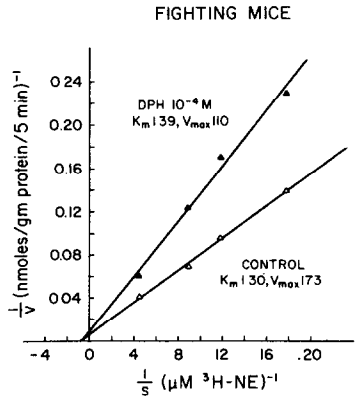

Fig. 2. Fighting mice. Double reciprocal plot of v (in nmoles of *l*-norepinephrine taken up per g of synaptosomal protein per 5 min) against s, the μM concentration of *l*-norepinephrine.

Figure 1 represents resting or non-fighting animals. Three facts are determined from this graphical analysis. (1) The K_m is the same for non-fighting animals with and without DPH (0·77 vs 0·74 μM); that is, the affinity of the NE carrier for its substrate is not affected by DPH. (2) The V_{max} values are different, the control value being higher than that obtained with DPH (61 per cent of control); this reflects DPH inhibition of NE uptake. (3) The type of inhibition is noncompetitive.

In Fig. 2 the kinetics of NE uptake in fighting mouse synaptosomes in the presence and absence of DPH are presented. It is immediately apparent that the relationship of the two lines is similar to that observed for non-fighting mice, as seen in Fig. 1. However, both K_m and V_{max} are increased compared to the non-fighting values. Increased K_m actually means decreased affinity of the carrier for the substrate, but V_{max} means a larger number of carrier sites are apparently available per unit time.

Again DPH inhibited V_{max} (64 per cent of control) but yielded no change for the apparent Michaelis constant (1·39 vs 1·30 μM). Therefore, the DPH inhibition of NE uptake was noncompetitive, as it was in experiments which employed non-fighting mice. Furthermore, the degree of inhibition of NE uptake in fighting (64 per cent of control) and non-fighting (61 per cent of control) synaptosome preparations was essentially the same. In both instances the inhibitory effect of DPH was significant. The regression analysis of the data to test for coincidence of the two lines indicated that DPH incubation did not result in the same linear function as that of untreated controls ($P < 0.01$ for non-fighting mice and $P < 0.0001$ for fighting mice).

These results demonstrate an adaptive change in the membrane affinity for NE after an acute stress situation. The adaptation is apparently at two loci. First, the increased K_m for NE in fighting as compared with non-fighting animals reflects a decreased affinity of the NE carrier for its substrate. The second change, increased V_{max}, may indicate an increased number of NE carriers or greater utilization of existing carriers.

The present data essentially confirm the NE uptake data of Welch *et al.* [1, 2], which were obtained by using mouse cortical homogenates. However, the values given by our preparation are higher, reflecting the use of purified synaptosomes from whole brain and measurements in terms of mg protein.

The observation that DPH inhibits NE uptake in synaptosomes incubated in physiologic media substantiates our previous findings [3, 4] and the present kinetic analysis demonstrates that this inhibition is noncompetitive. This implies that DPH does not alter uptake of NE by interacting directly with the active site of the NE carrier. Rather, a noncompetitive inhibition implies that there may be metabolic inhibition or alteration of the membrane in such a way as to impede the facilitated transport of NE across the membrane.

In previous communications we speculated upon possible mechanisms of DPH inhibition of NE uptake in synaptosomes [2, 3]. These included alterations in ATPase activity, which has been implicated in the transport and storage of catecholamines [14, 15]. Moreover, it has been shown that ATPase is both stimulated [16] and inhibited [16, 17] by DPH, depending upon the experimental conditions. Also considered was the possibility that alterations in electrolyte transport produced by DPH [18, 19] could be linked to changes in neurotransmitter kinetics [5].

In the present study the increase in uptake of NE in nerve terminals produced by fighting has been shown to be significantly decreased by DPH. This inhibition of NE uptake may represent the mechanism by which, in the studies previously cited, DPH abolished fighting in animals and in some instances reduced aggressive behavior and anger in humans.

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Accumulation of 4-hydroxyamphetamine by rat striatal homogenates*

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4-Hydroxyamphetamine (p-OHA) [1-(4-hydroxyphenyl)-isopropylamine], one of several amphetamine metabolites [1], is a sympathomimetic amine that has been implicated in some of the pharmacological actions of amphetamine [2–5]. The compound is a major metabolite of amphetamine in the rat and is found in the brain as well as the heart after systematic administration [4–6]. In addition, *p*-hydroxyamphetamine is an effective inhibitor of neuronal uptake of catecholamines in peripheral [7] and central neurons [8].

This report describes the behavior of *p*-hydroxyamphetamine-^{3}H(p-OHA-^{3}H) in rat striatal preparations that accumulate dopamine-^{3}H(DA-^{3}H). In this system, p-OHA is comparable to amphetamine as an inhibitor of dopamine uptake and is itself accumulated by the tissue. The nature of the accumulation suggests that it is the neuronal uptake process that is responsible.

The striata from six male Sprague–Dawley rats weighing 180–200 g were dissected and homogenized in 8 ml of 0·32 M sucrose. The homogenate was centrifuged for 10 min at 1000 *g* and the supernatant recentrifuged for 20 min at 11,000 *g*. The pellet obtained was resuspended in the same volume of isotonic sucrose and again centrifuged for 20 min at 11,000 *g*. The resulting pellet was resuspended by hand in 0·32 M sucrose to a final volume of 6 ml (protein concentration [9], 2·0–2·5 mg/ml).

A 0·1-ml aliquot of the suspension was added to 1·4 ml Krebs–Ringer phosphate containing 12·5 μM nialamide, 8·4 mM glucose, 1·1 mM l-ascorbate and the appropriate inhibitor. This mixture was allowed to preincubate for 3 min at 25° in a Dubnoff metabolic shaker. The tritiated amine was then added to a final concentration of 1 μM and the mixture incubated for 3 min at 25°. Uptake was quenched by the addition of 5 ml cold (0°) 0·9% NaCl that was 100 μM in unlabeled amine; this was followed immediately by filtration through a Millipore filter assembly. The incubation vessels and filters were washed with two 5-ml portions of quenching solution. The 0·8 μm Millipore filters were removed, placed in scintillation vials containing Bray's solution [10], and counted. Net uptake was calculated as the radioactivity retained by the filter less the radioactivity retained when the incubation was carried out at 0°. Each value was the mean of triplicates which usually had a standard deviation of 10 per cent.

Dopamine-^{3}H(10 Ci/m-mole) and amphetamine-^{3}H(6·2 Ci/m-mole) were obtained from the New England Nuclear Co., and *p*-hydroxyamphetamine-^{3}H (20 mCi/mg) was a generous gift from Dr. J. V. Dingell of the Department of Pharmacology, Vanderbilt University. The radiochemical purity of the *p*-hydroxyamphetamine was established to be greater than 95 per cent by thin-layer chromatographic (TLC) procedures.

The uptake described in these studies is the net accumulation occurring at 25° after subtraction of the accumulation at 0°. The rationale for this subtraction is the high temperature coefficient for membrane transport processes relative to diffusion [11]. In these experiments, the accumulation of DA and p-OHA at 0° was 10–20 per cent of that at 25°, while the accumulation of amphetamine at 0° was 90 per cent of that at 25°. The relative accumulations of DA, p-OHA and amphetamine are shown in Fig. 1. The uptake of DA is about 3 times that for p-OHA and 150 times that for amphetamine. The uptake of DA and p-OHA was not linear with time for more than 1 min, although uptake was still increasing at 3 min.

The effects of different inhibitory agents and experimental conditions on DA and p-OHA accumulation are summarized in Table 1. The accumulation was sensitive to osmotic shock, although the fraction liberated upon lysis was different for the two compounds in that p-OHA was much more completely released (77 per cent) than DA (46 per cent).

Table 1 also shows the results of experiments examining the interaction of the three compounds, p-OHA, ampheta-

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