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DISPOSITION AND BEHAVIORAL EFFECTS OF AMPHETAMINE AND β,β-DIFLUOROAMPHETAMINE IN MICE

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Abstract—β,β-Difluoroamphetamine, which has a lower pK value than amphetamine and so exists at physiological pH predominantly as a neutral molecule rather than as a cation, localized in epididymal fat to a greater degree than in brain in mice, in contrast to amphetamine. Difluoroamphetamine had a much shorter half-life in brain (19 min) than did amphetamine (51 min), and the half-life was not affected by a 4-chloro substituent (18 min), in contrast to that of amphetamine (261 min for 4-chloroamphetamine). The half-life of difluoroamphetamine and of 4-chloro-difluoroamphetamine was essentially the same in fat as in brain (16 and 20 min respectively). Due to its different distribution and its shorter half-life, difluoroamphetamine had to be given at higher doses to maintain brain levels comparable to those of amphetamine. Likewise, higher doses of the difluoroamphetamine were required for equivalent degrees of central nervous system (CNS) stimulation as measured by increased locomotor activity. The duration of CNS stimulation was shorter for difluoroamphetamine than for amphetamine, correlating with the more rapid removal of the former compound from brain. Two inhibitors of microsomal drug-metabolizing enzymes, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) and β-diethylaminoethyldiphenylpropylacetate (SKF 525-A), caused increased brain levels of difluoroamphetamine but not amphetamine, and desmethylimipramine (DMI) did not affect brain levels of either drug. The results suggest an alteration in metabolism as well as in tissue distribution resulting from the decreased basicity of the β,β-difluoro compound.

THE POTENT and varied pharmacological actions of amphetamine are the subject of continued investigation, and modification of the relatively simple structure of amphetamine has been one useful means for providing new tools in such investigation. Except for substitutions directly on the nitrogen of amphetamine, such as with benzylamphetamine or benzphetamine, no amphetamine derivatives that differ markedly in basicity appear to have been studied.[1] β,β-Difluoroamphetamine, which has a pK_a value (6·97) lower than physiological pH, in contrast to the high pK_a (9·45) of amphetamine, has recently been synthesized.[2] Difluoroamphetamine would exist at pH 7·4 predominantly as a neutral molecule, whereas amphetamine is nearly completely protonated at that pH. We have recently reported comparisons of amphetamine and difluoroamphetamine in rats.[2] Because of the species differences that exist in the effects of and particularly in the metabolism of amphetamine,[3] we also investigated the properties of difluoroamphetamine in mice and describe here a comparison of that compound with amphetamine.

MATERIALS AND METHODS

dl-Amphetamine sulfate was obtained from Chemicals Procurement Laboratories, and *dl*-4-chloroamphetamine hydrochloride from the Regis Chemical Company.

β,β-Difluoroamphetamine hydrochloride and 4-chloro-β, β-difluoroamphetamine hydrochloride were synthesized in these laboratories and used as the racemic mixtures.

Male albino Cox standard mice weighing 16–20 g were obtained locally and were used in all studies except as indicated. All drugs were injected i.p. Drug levels in tissues were determined by the methyl orange method.[4,5] All drug levels are expressed on the basis of wet tissue weight. Circular activity cages from the Woodard Research Corp., were used in measuring motor activity in the mice. In the behavioral studies, mice were placed in the cages for 30 min prior to injection of drugs.

RESULTS

Tissue levels of drugs. The relative distribution of amphetamine and of difluoroamphetamine in brain and in epididymal fat pads is shown in Fig. 1. Amphetamine was present in fat only at very low levels, less than one-tenth of the drug concentration in brain. In contrast, difluoroamphetamine was localized in fat at nearly double

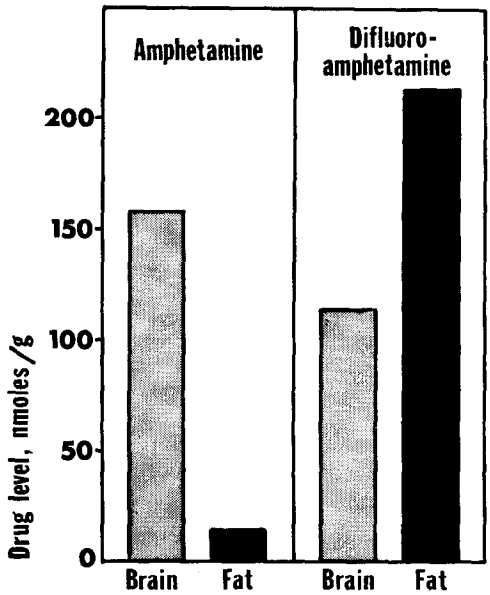

FIG. 1. Distribution of drug in brain and epididymal fat pads of mice given an i.p. injection 30 min earlier of amphetamine, 100 μmoles/kg, or difluoroamphetamine, 200 μmoles/kg. Mean values for six mice are shown. The standard errors were 8 and 7 per cent of the mean values, respectively, for amphetamine and difluoroamphetamine in brain; the fat pads were pooled prior to drug assay.

the concentration found in brain. Whereas the amount of the difluoroamphetamine in brain was lower than that of amphetamine even at twice the dose, the amount of the difluoro compound in fat was 15 times that of amphetamine at the doses used.

The disappearance of the difluoro compound from brain was much more rapid than that of amphetamine (Fig. 2). The half-life of amphetamine was 51 min, which was in good agreement with the recent results of Miller *et al.*[6] (50 min). The half-life of difluoroamphetamine, on the other hand, was only 18 min. Addition of a 4-chloro substituent prolonged the half-life of amphetamine to 261 min, presumably at least in part because *para*-hydroxylation is thereby prevented. Addition of a 4-chloro-substituent to the difluoroamphetamine did not alter its half-life (19 min).

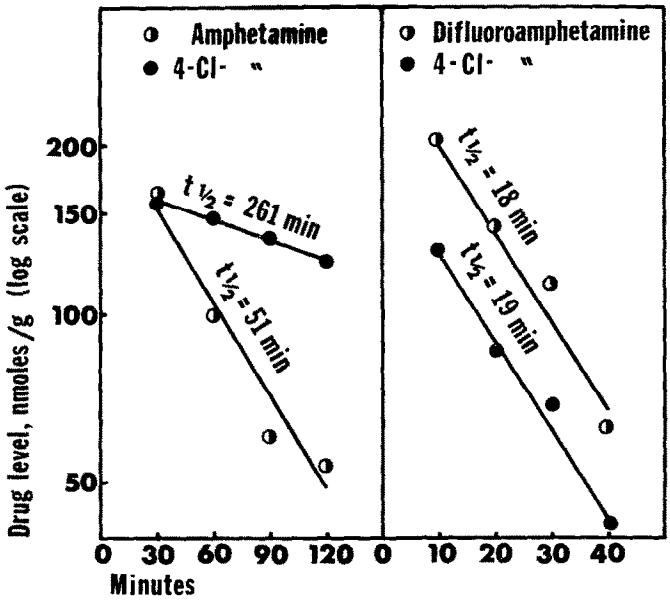

FIG. 2. Rate of disappearance of amphetamine and difluoroamphetamine from brain and the influence of 4-chloro substitution. All drugs were injected i.p. at zero time, the amphetamine at 100 μmoles/kg and the difluoroamphetamines at 200 μmoles/kg. The standard errors were between 4 and 15 per cent of the mean values. Half-life values were calculated by linear regression analysis of the slope of the line best fitting the experimental points. Each point is the mean value for six mice.

To determine if the high concentration of the difluoro compounds in fat might represent a more stable pool of drug, we determined the half-life of difluoroamphetamine and 4-chloro-difluoroamphetamine in fat (Fig. 3). The half-lives of difluoroamphetamine and of its 4-chloro analogue were essentially equal in fat to those found in brain, being 16 and 20 min respectively. Amphetamine and 4-chloroamphetamine were present in fat at concentrations too low to measure accurately enough for half-life determination. Comparing Figs. 2 and 3, one can also see that 4-chloro-difluoroamphetamine has an even greater tendency than difluoroamphetamine to localize in fat at the expense of tissues like brain. 4-Chloro-difluoroamphetamine concentration in fat was substantially higher, but in brain was consistently lower than that of difluoroamphetamine.

Figure 4 shows a comparison of amphetamine and difluoroamphetamine levels in brain 30 min after various doses of the two drugs. Approximately four to five times the dose of the difluoro compound was required to produce equivalent brain levels at 30 min.

To examine the proportion of injected drug found in brain in mice whose body composition included varying amounts of fat, we used C57BL6J ob/ob and C57BL6J ob^+/ob^+ mice.* Table 1 shows the concentration of drug in brain of mice with normal

* We thank Dr. T. T. Yen and Mrs. Jean Steinmetz for providing these mice, which were from the Jackson Laboratories, Bar Harbor, Me. The mice with the ob/ob phenotype of this inbred mutant C57BL6J strain are obese, whereas the ob^+/ob^+ littermates are not obese.

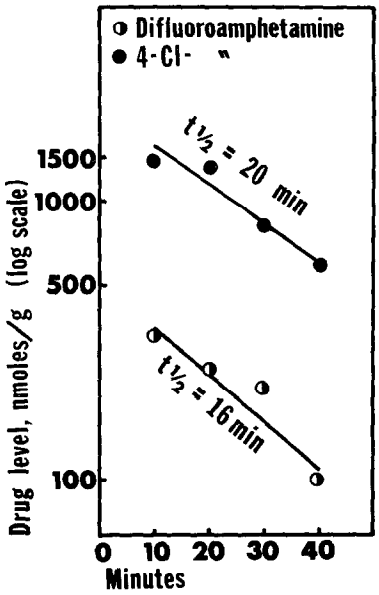

FIG. 3. Rate of disappearance of difluoroamphetamine and 4-chloro-difluoroamphetamine from epididymal fat. Experiment as in Fig. 2.

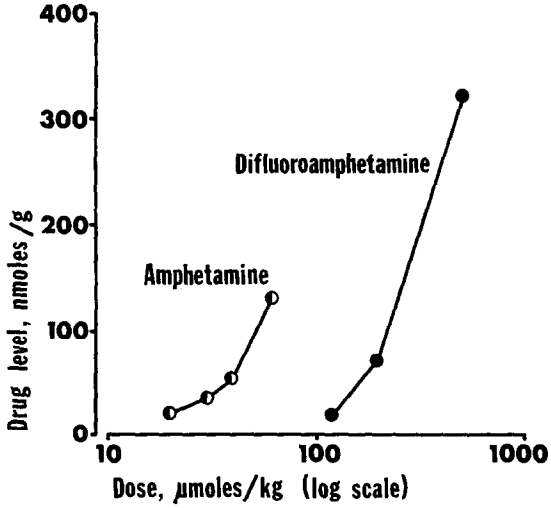

FIG. 4. Drug level in brain after various doses of amphetamine and difluoroamphetamine. Drug were injected i.p. 30 min before the mice were killed. Means for six mice per group are shown standard errors were between 5 and 19 per cent of the mean values.

body composition and of mice that were obese due to a genetic disorder. The con centration of drug in the brain of the obese mice was not significantly different from that in their non-obese littermates. Since the obese mice weighed twice as much a the non-obese mice, they received twice as much drug; the proportion of the total injected dose that was found in the brain was much less in the obese mice.

TABLE 1. BRAIN LEVELS OF DIFLUOROAMPHETAMINE IN MICE WITH DIFFERENT AMOUNTS OF BODY FAT*

Group	Body wt. (mg)	Drug concn. in brain (nmoles/g)	Per cent of injected dose found in brain
Lean	32·5 ± 1·3	50·9 ± 2·8	0·34 ± 0·02
Obese	68·2 ± 1·4	45·0 ± 5·5	0·12 ± 0·02
	$P_s < 0·001$	NS	$P < 0·001$

* Difluoroamphetamine was injected i.p. at 200 μmoles/kg 30 min before the mice were killed. Values are mean ± S.E.M. for six mice per group.

Behavioral effects. The degree of CNS stimulation produced by amphetamine and difluoroamphetamine was determined as the number of light beam interruptions by groups of six animals in activity cages. Figure 5 shows the duration of increased locomotor activity that resulted after equimolar doses of the two drugs. Whereas amphetamine caused increased motor activity lasting for at least 3 hr, the effects of the difluoro compound were over within 1 hr. The immediate effects of the two compounds, as indicated by the activity during the first 15-min interval after injection, were nearly identical, suggesting that the overall lower activity of difluoroamphetamine is due to its rapid disappearance from brain.

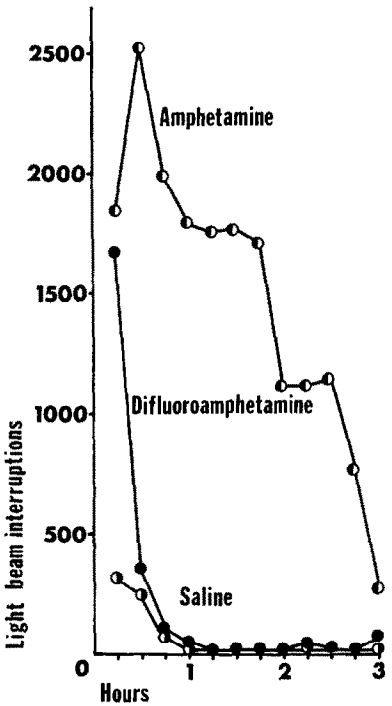

FIG. 5. Duration of increased locomotor activity in mice treated with amphetamine and difluoroamphetamine. Six mice per group were treated at zero time with 40 μmoles/kg, i.p., of amphetamine, difluoroamphetamine or with saline. Each point represents total light beam interruptions for each group during the preceding 15-min interval.

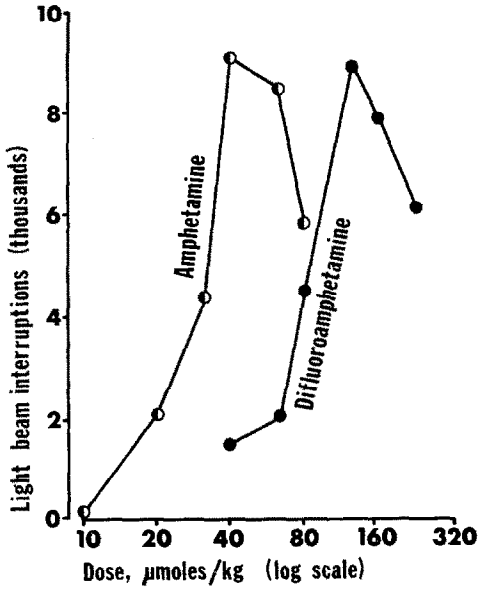

FIG. 6. Increased locomotor activity in mice treated with amphetamine or difluoroamphetamine, dose–response curves. Light beam interruptions for six mice per group during the 60-min period after drug treatment (with values for saline-treated mice subtracted) are shown. Drugs were injected i.p.

Dose–response curves for the two drugs are shown in Fig. 6. The slopes of the dose–response curves were strikingly similar, as was the tendency for light beam interruptions to decrease at excessive doses in both cases. Approximately three times as much difluoroamphetamine had to be injected to produce activity comparable to that of amphetamine. Again, part of the explanation for the higher dose requirement with the difluoro compound is that its activity declined to almost zero by the end of the 60-min period of measurement, as a consequence of its short half-life.

The decline in measured locomotor activity at very high doses of amphetamine is well known, and is due to a change in predominant behavioral effects from simply increased running to the so-called stereotyped behavior. The mice salivated profusely, sat on their hind legs and engaged in "face-washing" activity rather than in locomotion; there was marked exophthalmos and piloerection. At high doses of difluoroamphetamine, a similar decline in the number of light beam interruptions was observed, but the behavioral pattern was somewhat different. There was virtually no salivation or face-washing activity; chewing the bottom of the cage and biting each other were the predominant forms of activity among the mice. No piloerection was noticeable, and there was a moderate degree of ptosis.

Effects of microsomal inhibitors. Table 2 shows the effects of some known inhibitors of drug metabolism on the levels of amphetamine in brain. Desmethylimipramine (DMI), which increases the concentration of amphetamine in rat brain by blocking *para*-hydroxylation,[7–9] did not affect the levels in mouse brain of amphetamine (in agreement with the results of Lew *et al.*)[10] or of difluoroamphetamine. 2,4-Dichloro-6-phenylphenoxyethylamine (DPEA), an inhibitor of drug-metabolizing enzymes in hepatic microsomes, enhanced the concentration of difluoroamphetamine in brain but

TABLE 2. EFFECT OF MICROSOMAL INHIBITORS ON BRAIN LEVELS OF DRUG*

Experiment	Drug	Dose (μmoles/kg)	Brain level (nmoles/g)	Effect of inhibitor
1	Amphetamine	60	73 ± 7	NS
	Amphetamine + DMI	60	76 ± 6	
	Difluoroamphetamine	200	83 ± 9	NS
	Difluoroamphetamine + DMI	200	81 ± 5	
2	Amphetamine	60	75 ± 5	NS
	Amphetamine + DPEA	60	80 ± 5	
	Difluoroamphetamine	200	77 ± 11	$P < 0.01$
	Difluoroamphetamine + DPEA	200	142 ± 15	
3	4-Chloroamphetamine	60	91 ± 6	NS
	4-Chloroamphetamine + DPEA	60	90 ± 4	
	4-Chloro-difluoroamphetamine	200	63 ± 2	$P < 0.001$
	4-Chloro-difluoroamphetamine + DPEA	200	131 ± 7	
4	Amphetamine	60	75 ± 4	$P < 0.05 > 0.025$
	Amphetamine + SKF 525-A	60	95 ± 6	
	Difluoroamphetamine	200	59 ± 9	$P < 0.005$
	Difluoroamphetamine + SKF 525-A	200	121 ± 11	

* Drug levels at 30 min are given as mean ± S.E.M. for six mice per group. DPEA (10 mg/kg, 60 min before drug), DMI (10 mg/kg, 30 min before drug), or SKF 525-A (10 mg/kg, 30 min before drug) were injected, i.p.

had no effect on amphetamine levels. DPEA also increased the concentration of 4-chloro-difluoroamphetamine but not of 4-chloroamphetamine. β-Diethylaminoethyl-diphenylpropylacetate (SKF 525-A), another microsomal inhibitor, doubled difluoroamphetamine levels and had only a marginal effect on amphetamine levels.

DISCUSSION

The tendency of difluoroamphetamine to localize in adipose tissue in mice agrees with our earlier data in rats,[2] in which the concentration of that drug in epididymal fat was higher than in any other tissue. A probable explanation may be based on the decreased basicity of the difluoro compound, resulting in a predominantly neutral form of the drug at physiological pH. Adipose tissue may have affinity for a neutral molecule but not for the cation that amphetamine exists as, because of its stronger basicity. The difluoroamphetamine appears not to be tightly bound in the fat, but instead probably equilibrates rapidly with blood and other tissues, since its half-life in fat is essentially equal to its half-life in brain in the mouse, and with all other tissues studied in the rat.[2]

As a consequence of the altered distribution and of the rapid disappearance of the difluoro compound from tissues, higher doses of difluoroamphetamine than of amphetamine are required to produce comparable brain levels of the drug and comparable degrees of behavioral changes. Although difluoroamphetamine, like amphetamine, caused increased motor activity, the possibility that a different spectrum of

behavioral changes may be revealed by more sophisticated behavioral test systems deserves further investigation, in the light of observed behavioral differences at high doses. In view of the gross differences in organ distribution of the two compounds, one must consider that the regional and subcellular distribution of difluoroamphetamine in brain may differ from that of amphetamine and that behavioral differences may result.

The much faster disappearance of difluoroamphetamine from mouse brain and epididymal fat does not necessarily mean that the compound is metabolized differently from amphetamine, but other evidence suggests that this may be so. The addition of the 4-chloro substituent to amphetamine markedly delayed its disappearance from brain. The effect of the 4-chloro reported here confirms our earlier results[11] and is greater than that found by Miller *et al.*[6] in a different strain of mice. They reported that the half-life was increased only from 50 to about 120 min. The mouse is said to differ from the rat, which metabolizes amphetamine almost exclusively by *para*-hydroxylation, in that the mouse metabolizes amphetamine to a greater extent by deamination.[4] Our results with 4-chloroamphetamine may imply that these mice were substantially dependent on *para*-hydroxylation; there is not *a priori* reason for suspecting that the 4-chloro substituent would decrease the rate of oxidative deamination at the other end of the molecule. Thus the complete lack of effect of the 4-chloro substituent on the half-life of difluoroamphetamine, either in brain or in adipose tissue, implies a different pattern of metabolism of that compound. Preliminary metabolic studies in the rat, which almost exclusively *para*-hydroxylates amphetamine, have indicated that the difluoroamphetamine is metabolized by deamination.* Perhaps the tendency for difluoroamphetamine to be enzymatically deaminated in rats coupled with the ability of mice to deaminate amphetamine results in a very rapid rate of deamination of the difluoro compound. That possibility can at present only be viewed as speculation.

Lew *et al.*[10] suggested that the failure of desmethylimipramine to affect the disappearance of amphetamine from brain in the mouse may be due to the insignificance of *para*-hydroxylation as a route of metabolism for amphetamine in that species. However, our results, showing that 4-chloro substitution but not desmethylimipramine prolongs the half-life of amphetamine, suggest that *para*-hydroxylation may be a significant pathway and that DMI may be ineffective in preventing *para*-hydroxylation in mice as it does in rats. Alternatively, the effect of the 4-chloro substituent in slowing the disappearance of amphetamine may result from some effect other than prevention of *para*-hydroxylation. For instance, the tendency of 4-chloroamphetamine to bind particulate matter in brain to a greater extent than does amphetamine[11] might influence the half-life.

A precise comparison of the degree of CNS stimulation with the level of drug in the brain is difficult because of the grossly different half-lives of the two drugs. During any period of behavioral observation there is a large change in concentration of difluoroamphetamine relative to the change in concentration of amphetamine in the brain. As an approximation, though, the degree of CNS stimulation resulting from a given concentration of drug in the brain seems to be about the same for the two compounds. That is, the dose–response curves in Figs. 4 and 6 are separated by approxi-

* J. Parli, private communication.

mately the same extent. The lower potency of difluoroamphetamine as a CNS stimulant in mice and its shorter duration of activity compared to amphetamine seem to be understandable in the light of the lower drug levels in brain after equimolar doses and the rapid rate of disappearance of the difluoro compound.

The differences between amphetamine and difluoroamphetamine are not necessarily solely attributable to their different ionization constants. However, the relatively small size of the fluorine atoms and their location at a site of the molecule that is not a primary point of metabolic attack or a known site of interaction with receptors mediating amphetamine transport or action, do not suggest an explanation based on steric interactions. Differences in agonist–receptor combination might logically be expected between a charged amphetamine molecule and a neutral difluoroamphetamine molecule. The introduction of the strongly electron-withdrawing fluorine atoms β to the amine nitrogen may represent a means of presenting to the transport systems, degradative enzymes, and receptor systems one molecule that differs from another principally only in the presence or absence of a charge on the amine function. If so, the striking differences between amphetamine and difluoroamphetamine fulfill what one might have predicted about the importance of the charge on the amino group in the biological properties of amphetamine.

REFERENCES

1. T. B. Vree, A. T. J. M. Muskens and J. M. Van Rossum, *J. Pharm. Pharmac.* **21,** 774 (1969).
2. R. W. Fuller and B. B. Molloy, *Pharmacologist* **13,** 294 (1971).
3. L. G. Dring, R. L. Smith and R. T. Williams, *Biochem. J.* **116,** 425 (1970).
4. J. Axelrod, *J. Pharmac. exp. Ther.* **110,** 315 (1954).
5. B. Dubnick, G. A. Leeson, R. Leverett, D. F. Morgan and G. E. Phillips, *J. Pharmac. exp. Ther.* **140,** 85 (1963).
6. K. W. Miller, E. Sanders-Bush and J. V. Dingell, *Biochem. Pharmac.* **20,** 500 (1971).
7. F. Sulser, M. L. Owens and J. V. Dingell, *Life Sci.* **5,** 2005 (1966).
8. S. Consolo, F. Dolfini, S. Garattini and L. Valzelli, *J. Pharm. Pharmac.* **19,** 253 (1967).
9. T. Lewander, *Eur. J. Pharmac.* **6,** 38 (1969).
10. C. Lew, S. D. Iversen and L. L. Iversen, *Eur. J. Pharmac.* **14,** 351 (1971).
11. R. W. Fuller and C. W. Hines, *J. pharm. Sci.* **56,** 302 (1967).