"Aggregation" Phenomenon and Some Ephedrine Isomers

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Experiments have shown that three ephedrine isomers, D(−) ephedrine, L(+) ephedrine, and D(−) pseudoephedrine, are significantly more lethal to grouped *versus* isolated mice. The magnitude of this effect is considerably less than that observed with racemic amphetamine. A fourth isomer, L(+) pseudoephedrine, did not demonstrate this phenomenon.

OUR INTEREST in the pharmacology of a series of ephedrine isomers was first stimulated by the findings of LaPidus, *et al.* (1), who reported that D(−) pseudoephedrine blocked the pressor activity of D(−) ephedrine. It appeared worthwhile to initiate a study into the central nervous system activity of these compounds and to compare critically their activity with that of racemic amphetamine as well as with that of two additional ephedrine isomers which were available—L(+) ephedrine and L(+) pseudoephedrine.

As a starting point for these studies, a series of experiments was designed to evaluate the incidence of lethality to these agents observed in an isolated *versus* aggregated environment; the object of this work was to establish the presence or absence of the well-known "aggregation" phenomenon (2–7) with these isomers. The results obtained constitute the basis for this note.

EXPERIMENTAL

Novice, adult, male, albino mice of a random bred Swiss strain (Maxfield Animal Supply, Cincinnati, Ohio) were used as experimental animals. These mice, which ranged in weight from 17 to 23 Gm., were housed in groups of 16 to 22 animals each in metal cages 24 × 23 × 18 cm. They were maintained on Purina laboratory chow and had free access to food and water except during the experimental test periods.

All experiments were conducted in both an aggregated and isolated environment. Aggregation was defined as placing three mice in a metal cage 7 × 7 × 7.5 cm. with a wire mesh bottom; isolation consisted of placing a solitary mouse in a similar cage. The room temperature was maintained at approximately 25° throughout the study.

Aqueous solutions of all drugs were administered intraperitoneally in a constant volume of 1 ml. per 100 Gm. body weight. With the exception of L(+) ephedrine, which was obtained as the hydrochloride salt, the free base of the ephedrine isomers was dissolved in a few drops of 1:20 hydrochloric acid and the volume of the resulting solution adjusted with distilled water. Amphetamine[1] was administered as the sulfate salt. The pH of the drug solutions was approximately 5. In all instances drug dosage was calculated as free base.

To determine the dose lethal to 50% of animals (LD_{50}) for each drug, groups of nine animals were randomly selected, injected with various doses of the agent under investigation, and placed in either an aggregated or isolated environment. The incidence of lethality and general behavior of the animals were recorded every 15 minutes for a period of 3 hours. Dead animals in the group situation were replaced with noninjected animals to maintain a constant degree of aggregation. This procedure was repeated until a minimum of three points were established in the range between 0 and 100% lethality in both environmental situations. The results obtained were then plotted on logarithmic probability paper, and a regression line was fitted to the plotted points by eye. From this plot of the data, the LD_{50}, 95% fiducial limits, and the lethality potency ratio, (LPR = LD_{50} isolation/LD_{50} aggregation) were calculated by the method of Litchfield and Wilcoxon (8).

RESULTS AND DISCUSSION

The LD_{50}'s and LPR's obtained for the agents under investigation are shown in Table I. It is evident from these results that, except for L(+) pseudoephedrine, all drugs were significantly more lethal to grouped *versus* isolated mice. Thus an "aggregation" phenomenon was demonstrated with D(−) ephedrine, L(+) ephedrine, D(−) pseudoephedrine, and racemic amphetamine. The findings pertaining to D(−) ephedrine are in agreement with those reported by Chance (3), who also found this agent to be considerably more lethal in animals subjected to aggregation. To our knowledge, the central nervous system activity of the remaining ephedrine isomers has never been critically investigated.

Although three of the four ephedrine isomers tested demonstrated the "aggregation" phenomenon, an examination of the LPR's reveals that the magnitude of these responses was significantly less than that observed with amphetamine. Furthermore, no statistical difference was apparent among the LPR's of those ephedrine isomers which were shown to be more lethal in an aggregated environment.

In addition to the quantitative differences in

TABLE I.—LETHALITY OF SELECTED STIMULANTS IN AGGREGATED AND ISOLATED MICE[a]

Drug	LD_{50}[b] Aggregated	LD_{50}[b] Isolated	LPR[c]
D(−) Ephedrine	177 (138–209)	270 (229–319)	1.53 (1.13–2.06)
L(+) Ephedrine	255 (234–278)	323 (299–349)	1.31 (1.19–1.43)
D(−) Pseudoephedrine	248 (214–288)	312 (292–324)	1.26 (1.07–1.49)
L(+) Pseudoephedrine	220 (194–248)	224 (193–260)	1.02 (0.76–1.38)
(±) Amphetamine	12.2 (9.5–15.5)	104 (97.1–110)	8.5 (6.6–11.1)

[a] Three-hour observation period. [b] mg./Kg. Values in parentheses are 95% fiducial limits. [c] Lethality potency ratio = LD_{50} isolation/LD_{50} aggregation. Values in parentheses are 95% fiducial limits.

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[1] Marketed as Benzedrine Sulfate by Smith Kline and French.

lethality described above, the agents investigated manifest some striking qualitative differences in toxicity. Although the design of the test cages did not permit continuous observation, it was possible to make intermittent examinations of general behavior. On the basis of these observations it was apparent that the toxicity of racemic amphetamine and D(−) ephedrine could be qualitatively differentiated from that of the other ephedrine isomers employed. For example, pilo erection was commonly observed in mice which had been treated with either racemic amphetamine or D(−) ephedrine and then placed in either isolation or aggregation. In contrast, little pilo erection was observed in animals subjected to similar environmental conditions after having been treated with L(+) ephedrine, D(−) pseudoephedrine, or L(+) pseudoephedrine.

Animals injected with lethal doses of either racemic amphetamine or D(−) ephedrine and then placed in an isolated environment demonstrated tremors, loss of righting reflex, clonic and tonic convulsions, and acute respiratory failure leading to death. These animals invariably died within 1 hour after drug administration, usually in the throes of a convulsion. On the other hand, there appeared to be two distinct causes of death in mice treated with lethal doses of either of the above agents and then placed in a crowded environment. Those animals that died within the first hour succumbed to convulsions (as described above), whereas exhaustion due to prolonged hyperactivity or repeated episodes of clonic convulsions was the apparent cause of death in animals that expired 1 or more hours after drug administration. These mice lost their righting reflex, appeared comatose, and experienced progressive respiratory difficulty until apnea occurred and death ensued.

In contrast to the above, the pattern of toxicity observed in mice treated with L(+) ephedrine, D(−) pseudoephedrine, or L(+) pseudoephedrine was essentially the same for both environmental situations. Death was caused by acute respiratory failure preceded by clonic or tonic convulsions, and always occurred within 1 hour after drug administration.

An explanation for the failure of L(+) pseudoephedrine to demonstrate an "aggregation" phenomenon is not readily available. However, a number of preliminary experiments conducted in our laboratory, *e.g.*, determination of the behavioral changes accompanying drug administration, low frequency electroshock seizure threshold studies, and examination of drug-induced alterations in levels of central nervous system activity measured by chemoshock techniques indicate that several major quantitative and qualitative differences exist among these four ephedrine isomers. It is anticipated that further investigation into the exact nature of central stimulation induced by these agents will help elucidate the findings reported herein.

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Colorimetric Microdetermination of Plasma Free Fatty Acids

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The application of a colorimetric method to the determination of free fatty acids in plasma is described. The method is simple, sensitive, and yields results comparable to those obtained with the titrimetric procedure.

THE MEASUREMENT of unesterified fatty acid levels in biological samples is usually accomplished by microtitration of the acidity present in suitably prepared extracts (1). Such microtitration techniques require special equipment and even with care and experience may give variable results. Ayers reported a colorimetric method for the estimation of fatty acids based upon the finding that copper or cobalt salts of monocarboxylic acids dissolve in chloroform to give colored solutions (2). Iwayama subsequently developed an essentially new procedure, also based upon the solubility of the copper salts of C_{10}–C_{22} monocarboxylic acids in chloroform, but its sensitivity was not great enough to be useful for the determination of plasma free fatty acids (3). The sensitivity of the latter procedure was greatly increased by Duncombe, who measured the concentration of copper in the chloroform solution with diethyldithiocarbamate (4). We have adapted this colorimetric method to the determination of plasma free fatty acids following their extraction by the technique of Dole and Meinertz (1). The resulting procedure is simple, very sensitive and reproducible, and uses commonly available laboratory equipment.

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EXPERIMENTAL

Reagents.—The following reagents were used.

Extraction Solution.—Mix 1 *N* H_2SO_4, hexane, and isopropyl alcohol in the volume ratio 20:200:780.

Cupric Nitrate Reagent.—Mix 450 ml. of 1 *M* triethanolamine, 50 ml. 1 *N* acetic acid, and 500 ml. 5% cupric nitrate solution.

Sodium Diethyldithiocarbamate, 0.1%.—Dissolve 100 mg. $(C_2H_5)_2NCS_2Na \cdot 3H_2O$ in 100 ml. normal butanol (prepare fresh daily).

Stock Standard Fatty Acid Solution.—Dissolve 56.8 mg. stearic acid in 100 ml. of hexane.

Working Standard Fatty Acid Solution.—Dilute 10 ml. of stock standard to 100 ml. with hexane. This solution contains 0.2 μmoles/ml.