cluded that if K' (2%) = 4 K' (1%) the difference in K' values is solely due to moisture. Such a conclusion could not have been reached without knowledge of the apparent (third) order of reaction.

SUMMARY

1. Vitamin A acetate has been used as an example to demonstrate "order of interaction" in solid dosage forms.

2. Since moisture is a variable seldomly controlled within extremely narrow limits in stability programs, it may prove useful, in many cases, to establish the "order of interaction" between moisture and active component(s).

3. Screening bases for such studies can be selected by choosing the worst offenders in preliminary compatibility programs (4).

4. If a high-order interaction is established, stringent moisture control data should be obtained in the stability program.

5. The study of "order of interaction" may be carried out in a relatively short span of time (30 days).

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New Pharmacologic Aspects of β-Diethyl-aminoethyl 2,2-Diphenylpentanoate

By RICHARD A. CARRANO* and MARVIN H. MALONE

In a comparison with atropine and structurally related adiphenine using a gross *in vivo* screen in rats, β-diethyl-aminoethyl 2,2-diphenylpentanoate (SKF 525-A) appeared to act peripherally as a parasympatholytic and/or sympathomimetic. SKF 525-A apparently has some selective activity on the central nervous system (blepharoptosis, hypothermia) indicating a capacity to cross the blood-brain barrier. Drug–receptor interactions were studied on the isolated rat jejunum using furtrethonium as the agonist. SKF 525-A was primarily a noncompetitive antagonist with a competitive component and qualitatively different from the activities of atropine, adiphenine, and papaverine. The respective pA_2 and pD'_2 values are reported. The SKF 525-A receptor appears composed of the cholinergic receptor plus another spasmogen receptor. SKF 525-A did not inhibit the action of acetylcholinesterase, but was a potent inhibitor of monoamine oxidase at physiological concentrations.

MUCH OF the work previously reported on β - diethyl - aminoethyl 2,2 - diphenyl pentanoate (SKF 525-A) has been concerned with its ability to act as a multipotent inhibitor of various liver microsomal degradation reactions (1). The original observations made for SKF 525-A concerning potentiation of barbiturates and other central nervous system depressants (2–5) suggested possible potentiation by CNS mediation; however, Brodie (1) demonstrated that this agent was able also to prolong the activity of the central stimulant, amphetamine. This study was prompted by the chemical similarity between SKF 525-A and adiphenine, and also by the lack of comprehensive screening in the literature.

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EXPERIMENTAL

In Vivo Hippocratic Screening.—In accordance with the method of Malone and Robichaud (6), nonfasted albino rats (Wistar strain) in the weight range of 150–250 Gm. were injected intraperitoneally with 5 logarithmetically spaced doses of each drug tested (1 lethal, 1 essentially ineffective dose, and 3 effective log-dosages between those two). Observations were made using the standard worksheet (6) at 5, 10, 15, 30, and 60 min. postinjection, 2, 4, and 24 hr. postinjection, and 2, 4, and 7 days postinjection.

Mechanism of Drug-Receptor Interaction.—Using the methods of Ariëns (7), van Rossum (8), and van Rossum and van den Brink (9), cumulative dose-response curves were made utilizing rat jejunum and furtrethonium iodide as the reference agonist. The bath solution was standard Tyrode's oxygenated with 95% oxygen and 5% CO_2 and containing the calcium disodium salt of ethylenediaminetetraacetic acid in a concentration of 1×10^{-5} Gm./L. The jejunum was mounted in the bath (37.5°) using a modified Magnus technique. All drug concentrations were calculated in terms of drug base. When an antagonist was tested it was

allowed to incubate with the tissue for 10 min. prior to the production of the cumulative dose-response curve.

In Vitro Acetylcholinesterase Inhibition Studies.—The manometric techniques of Umbreit *et al.* (10) were used for this study with acetyl-β-methylcholine (0.03 *M*) as the substrate for acetylcholinesterase. Physostigmine was used in concentrations ranging from 10^{-6} to 10^{-9} *M* as a standard acetylcholinesterase inhibitor. SKF 525-A was used in concentrations ranging from 10^{-3} to 10^{-6} *M*. The inhibitor and acetylcholinesterase were incubated (37°) together for 20 min. prior to mixing with the substrate. The total flask volume was 3 ml. and pH was kept at 7.4 with Krebs-Ringer bicarbonate buffer. Molar I_{50} and I_{80} values were calculated. All drug concentrations were expressed in terms of drug base.

In Situ Monoamine Oxidase Inhibition Studies.—Rat liver MAO activity was determined by the spectrophotometric disappearance of the substrate kynuramine (11). Iproniazid was used as the reference MAO inhibitor. All concentrations were calculated in terms of the drug base.

Radiometric determinations were carried out using the method of Wurtman and Axelrod (12) with incubations at 37° in 15-ml. glass-stoppered centrifuge tubes for 20 min. A young male rat was sacrificed by decapitation, and 200 mg. of liver rapidly excised and homogenized thoroughly with 10 ml. of cold isotonic KCl. One milliliter of this homogenate was diluted to 10 ml. with isotonic KCl and rehomogenized so that a final concentration of 2 mg./ml. was obtained. The total volume of each incubation tube was 3 ml. made up of the appropriate ingredients. At the termination of the incubation, 0.2 ml. of 2 *N* HCl was added to each tube followed by 6 ml. of toluene. The ^{14}C derivative was extracted into the toluene layer by shaking and the two layers separated by centrifugation. Four milliliters of the toluene layer was taken from each tube and placed into a liquid scintillator vial containing 10 ml. of liquid scintillator solution (0.5 Gm. POPOP, 4 Gm. PPO, add 1 L. of toluene), and counted for 10 min. in a Packard tricarb counter. Counting efficiency of each vial was determined by the internal standard method. A wide range of log-concentrations was used for each agent with concentrations expressed in terms of drug base.

RESULTS AND DISCUSSION

In Vivo Hippocratic Screening.—Dosages of 32, 56, 100, 178, 316 mg./Kg. were injected for each of the following: SKF 525-A, structurally related adiphenine, and atropine. The qualitative nature of the observable symptoms which exhibit dose-response relationships permit an estimate of possible mechanism of action (6).

Atropine produced excellent dose-response activity for the following acute effects: mydriasis, decreased skeletal muscle coordination and tone, lowering of rectal temperature, tremors and convulsions coupled with xerostomia, and decreased lacrimation. An increase in blood pressure was suggested by the presence of exophthalmos. The well-known parasympatholytic activity appeared to be detected by the screening technique and all activities were in agreement with reports in the literature. At 100–316 mg./Kg. dosages, there was an apparent blockade of transmission at the level of the motor end plate of skeletal muscle. The dosage of 316 mg./Kg. was lethal within 30 min. Recovery from 178 mg./Kg. of atropine was essentially complete by +24 hr.

Adiphenine produced effects very similar to those observed with atropine. However, mydriasis was not as pronounced and stimulation of both salivation and lacrimation was noted at doses of 100 and 178 mg./Kg. Lack of xerostomia suggests a different mechanism of action at these higher dosage levels, possibly indicating a decreased affinity of the adiphenine molecule to the parasympathetic receptor, and possibly the extension of its action to another closely related receptor or receptor system.

SKF 525-A was found to be a strong mydriatic but not as powerful as atropine. It had the ability to decrease motor activity, although paralysis of skeletal muscle and loss of screen grip (6) were not seen. Fine tremors of skeletal muscle were pronounced at the two lower dosages. Lacrimation was greatly increased. The most striking effect of SKF 525-A and with an excellent dose-response relationship was hypothermia (−6.9° at +1 hr. with a dosage of 178 mg./Kg.). At the lowest dosage level of 32 mg./Kg., SKF 525-A produced a hypothermia (−3.3° at +1 hr.) equivalent to that noted with 178 mg./Kg. of atropine and adiphenine. Associated with hypothermia was an effect of palpebral ptosis and enophthalmos. The dosage of 316 mg./Kg. was lethal within 30 min. and was accompanied by convulsions. The animals receiving 178 and 100 mg./Kg. died within 60 and 95 hr. of dosing and death was associated with increased skin plasticity, pilomotor erection, and profound loss of body weight. Since only 1–2 rats per dosage are required for the Hippocratic screening (6), it is a qualitative technique whereby the evaluation of the activity of the test material is based on the complete log-response profile of the entire animal population receiving the 5 log-dosages rather than on one animal or on one selected log-dosage. The Hippocratic technique is used as an initial directive screen to suggest avenues for further more specific pharmacologic evaluation. In considering the total profile of acute symptoms produced by SKF 525-A, one could postulate either parasympatholytic or sympathomimetic activity. Increased lacrimation and mydriasis accompanied by skeletal muscle tremors suggest sympathomimetic activity. Salivation was not stimulated concurrent with lacrimation. The hypothermia appeared due to a selective CNS activity—this was suggested considering two factors. First, the "true" palpebral ptosis indicative of possible hypothalamic depression was correlated directly with the period of strong hypothermic activity; and second during the ptotic effects the animals were obviously conscious. Since death with SKF 525-A came on slowly accompanied by weight loss, this agent could produce general metabolic inhibition.

It was evident from this gross *in vivo* screen that SKF 525-A may have important pharmacologic actions independent of metabolic inhibition and actions quite different from those seen with atropine and adiphenine. In general, the data indicated a decrease in parasympatholytic activity stepwise

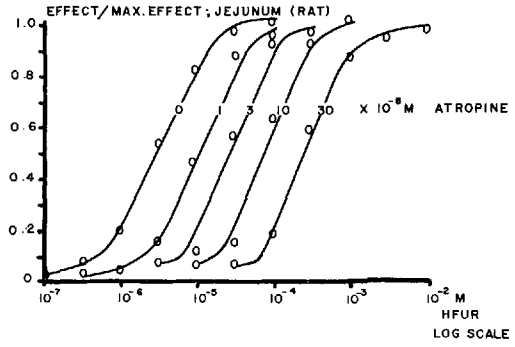

Fig. 1.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of atropine.

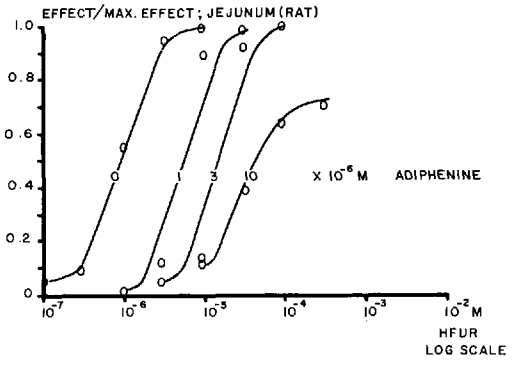

Fig. 2.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of adiphenine.

from atropine to adiphenine to SKF 525-A. A shifting of mechanism of activity or site of action may be pertinent to this structural progression.

Drug Receptor Interactions.—The results for SKF 525-A were compared with those for atropine, adiphenine, and papaverine. Figure 1 illustrates the cumulative dose-response curves for furtrethonium in the presence of increasing log-increments of atropine. A constant level of atropine was maintained for each dose-response curve of furtrethonium. The conclusion that must be drawn is that atropine is a pure competitive antagonist of furtrethonium for the cholinergic receptor of rat jejunum. There is a concentration-related decrease in the affinity of the furtrethonium molecule for the cholinergic receptor. The influence of atropine can be overcome by an increase in the concentration of furtrethonium and eventually furtrethonium occupies all receptors, thus producing a maximal effect. The complementarity between the cholinergic receptor site and the atropine molecule is similar to that between furtrethonium and the cholinergic receptor site, but not equivalent since atropine has no intrinsic activity.

Figure 2 illustrates similar cumulative dose-response curves with adiphenine as the antagonist. Here the competitive nature of the drug–receptor interactions can also be seen. However, with a concentration of 10×10^{-6} *M* for adiphenine, the maximum response obtainable for the furtrethonium cumulative dose-response curve is reduced. This implies not only a decrease in the affinity, but also a decrease in the intrinsic activity of the furtrethonium molecule for the cholinergic receptor. A noncompetitive component has been introduced into adiphenine's action. This noncompetitive component may be the determinate factor responsible for the decrease in gross parasympatholytic activity (as compared to atropine) noted in the *in vivo* screening data.

Figure 3 demonstrates the furtrethonium dose-response curves in the presence of increasing log-increments of the musculotropic antagonist papaverine. A "pure" noncompetitive antagonism is illustrated. The papaverine molecule and the cholinergic receptor do not have any degree of complimentarity; however, the interaction of papaverine with components (7) of the contractile mechanism of the muscle has a blocking influence on the action produced by furtrethonium on the cholinergic receptor of the jejunum.

Figure 4 illustrates that SKF 525-A, in part, appears to be a noncompetitive antagonist of furtrethonium on the cholinergic receptor. It is not correct to assume that SKF 525-A acts in the same way as papaverine on this biological preparation. Examination of the curves in Fig. 4 reveals that there is a shift in the dose-response curves for furtrethonium occurring along the log-dose axis

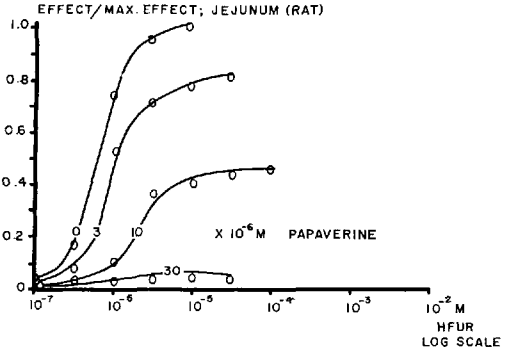

Fig. 3.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of papaverine.

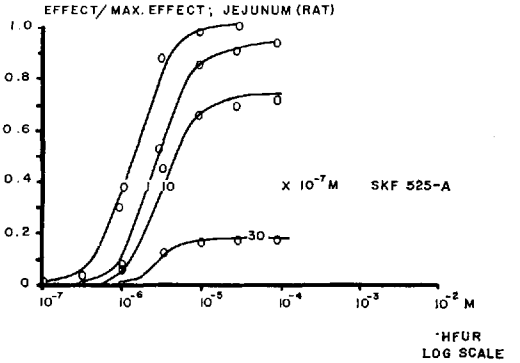

Fig. 4.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of SKF 525-A.

TABLE I.—PARAMETERS OF PARASYMPATHETIC AGENTS TESTED ON ISOLATED RAT JEJUNUM (10 min. INCUBATION) WITH FURTRETHONIUM AS THE AGONIST

	Intrinsic Activity	Log Affinities		
		pD_2	pA_2	pD'_2
Furtrethonium[a]	1	5.90 (5.90)[b]	...	...
Atropine	0	...	8.42 (8.80)[b] (8.37–8.77)[c]	...
Adiphenine	0 (−1)	...	6.69 (6.80)[b]	4.58[d] (4.70)[e]
SKF 525-A	?[f]	...	...	6.06
Papaverine	−1	...	...	5.00 (4.80)[b]

[a] While all calculations are in terms of the base molecules, furtrethonium iodide and atropine sulfate were the respective salts used with all other agents as hydrochloride salts. [b] Parenthetical value was reported by van Rossum and van den Brink (9); the incubation time was not listed. [c] Parenthetical values were reported by Schild (17, 18) for a 2 and 14-min. incubation time, respectively. [d] Incubated at 1×10^{-5} *M* concentration. [e] Parenthetical value was reported by Ariëns and van Rossum (13); the incubation time was not listed. [f] See text for discussion.

prior to the depression of maximal effect. This is not demonstrated in Fig. 3 with papaverine. According to Ariëns and van Rossum (13), van Rossum (8), and Ariëns (7), when a noncompetitive antagonist interferes with the induction of the stimulus its presence may result in a shift of the dose-response curves of the agonist followed by a decline of the maximum attainable effect. In such a case the antagonist may be expected to exhibit a certain specificity with respect to the agonist. A reserve in receptors for the agonist may be the cause of this shift. Since SKF 525-A is a reversible antagonist, the small shift noted is significant. Therefore, SKF 525-A although noncompetitive in action like papaverine acts by way of a different mechanism. Papaverine's action is nonspecific whereas SKF 525-A has a certain specificity with respect to the furtrethonium molecule and the cholinergic receptor and, therefore, may be considered to interfere with the actual induction of the stimulus itself. Table I gives the values for the log-affinities calculated according to the method of van Rossum and van den Brink (9). An examination of Table I shows that the values obtained in this laboratory are in agreement with those reported by others. Atropine has the greatest affinity (pA_2) for the cholinergic receptor, even greater than furtrethonium. However, since its intrinsic activity is equal to zero it cannot cause an effect. The pronounced affinity of atropine to the cholinergic receptor is understood in light of the fact that it is effective in concentrations around 1×10^{-8} *M*. Furtrethonium produces its effects at about 3×10^{-6} *M*; but since it has an intrinsic activity, it is capable of eliciting a stimulus and a subsequent response of the biologic object. Adiphenine has an affinity (pA_2) to the cholinergic receptor less than atropine but greater than furtrethonium. The inability of adiphenine to produce a stimulus is due to a molecular configuration that destroys its intrinsic activity. The affinity of SKF 525-A to the cholinergic "neighboring" receptor site (pD'_2) is higher than both papaverine and adiphenine. The value for the affinity is indirectly related to the effective concentrations. Table I lists a question mark for the intrinsic activity of SKF 525-A. As a strong noncompetitive antagonist with some competitive characteristics, it may have two intrinsic activities of 0 and −1, as does adiphenine. On several occasions when SKF 525-A was introduced into the bath, it initiated a stimulus and produced an effect which rapidly subsided. Whether this stimulatory effect was induced by interaction with receptors is questionable. However, such a response was never noted for any of

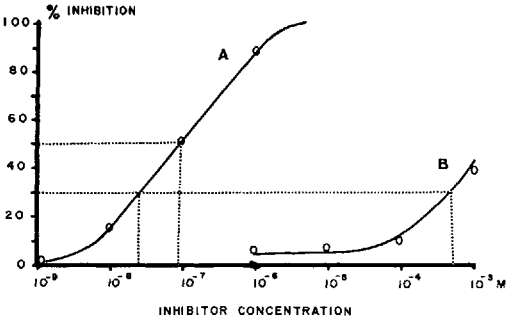

Fig. 5.—Acetylcholinesterase inhibition by physostigmine and SKF 525-A. Key: A, physostigmine ($M\ I_{50} = 9.50 \times 10^{-8}\ M$; $M\ I_{30} = 2.65 \times 10^{-8}\ M$); B, SKF 525-A ($M\ I_{30} = 8.25 \times 10^{-4}\ M$).

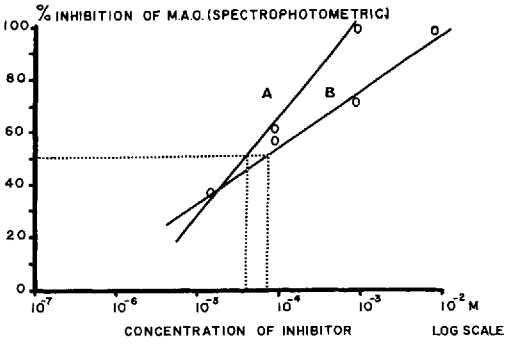

Fig. 6.—Spectrophotometric determination of $M\ I_{50}$ for iproniazid ($8.5 \times 10^{-5}\ M$) and SKF 525-A ($5.3 \times 10^{-5}\ M$) on monoamine oxidase. Key: A, SKF 525-A; B, iproniazid.

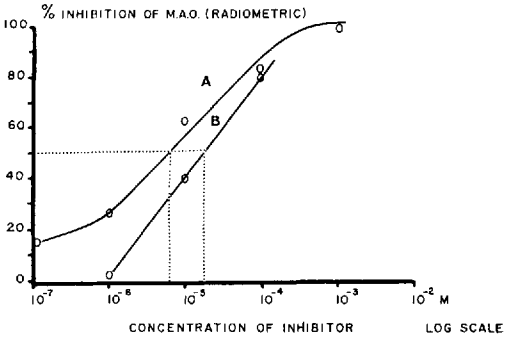

Fig. 7.—Radiometric determination of M I_{50} for iproniazid (7.5×10^{-6} M) and SKF 525-A (1.9×10^{15} M) on monoamine oxidase. Key: A, iproniazid; B, SKF 525-A.

the other antagonists utilized. Important implications may be derived from these observations in connection with the rate theory proposed by Paton (14). In reference to this theory, Paton has suggested that if the rate of occupation of the receptor is an important factor for the intrinsic activity, it may be expected that not only the mimetics but also the lytics will induce an initial response (contraction) before acting as a blocking agent. The unique action of SKF 525-A in blocking the interactions of furtrethonium with the cholinergic receptor is strong indication that the receptor site for SKF 525-A is composed of certain components of the cholinergic receptor and another spasmogen receptor.

In Vitro Acetylcholinesterase Inhibition.—Since SKF 525-A is considered to be a multipotent enzyme inhibitor and since most of the antiacetylcholinesterases, especially the reversible ones, are structurally related to acetylcholine, the action of SKF 525-A on acetylcholinesterase was compared with physostigmine. Figure 5 shows that SKF 525-A was not an effective acetylcholinesterase inhibitor at physiological concentrations. The data for the standard inhibitor, physostigmine, were in excellent agreement with that reported in the literature (15).

In Situ Monoamine Oxidase Inhibition.—The possibility of sympathomimetic effects due to MAO inhibition was considered to be possible in light of the Hippocratic screening. Figure 6 illustrates the data obtained from the spectrophotometric method and shows that SKF 525-A has a lower molar I_{50} than the standard, iproniazid. Figure 7 illustrates the results from the radiometric technique for SKF 525-A and iproniazid. The molar I_{50} values for both procedures are in good general agreement. The concentrations used are in the physiological range. The radiometric method is regarded generally to be the more sensitive and more reliable of the two assays. Allmark (16) demonstrated that large doses of isoniazid and related compounds (iproniazid) prolonged the sleeping time of barbiturates. This provides a correlation between SKF 525-A activity and MAO inhibition since the first major action of SKF 525-A that was studied was its capacity for barbiturate potentiation. In any case it is possible that inhibition of MAO may account for the sympathomimetic effects seen when SKF 525-A alone is administered to intact unanesthetized rats.

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