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Principal Investigator: Fridolin Sulser, M.D.
Professor of Pharmacology
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Paul Gazzerro, Jr.
Associate Vice Chancellor for
Medical Affairs, Operations &
Fiscal Planning

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During the tenure of contract DADA-17-73-C-3130, a number of significant findings have emerged which have been reported at National and/or International meetings, are published or are contained in manuscripts submitted and/or accepted for publication (Number of publications and abstracts: 17). Briefly, studies on the mechanism of action of amphetamines have provided evidence that the central stimulatory action of amphetamine is mediated through newly synthesized catecholamines, predominantly dopamine, whereas the action of p-chloroamphetamine depends on the store of catecholamines. We have shown that

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20) (S)-amphetamine is predominantly hydroxylated in the periphery to (S)-p-hydroxyamphetamine which is then transported to the brain, taken up by noradrenergic neurons and converted by β-hydroxylase to (α-S, β-R) p-hydroxynorephedrine (PHN). Evidence is accumulating that PHN could be involved in the development of specific noradrenergic tolerance to (S)- amphetamine. Introduction of a Cl into para-position of the aromatic ring of amphetamine causes a ~~remarkable~~ shift of uptake blocking properties of amphetamine from catecholaminergic to serotoninergic neurons and produces long lasting neurotoxic effects on central serotoninergic neurons. Behavioral studies in rats have provided an animal model for 3 distinct phases of the action of amphetamine: facilitation, tolerance and post-amphetamine depression. They prompted the search for biochemical correlates and for the development of new anti-amphetamine drugs which do not elicit extrapyramidal symptoms. Studies on non-hepatic metabolism of drugs of abuse have been initiated during the tenure of this contract and a number of still ongoing studies on the limbic norepinephrine receptor coupled adenylate cyclase system are the result of studies partially supported by this contract (now supported by USPHS grants).

Name, Title, Address and Telephone Number of Principal Investigator.

Fridolin Sulser, M.D., Director
Tennessee Neuropsychiatric Institute
Professor of Pharmacology
Vanderbilt University School of Medicine
Nashville, Tennessee 37232
Telephone: 615-741-7431
615-322-2207

Name, Title, Address and Telephone Number of Department Chairman.

Joel G. Hardman, Ph. D.
Department of Pharmacology
Vanderbilt University School of Medicine
Nashville, Tennessee 37232
Telephone: 615-322-2207

Name, Title, Address and Telephone Number of Official Authorized to sign for Applicant Organization

Paul Gazzerro, Jr.
Associate Vice-Chancellor for
Medical Affairs, Operations and Fiscal Planning
Vanderbilt University Medical Center
Nashville, Tennessee 37232
Telephone: 615-322-3335

A. SUMMARY OF PROGRESS

During the tenure of contract #DADA 17-73-C-3130, a number of pertinent findings have emerged which have been reported at national and international meetings, are published or are contained in manuscripts submitted and/or accepted for publication. These studies can be summarized as follows:

1) Studies on the relative role of storage versus synthesis of catecholamines in the central action of amphetamines.

d-Amphetamine and dl-p-chloroamphetamine (PCA) were studied in animals whose noradrenergic terminals in brain had been destroyed by intraventricular 6-hydroxydopamine (2 doses of 250 μg, spaced one day apart). This procedure reduced the level of NE in brain to about 16% of its control value while that of dopamine (DA) in the striatum was only slightly reduced (65% of its control value). The activity of tyrosine hydroxylase, measured by the coupled assay of Waymire et al. (1971) and expressed as $nc^{14}CO_2$/30 min/20 mg tissue decreased in the striatum from 22.8 ± 1.1 to 7.7 ± 0.9 and in the diencephalon from 3.5 ± 0.1 to 2.1 ± 0.1. It is of interest that such a procedure does not alter the central action of PCA and only slightly reduces that of amphetamine. Reserpinization of animals (depletion of remaining stores) whose noradrenergic neurons were preferentially destroyed by 6-hydroxydopamine, completely blocks the action of PCA and either enhances or does not change that of amphetamine. The psychomotor stimulation and stereotyped behavior elicited by amphetamine are also blocked in animals whose dopaminergic terminals have been selectively destroyed by 6-OHDA injected intraventricularly following desipramine. These data provide more direct evidence that the central stimulatory action of amphetamine is mediated through newly synthesized catecholamines, predominantly DA, whereas the action of PCA depends on the store of catecholamines. These results are compatible with data showing that motor activity of rats can be increased by doses of PCA which increase the turnover rate of DA but not necessarily that of NE in various parts of the brain. Since the steady-state concentration of DA remains unchanged after PCA, the data might suggest that PCA alters the synthesis of catecholamines, particularly DA in nerve terminals and cell bodies in brain. However, we found that PCA does not change the activity of tyrosine hydroxylase in the striatum or diencephalon if the drug is administered in vivo. Moreover, the in vitro addition of PCA over a 10,000-fold range does not alter the activity of tyrosine hydroxylase in preparations from striatum or diencephalon. Since we measured total enzyme activity, we cannot rule out that the drug may have caused a shift in the activity of tyrosine hydroxylase from the soluble to the particulate synaptosomal fraction as has been reported to occur with methamphetamine. It is noteworthy in this regard that the two hydroxylated metabolites of amphetamine, p-hydroxyamphetamine (POH) and p-hydroxynorephedrine (PHN), decreased the turnover of NE in brain in vivo and slightly inhibited tyrosine hydroxylase in striatum and diencephalon in vitro.

2) Studies on the formation of p-hydroxynorephedrine in brain following intraventricular administration of p-hydroxyamphetamine.

We have shown that the brain of rats in vivo is capable of forming and accumulating large amounts of p-hydroxynorephedrine (PHN) following the intraventricular administration of dl-p-hydroxyamphetamine (POH) whereas negligible

amounts of PHN could be detected following the intraventricular administration of d-amphetamine. Thus, 57% and 65% of the total radioactivity at 4 and 12 hours, respectively, following the intraventricular administration of POH consist of PHN. While the amount of POH declines from 1229 to 93 mμc, its β-hydroxylated derivative PHN accumulated to the level of approximately 300 mμc. At 4 hours, PHN represented 6% of the initial intraventricular dose. By 12 hours, the amount of PHN had declined only slightly to 5% of the initial dose. Following the intraventricular administration of ^{3}H-d-amphetamine, the drug disappeared rapidly in accordance with its very short half-life following intraventricular administration. Only residual amounts of amphetamine remained at the end of 4 and 12 hours and only small amounts of radioactivity were found in the POH and PHN fractions. Moreover, PHN did not accumulate in brain tissue as it did following the intraventricular administration of POH. These studies support the view that the aromatic hydroxylation of amphetamine occurs in the periphery, that POH is then transported to the brain, taken up by noradrenergic neurons and converted to PHN by dopamine β-hydroxylase. Since the accumulation of PHN in noradrenergic neurons may be involved in the development of tolerance to certain actions of amphetamine, it will be pertinent to determine the stereoselectivity or stereospecificity of the in vivo oxidation of (S)-(+)-amphetamine and to determine the absolute configuration of PHN.

3) The action of (S)-(+)- and (R)-(-)-p-substituted amphetamine hydrochlorides and (αS)- and (αR)-p-chloronorpseudoephedrine hydrochlorides on the level of 5-hydroxytryptamine and the activity of tryptophan hydroxylase in rat brain.

The optically pure isomers (S)-(+)- and (R)-(-)-p-chloroamphetamine (PCA) as well as the optically active enantiomers of p-chloronorephedrine or p-chloronorpseudoephedrine as possible active metabolites of the parent compound have been synthesized and the absolute configurations were established. On the biological side, we found that the long-term effects of the two isomers of PCA on serotonergic neurons were quite different. The effect of (R)-(-)- PCA on tryptophan hydroxylase has disappeared two weeks following its administration while the effect of (S)-(+)-PCA persists for many months. The availability of (S)-(+)-PCA thus offers an excellent tool to study the role of central serotonergic neurons in the action of drugs such as hallucinogens, narcotic analgesics, etc

Since the racemic mixture of PCA is a substrate for dopamine β-hydroxylase, it seemed possible that the β-hydroxylated metabolite, p-chloronorephedrine, might be responsible for the long-term effects of PCA and particularly of (S)-(+)-PCA. Our investigation of all four isomers of the β-hydroxylated compounds showed, however, that they do not decrease the level of 5 HT in brain either at 4 hours or 2 weeks after a large dose. The activity of tryptophan hydroxylase was not appreciably changed. Therefore, it appears that β-hydroxylation of either isomer of PCA is not required or responsible for either the short or long-term effects of the drug on serotonergic neurons. The β-hydroxylated metabolites of PCA are also less active than the parent compound in blocking the uptake of NE, 5HT and dopamine into synaptosomes prepared from various brain areas. It is pertinent, however, that the introduction of an OH or a Cl into the p-position of the aromatic ring of amphetamine increases the activity of these drugs in blocking the neuronal membrane pump for catecholamines and causes a remarkable shift of the blocking activity from catecholaminergic to serotonergic neurons.

4) On the significance of the increase in HVA caused by antipsychotics in corpus striatum and limbic forebrain.

The effect of various antipsychotics on the blockade of dopaminergic receptors in striatum and limbic forebrain was examined by establishing dose-response curves for the increase in HVA and for the antagonism of d-amphetamine induced rotations in rats with unilateral lesions of the substantia nigra. A good quantitative correlation was found between dopaminergic blockade in the striatum as reflected by the ED_{100} for striatal HVA increase and the ED_{50}s for rotational antagonism and the occurrence of extrapyramidal side effects in man. The ED_{100} for HVA increase in the striatum by the various antipsychotics in μmole/kg were: haloperidol < 0.7; pimozide < 1.1; chlorpromazine 3.3; thioridazine 14.2; clozapine 44.0. The potency ratios of the different antipsychotics for blocking DA receptors in the striatum (biochemical) and for blocking rotational behavior due to amphetamine (physiological) are for all practical reasons identical. The ED_{100} for the increase in HVA in the limbic forebrain showed the same rank order of potency as those in the striatum: haloperidol > pimozide > chlorpromazine > thioridazine > clozapine. The results thus demonstrate an excellent correlation between the degree of dopaminergic blockade and the incidence of extrapyramidal side effects in man.

The relative ineffectiveness of thioridazine and clozapine in elevating HVA in both corpus striatum and limbic forebrain or in antagonizing amphetamine-induced stereotyped and rotational activity suggests a dissociation between dopaminergic blockade and antipsychotic efficacy. It is also pertinent in this regard that clozapine is totally ineffective in antagonizing the action of the dopamine agonist apomorphine. These studies raise the important question of why d-amphetamine can induce a psychosis indistinguishable of paranoid schizophrenia and seriously question existing hypotheses on the mode of action of antipsychotics. Obviously, the discovery of a behavioral parameter of amphetamine which is unrelated to dopaminergic mechanisms and which is blocked by clozapine, will be important for the design of experiments to unravel the crucial psychotomimetic action of amphetamines.

5) Non-hepatic metabolism of drugs of abuse and possible mechanisms for the development of tolerance.

During much of the initial period covered by this report, Dr. James V. Dingell was on sabbatical leave with R. T. Williams in the Department of Biochemistry of St. Mary's Hospital Medical School, University of London, London, England. Before his departure in February of 1973, his studies were concerned with the applicability of the methodology described in the original application to the measurement of the metabolism of morphine and meperidine by preparations of lung and brain. Included among these was a preliminary study which showed that microsomal preparations of rat brain cannot metabolize morphine by conjugation with glucuronic acid. Recent studies have now shown that when labelled p-hydroxyamphetamine was incubated with hepatic microsomes and UDP-glucuronic acid, a metabolite was formed which was not extractable into butanol. After reincubation of the mixture with β-glucuronidase, all of the radioactivity could be extracted into butanol, but its release by β-glucuronidase could be prevented by reincubating in the presence of saccharolactone, a specific inhibitor of β-glucuronidase.

The formation of POH-glucuronide was proportional to the incubation time when mixtures were incubated for less than 30 minutes; zero order kinetics were

approached when 661 mμmol of POH were incubated. The maximum rate of conjugation of POH required more than 20 μmol of UDP-glucuronic acid. Interestingly, we have previously observed that the maximum rate of conjugation of tetrahydrocortisone could be achieved with only 3 μmol of UDP-glucuronic acid. The rate of formation of the glucuronide of POH by hepatic microsomal preparations from female rats was approximately twice that of similar preparations from male rats and was stimulated four-fold when incubations were carried out in the presence of Triton X-100 (0.05% v/v).

The glucuronide metabolite of POH was formed in microsomal preparations of the livers of rats, mice, rabbits and guinea pigs; but its sulfate conjugate was not formed by the soluble fraction although the soluble fraction of liver actively conjugated α-naphthol with sulfate. None of the other tissues examined including brain, lung, heart, kidney, intestine and spleen possessed enzymes for the conjugation of POH by either pathway. The livers of rats which had been made tolerant to the anorectic action of amphetamine showed no alteration in their ability to conjugate POH with glucuronic acid and also showed no ability to form its sulfate conjugate. Pretreatment of rats for one week with POH (50 mg/kg, i.p., daily) likewise did not alter its conjugation by liver preparations, in vitro.

With the exception of the small percentage of a dose of morphine which is metabolized by demethylation, the drug is metabolically inactivated almost exclusively by conjugation with glucuronic acid. The results of preliminary studies to describe the basic properties of the glucuronyltransferase system which is involved in the biotransformation of the morphine also suggest that several species of the transferase may develop with maturation. For example, like the conjugation of 7-hydroxychlorpromazine, but unlike that previously observed with steroids, the glucuronidation of morphine is stimulated by high concentrations of substrate. Again, when high concentrations of morphine were incubated with microsomal preparations of the livers of adult rats, two pH optima were observed; one at pH 8.5, the other at pH 9.5. Under similar conditions but with Triton activated preparations, only the pH optimum at 8.5 was apparent. However, when low concentrations of morphine were incubated with hepatic microsomes from mature rats, a single pH optimum was observed at 9.5 which was identical to that found with preparations of livers from immature animals at all concentrations of substrate.

Although the process of glucuronidation by hepatic microsomes is generally stimulatable by Triton X-100, there appear to be fundamentally important differences between the transferase which conjugates exogenous substrates, such as 7-hydroxychlorpromazine and morphine and that which forms the glucuronide of steroids such as tetrahydrocortisone, estrone, and estradiol. For example, we have found that the maximum rate of conjugation of steroids can be attained by incubation with concentrations of UDPGA which are but a fraction of those which are required to saturate the system for the conjugation of p-hydroxyamphetamine, 7-hydroxychlorpromazine, or morphine. Moreover, unlike the conjugation of 7-hydroxychlorpromazine and morphine, that of the steroids does not appear to be stimulated by incubation of high concentrations of substrate. In view of these observations, it does not appear unreasonable to speculate that although several forms of glucuronyltransferase may be involved in the conjugation of the exogenous substrates, the glucuronidation of endogenous

substrates such as steroids may be mediated by a single and perhaps distinct species of the enzyme. Since conjugation processes such as glucuronidation can be viewed as true detoxification mechanisms, a greater understanding of the enzymatic processes involved can be expected to have both pharmacological and clinical relevance. Of perhaps equal importance however, a greater understanding of the basic mechanism of glucuronidation may provide the necessary information to resolve much of the frequently bewildering confusion concerning the response of the glucuronltransferase system to various stimulators, inducers, and inhibitors.

6) Biochemical and behavioral studies with d- and l-PCA.

Previous studies have shown that the administration of dl-p-chloro-amphetamine (PCA) to rats causes a long-lasting decrease in serotonin (5HT), 5-hydroxyindole acetic acid (5HIAA) and tryptophan hydroxylase activity (TH) in brain. It was, therefore, of interest to compare the biochemical effects of the pure isomers with the levels of the unchanged drug. A single dose of d- or l-PCA (5 mg/kg) was injected i.p. into male Sprague-Dawley rats. Brains were assayed for 5HT, TH, 5HIAA or synaptosomal uptake of 5 HT by standard methods. Both isomers caused a rapid and profound reduction in the brain levels of 5HT, 5HIAA and TH. Significant reductions were maintained for at least 2 weeks after the d-isomer. However, partial recovery occurs within 2 days after the administration of the l-isomer, with almost complete recovery after 2 weeks (5HT, 82%, 5HIAA, 130%; TH, 103% of control). The synaptosomal uptake of 5HT was also markedly reduced within 4 hours after treatment with d- and l-PCA: however, significant recovery has occurred within 4 days after injection of l-PCA, but not after the d-isomer. These differences in the duration of the biochemical effects may be related to differences in the rate of disappearance of the two isomers from the brain. In order to investigate this possibility we have developed a gas chromatographic method for the assay of brain levels of PCA.

The behavioral effects of the d- and l-isomer of PCA have been investigated, using the Sidmann avoidance paradigm. Both isomers increase response rate in this task; however, d-PCA is 5-10 times more potent than the l-isomer. A similar difference in potency was found when total motor activity was measured using Williamson jiggle cages. In order to test the relative importance of changes in the turnover and metabolism of DA and NE in the mediation of the stimulatory effects, we plan to study the effects of both isomers on the turnover rate of NE and DA in brain.

The studies initiated in our laboratory and supported in the past by this contract, have triggered a number of studies in other laboratories and it becomes apparent that PCA-like compounds represent interesting new tools to study serotonergic mechanisms in the CNS and their involvement in a number of biologically important functions including the development of tolerance and physical dependence (see for instance Lal et al., Federation Proceedings XXXI, 487, 1974; Morphine withdrawal syndrome in mice and rats: Partial blockade by PCA).

7) Detailed study of the biological disposition of PCA in mice and rats.

The brain levels of p-chloroamphetamine (PCA) were estimated by a gas chromatographic method developed in this laboratory. Brains were rapidly

removed after decapitation, washed with cold saline, weighed, and stored at -5° until analyzed. For analysis, brains were placed in 30 ml centrifuge tubes containing a known amount of p-chloromethamphetamine (PCMA), which serves as an internal standard. The brains were homogenized in 10 ml 0.6N cold perchloric acid using a Polytron homogenizer (Brinkman Instruments). Following centrifugation at 20,000 rpm for 20 minutes in a Sorvall high speed centrifuge, duplicate 4 ml aliquots of the supernatants were placed in 45 ml conical centrifuge tubes containing 1 ml of 5N NaOH and 5 ml of hexane (Nanograde, Burdick and Jackson Company). PCA and PCMA were extracted into the hexane layer by shaking on a Eberbach reciprocal shaker for 30 minutes. After centrifugation for 10 minutes at 1800 rpm, 4 ml aliquots of the hexane layers were transferred to 13 ml centrifuge tubes containing 1 ml of 1N HCl. The layers were equilibrated by mechanical shaking for 30 minutes and centrifugated for 10 minutes as above. The hexane hayer was carefully aspirated off and 0.9 ml of the acid layer was transferred to a 3 ml conical glass-stoppered centrifuge tube containing 100 µl of 10N NaOH. This aqueous layer was extracted with 500 µl of cyclopentane (Nanograde - Burdick and Jackson) by vigorous swirling on a Vortex mixer for 3 minutes. The tubes were centrifugated for 10 minutes and 400 microliters of the cyclopentane layer were transferred to 1 ml Reactivials. Ten µl of a 1% trichloracetyl chloride solution in cyclopentane were added. The samples were gently shaken and were allowed to stand for 20 minutes at 40°C. The samples were evaporated to near dryness by blowing with hot air, then placed in vacuo for 20 minutes to remove excess reagents. The residues were dissolved in cyclopentane and analyzed by gas chromatography (GC).

GC analyses were performed on a Varian 2100 gas chromatograph equipped with a ^{63}Ni electron capture detector, using a 6-foot 1% OV-17 column (applied Science). The conditions were : column temperature 180°C; injector temperature 200°C; and detector temperature 250°C. N_2 carrier gas was maintained at a flow rate of 50 cc/minute. Under these conditions, the retention times of PCA and PCMA were 4 minutes and 7 minutes, respectively. A standard curve was prepared by adding known amounts of PCA and PCMA to brain homogenates and by determining the ratio of PCA peak heights/PCMA peak heights. When peak ratios were within the range 0.2-4.0, a linear relationship was found between the ratios and the concentration of PCA added. Recovery of known amounts of PCA added to blank brain was 75% ± 5%. Variation among brain levels of PCA for animals treated similarly was found to be less than 10%. GC spectra of blank brains had a number of small peaks with retention times varying from 30 seconds to 16 minutes, but these did not significantly interfere with the quantification of PCA even at the lowest levels.

Combined extracts of brains of rats killed 2 hours after 1-PCA were concentrated for analysis by gas chromatography-mass spectrometry. The GC peak identified previously as PCA gave a spectrum identical to authentic PCA trichloracetamide. The base ion was m/e 152, equal to approximately 13% of the total ion current. This peak represents the p-chlorophenylpropylene ion. The other major peaks were m/e 188 and m/e 127, representing fragmentation between the α- and β- carbons. PCMA was also confirmed by comparison of the peaks in extracted samples with authentic material. Ions with m/e 127 and 152 were also present in this compound, but the ion at m/e 188 shifted to m/e 202 (+ 14) and became the base peak, accounting for approximately 13% of the total ion current.

The use of the ^{63}N electron capture detector allowed detection of PCA

from 20 ng to 40 µg per gram brain. However, the very limited linear range of this detector (theoretically 1:50 - in our hands 1:8) presents some difficulty. If the amount of either PCA or PCMA which is injected in the GC is sufficiently high, the detector will become "saturated", resulting in erroneous ratios.

The rate of decline of levels of both d- and l-PCA in brain was determined. Male Sprague-Dawley rats were injected i.p. with either d- or l-PCA HCl at a dose equivalent to 5 mg/kg of the free base. Groups of 4 animals were sacrificed at 4, 8, 24, 36 and 48 hours after injection, and the brain levels were analyzed by the method previously described. The brain levels of the two isomers were the same at each time period analyzed. From 4-36 hours, the brain levels of both isomers declined in a monophasic-exponential manner, with a half-life of 8 hours. The 48 hour levels were slightly above the extrapolated linear line, indicating the possibility of a second phase in the fall-off curve. In order to test this possibility we plan to extend the time-course. Since the 48-hour value was the same for both d- and l-PCA, it appears that the differences in the duration of the effects of serotonergic neurons are not explained by differences in half-lives.

8) Neuropnysiological studies on the mode of action of morphine.

We have been studying the effects of intravenously administered morphine on single unit activity of neurones within the raphé dorsalis and medianus of male Sprague-Dawley rats. Both tungsten and NaCl-filled glass microelectrodes have been used in our initial studies. However, the NaCl-filled glass electrodes have been found to give the best recordings and have been utilized for most of the experiments.

Since serotonergic mechanisms have been implicated in temperature regulation, it is not surprising to find alterations in neuronal activity within the raphé nuclei after alterations in body temperature. Our initial studies thus indicated a need to maintain the body temperature of our preparation within normal limits and to maintain all injection solutions at body temperature. We have now accumulated several experiments on the effects of systemic administration of morphine on neuronal activity of raphé cells. As reported by others, the spontaneous firing rate of cell within the raphé nuclei was in the range of 1-3/sec. After administration of morphine (8 or 16 mg/kg, i.v.) there was a marked (4-10 fold) decrease in single unit activity. Firing rate was lowered so greatly that we became concerned over the possibility that the cell had been "lost". However, after injection of the narcotic antagonist naloxone (5 mg/kg, i.v.) the firing rate not only returned but was increased above the normal rate. Thus, the effects of morphine on single unit activity do appear to be related to a narcotic effect.

9) Effect of partial disruption of serotonergic mechanisms on morphine-induced 5-HT turnover.

Our previous work raised the possibility that pharmacological or mechanical insults to serotonergic mechanisms within the CNS which did not produce any decrease in the levels of 5-HT within the forebrain might still cause a disruption of serotonergic function under certain conditions. Thus, PCA (0.1 mg/kg) which does not alter the levels of 5-HT in the brain still antagonized morphine-induced hypoactivity, which is proposed to be dependent upon

an increase in 5-HT turnover. We have, therefore, studied the effects of PCA (0.1 mg/kg) on 5-HT turnover using the isotopic technique of Neff et al. (1971). We also studied the effects of morphine on 5-HT turnover in animals pretreated with saline or PCA (0.1 mg/kg) 24 hours previously. The turnover of 5-HT in PCA-treated animals was not significantly different from that of saline-treated animals. Morphine-treated animals showed a significant increase in 5-HT turnover. However, in PCA-pretreated animals the morphine-induced increase in 5-HT was reduced so that the 5-HT turnover was now not significantly different from saline-treated animals. However, the turnover was not different from that seen in animals receiving morphine alone. Thus, doses of PCA which do not alter levels of 5-HT in the brain do appear to reduce the increase in 5-HT turnover normally produced by morphine administration. Further studies are being carried out to confirm or further define these initial observations.

10) Studies on the development of tolerance to d-amphetamine, and on post-drug depression.

Since our initial finding that, following chronic administration, tolerance develops in rats to the facilitation of self-stimulation behavior, we have initiated several studies which hopefully would help to unravel the biochemical and anatomical basis of the phenomena. Our first study was aimed at determining the possible role of PHN in tolerance. To do this we tested the effect of chronic administration of l-amphetamine, since PHN is not a metabolite of this compound.

We first tested several doses of l-amphetamine (0.5 - 2.0 mg/kg) to determine the dose which would produce the most reliable facilitation of this particular behavior. Form these data we decided to use 1.0 mg/kg as our test dose. This dose produced a marked and reliable facilitation of responding similar to that seen following administration of 0.5 mg/kg d-amphetamine. Although the magnitude of the effect with l-amphetamine was less than with d-amphetamine, increasing the dose of the l-isomer resulted in a decrease of facilitation, probably because other effects of the drug (e.g. stereotyped behavior) which occur at these higher doses were disruptive.

Two groups of animals were then trained to respond for self-stimulation in the medial forebrain bundle (MFB) and were tested 3 or 4 times for facilitation of responding with either d- or l-amphetamine to assure that the drug effect was reliable. At this point testing was terminated and increasing doses of d- (1.0 - 12/0 mg.kg) or l- (2.0 - 24.0 mg/kg) amphetamine were administered 3 times daily for 4 days. On the fifth day, the animals were again tested for facilitation of responding following the administration of the test doses of the drugs. Much to our surprise, the animals which had received the chronic d-amphetamine treatment did not show tolerance to the test dose of that drug. These data were in contrast to our previous data with 6 animals, which all showed tolerance after identical treatment. In the case of l-amphetamine, 3 animals were tested. Two of these showed tolerance following chronic administration while the third showed a very marked decrease in the drug effect. However, since the d-amphetamine animals had not shown tolerance, we could not reach a conclusion concerning similarities or differences between the effects of the two isomers.

At this point, we decided to test a larger group of animals to determine whether there would be any behavioral differences between animals which do or

do not develop tolerance which might enable us to predict a priori whether or not tolerance would develop. To date we have tested 24 animals, 16 of which have developed tolerance while 8 have not.

Careful examination of the data has not revealed any reliable behavioral differences between the two groups. However, histological examination of the brains of these animals has revealed that there are reliable differences in the exact site of the electrodes. In all animals, the electrode placements were in approximately the same anterior-posterior region of the brain, being around 4.0 mm anterior to the interaural line, when the skull is leveled between bregma and lambda. However, in the group of animals which showed tolerance, most had electrodes terminating in or very close to the zona incerta, an area of AChE - containing fibers as well as catecholamine fibers. The non-tolerance animals had their electodes terminating approximately 1.0 mm more ventral, lying in or very close to the fornix. Having determined these placement differences, we are now ready to repeat the work comparing d and l-amphetamine using animals implanted in the zona incerta region, where tolerance to d-amphetamine does develop.

Other work aimed at gaining more information concerning amphetamine tolerance and depression has involved training animals to respond for stimulation in the locus coeruleus, a site of NE cell bodies. When tested following chronic d-amphetamine treatment, the animals did not show tolerance but did exhibit post-drug depression. Histological examination of the brains of these four animals revealed, that the electrodes were medial to the locus coeruleus, terminating primarily in the dorsal tegmental nucleus. Howver, it is still possible that the effects were due to stimulating the locus coeruleus since the two structures lie very close and the current does spread to some extent. Therefore, we have readjusted our coordinates and implanted animals with electrodes which should be closer to the locus coeruleus. We will repeat the study to determine which site was really responsible for the effects seen.

The final study which we were able to undertake in part was concerned with possible cross tolerance between d- amphetamine, cocaine and methylphenidate. Since these drugs all facilitate self-stimulation responding similarly but have different mechanisms of action, we reasoned that cross-tolerance studies might provide information on the mechanism (presynaptic or postsynaptic) for development of non-metabolic tolerance to amphetamine. To date we have completed the work with cocaine. Animals which were administered increasing doses of d-amphetamine and showed tolerance to that drug were also tolerant to cocaine. We are presently beginning further studies which will involve testing for cross-tolerance to methylphenidate as well as administering cocaine or methylphenidate chronically to determine if tolerance develops to these drugs and, if so, to test for cross-tolerance to d-amphetamine.

11) Development of new methodology for the analysis of the level and turnover of acetylcholine (ACH)

In order to meaningfully investigate the cholinergic system it was necessary to be able to assess ACh turnover, rather than just measure steady state level The initial work was therefore methodological. A simultaneous assay for ACh and choline was developed for use in conjunction with radio-labelling techniques for measuring ACh turnover. It was also necessary to improve the microwave sacrifice techniques to prevent post mortem changes in the levels of ACh and choline.

However, the complicated nature of the kinetics of the cholinergic system and the inability to accurately measure the specific activity of the choline precursor pool makes interpretation of isotopic experiments difficult. Work was therefore undertaken to develop an alternate method for ACh turnover.

Another approach to assessing turnover is to measure the decline of ACh levels following blockade of synthesis. We attempted to use the choline-acelytransferase inhibitor, bromoacetylcholine for such a method but, it proved to be unsuitable. A second approach has proved valuable. We and others have shown that intraventricular administration of hemicholinium - 3 (HC-3) causes a dose and time dependent decrease in ACh levels in various brain regions. Work in other laboratories has shown that the decline is dependent on and proportional to the rate of firing of cholinergic neurons. Therefore, drug induced changes in the rate of HC-3 induced decline of ACh levels is probably proportional to their effect on cholinergic neurons and this method therefore constitutes an indirect method for assessing ACh turnover. We have used the HC-3 method to measure the effect of morphine on ACh turnover.

12) Effect of morphine and amphetamine on the level and turnover of ACh.

Using the focused microwave system, doses of morphine as high as 128 mg/kg did not produce changes in levels of the ACh in any brain region measured. We have measured, 1 hr. after administration, the effect of 2, 4, 8, 16, 32, 64 and 128 mg/kg of morphine on ACh turnover in specific brain regions. There is a large and very significant ($p < .001$) dose dependent decrease in the turnover of ACh in the hippocampus and hypothalamus; there is a much smaller, and less significant ($p < .10$) decrease in the turnover of ACh in mid-brain. The hypothalamus appears to be more sensitive than either the hippocampus or mid brain and effects of morphine appear to be maximum in all three areas at doses of 16 - 32 mg/kg. Surprisingly, morphine does not effect ACh turnover in the striatum. The decrease in turnover of the ACh induced by morphine is blocked in all areas by pretreatment of the animals with naloxone.

We have also looked at the time causes of morphine induced changes in ACh turnover following either 4 or 16 mg/kg. Preliminary results indicate that the effects following 4 mg/kg are evident from 1 to 5 hrs. after administration, but have disappeared 7 hrs. after administration of the drug. Following 16 mg/kg, the decrease in turnover persists for 7 - 10 hrs. in the mid-brain and hypothalamus, but it appears that there may be a "rebound" increase in ACh turnover in the hippocampus.

We have also measured the rate of high affinity choline uptake by synaptosomes from hippocampus and striatum of rats given morphine in vivo. Doses of morphine of either 16 or 32 mg/kg did not affect choline uptake 1 hr. after administration.

Studies with amphetamine indicate that the drug can induce simultaneously increases and decreases in the level of ACh, depending on the area of the brain.

Preliminary results using the HC-3 method indicate that those areas which show an increase in the level of ACh show a decrease in the turnover of ACh (Striatum and mid brains) and those areas which show a decrease in levels of ACh appear to have a increased ACh turnover.

13) Studies on the norepinephrine sensitive cyclic AMP generating system in the limbic forebrain.

Although these studies have been chiefly supported by PHS grant MH-11468, the data are pertinent to this proposal as they draw attention to the crucial role of noradrenergic mechanisms in CNS function and particularly in the mode of action of amphetamine - and possibly LSD - antagonists. The studies are thus briefly summarized:

Because the limbic forebrain receives ascending norepinephrine (NE) fibers originating in cell bodies of the pons and medulla oblongata, dopamine fibers from the A-10 region and 5-HT fibers from the anterior raphé complex, it was of interest to know whether or not an adenylate cyclase system sensitive to 1, 2, or all 3 of the putative neurotransmitters was present in slices of the limbic forebrain. We found that slices of the rat limbic forebrain contain at least two different cyclic AMP generating systems: 1) A noradrenergic system which responds to NE (K_a approximately 5 μM: maximal stimulation occurring at 10 to 50 μM) and isoproterenol (K_a approximately 5 μM). Although the rise of the nucleotide elicited by isoproterenol is more rapid than that caused by NE, the maximal effect is less than half of that induced by NE. 2) An adenosine dependent system (K_a approximately 30 μM). The noradrenergic cyclic AMP generating system in the limbic forebrain displays a number of properties of a central NE receptor: It develops supersensitivity to NE and isoproterenol following prolonged deprivation of NE at postsynaptic sites (chronic treatment with reserpine, chemosympathectomy with 6-OHDA, surgical denervation). When noradrenergic terminals are protected from 6-OHDA by DMI, the responses to NE are not enhanced. Responses to NE are blocked by both propranolol and phentolamine, while responses to isoproterenol are blocked by propranolol but not by phentolamine. While the presence of classical β-receptors is fairly well established, the blockade of the NE response by phentolamine does not necessarily imply the presence of classical α-receptors as α-stimulants, such as phenylephrine and clonidine, do not increase the level of the nucleotide in this area of the brain. The adenosine dependent system does not develop supersensitivity after central chemosympathectomy and is not blocked by either α- or β-antagonists. Interestingly, dopamine, (DA) and serotonin (5HT) were without effect in all concentrations tested. While not altering the basal level of the nucleotide, clinically effective anti-psychotic drugs caused a dose dependent inhibition of the limbic noradrenergic cyclic AMP response with clozapine and pimozide being particularly potent (IC_{50} 0.06 and 0.08 μM respectively). Antipsychotic drugs do, however, not affect cyclic AMP responses elicited by adenosine. The results are compatible with the view that the central NE receptor is closely related to or may be an intregral part of an adenylate cyclase system and that its blockade in the limbic forebrain by antipsychotic drugs may contribute to their therapeutic action.

More recent studies in our laboratories have revealed that, independent of the actual concentration of NE in brain, a persistent increase in the availability of NE (MAO inhibition, blockade of presynaptic neuronal uptake, ECT) leads to a marked decrease in the activity of the cyclic AMP generating system. The results provide evidence for an additional regulatory mechanism in the CNS involving the noradrenergic receptor that adapts its sensitivity to NE in a manner inversely related to the degree of its stimulation by the catecholamine. On the practical side, the demonstrated decreased reactivity to NE following ECT may provide the biochemical basis for the observed antagonism by ECT of stimulatory effects elicited by amphetamine (Pepeschi et al. Psychopharmacologia, 35: 149, 1974) and provokes some novel thoughts on the

development of clinically (and thus militarily) relevant antiamphetamine drugs.

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C. PERSONNEL SUPPORTED BY CONTRACT DADA 17-73-C-3130

1.	Fridolin Sulser, M.D., Principal Investigator July 1, 1973 - October 31, 1975	10%
2.	Jerzy Vetulani, Ph.D., Assistant Professor of Pharmacology December 7, 1973 - June 30, 1975	30%
3.	H. Joseph Sekerke, Ph.D., Instructor in Pharmacology July 1, 1973 - August 31, 1974	100%
4.	Robert J. Stawarz, Ph.D., Instructor in Pharmacology July 1, 1973 - October 31, 1975	75%
5.	Michael Laskowski, Ph.D., Instructor in Pharmacology July 1, 1973 - December 31, 1973	32%
6.	Elizabeth Burrows, Ph.D., Research Associate in Chemistry May 1, 1974 - August 31, 1974	100%
7.	Nancy Leith, Ph.D., Research Associate in Pharmacology October 1, 1973 - June 30, 1975	50%
8.	Jon Neergaard, Ph.D., Research Associate in Chemistry March 1, 1974 - May 31, 1974 June 1, 1974 - June 30, 1974	 53% 100%
9.	Sharon Buff, Research Assistant July 1, 1973 - August 25, 1974	100%
10.	Eve W. Cole, Research Assistant September 10, 1973 - October 11, 1975	50%
11.	William Pamplin, Research Assistant March 18, 1974 - June 30, 1975	100%
12.	Craig Lewis, Research Assistant October 27, 1974 - June 30, 1975	100%
13.	Stuart Berney, Research Assistant February 8, 1974 - June 30, 1974	100%
14.	Ronnie Nixon, Research Assistant September 2, 1973 - June 7, 1975	27%
15.	Charles Mount, Research Assistant July 1, 1973 - August 27, 1974	100%

16. George Jones, Animal Care Technician 25%
March 3, 1974 - June 30, 1975

17. Linda D. Brush, Secretary 15%
May 31, 1974 - June 30, 1975

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