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Report No. 2

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U.S. Army Chemical Center

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Title: Basic Psychological Studies of the Effects of Incapacitating Agents

Contractor: Indiana University Foundation

Research Division

Bloomington, Indiana

Principal Investigator: Roger W. Russell

1. Nature of the present report. The present report covers activities during the second six months of the project. Report No. 1 described the objectives of a program for identifying and evaluating chemical agents for potential incapacitating properties and specified requirements for achieving the objectives. The present report describes the first six months work on the design of specific screening techniques and the experimental testing of their adequacies in operation, research which will continue during the remaining year of the contract.

Report No. 2 has been reviewed and approved by Dr. Stanley S. Pliskoff, Contract Project Officer.

2. General considerations. Beginning in September 1960 two senior investigators were added to the project's staff. Dr. Ethelda N. Sassenrath (Ph.D. Biochemistry) is responsible for the biochemical and pharmacological studies; Dr. Frank Dalziel (Ph.D. Psychology), for the development of behavioral techniques. Both will continue with the project during the remainder of the contract period. In addition we have the services of two graduate students on university assistantships, whose research interests are in the general field of the project.

During the second six months, work on the project has emphasized two phases of the general program outlined in Report No. 1: the development of techniques for screening in depth using animal subjects and the study of basic biochemical and pharmacological characteristics of a prototype chemical agent.

Librium (7-chloro-2-methylamino-5-phenyl-3H-1, 4-benzodiazepine-4-oxide hydrochloride) was chosen as the first prototype agent because of the fact that this drug was already being used in other studies in the department's laboratories, studies involving both human and infrahuman animal subjects. By coordinating our research in this way at this time it is possible to use data from the other studies for our purposes, thus essentially extending our program without additional time and cost to the project. By agreement with other investigators we intend to continue to augment our own work in this way whenever possible. We will include brief references to the supplementary work in the present report.

3. Preliminary screening: animal. Report No. 1 argued for the importance of preliminary screening as the first phase of a general screening program for the identification and evaluation of incapacitating agents. Such screening would involve techniques which are "quick" in the sense of requiring only one day or a very few days to complete; they are "limited" because they do not allow study in depth and because they provide results which depend heavily upon the observational skill of the investigator under very restricted conditions of observation.

During the period covered by this report we have obtained more detailed information from workers who have developed and are using preliminary screening techniques. Many of these are investigators in laboratories supported by pharmaceutical companies. We have visited one such laboratory

in order to observe the techniques in operation.

Our plans are to evaluate a selected combination of the techniques in terms of the requirements for behavioral measures described in Report No. 1. The evaluation should begin during the next six-months period of the contract.

4. Screening in depth: animal. We have concentrated much of our attention during the second six months of the contract period on the development of instrumentation and procedures for animal screening in depth. The work has been within the program outlined in Appendix A of Report No. 1.

a. Research under the present contract. The work has given particular emphasis to instrumentation required for the observation of behavior in three general types of situations, each of which has advantages in permitting the recording of a variety of different measures of behavior. There are obvious advantages in selecting multiple-purpose equipment for screening programs.

(1). Conditioned emotional response (CER). The measurement of this response requires the use of operant conditioning equipment, equipment which can also be used for observing discrimination behavior, operant responses under different conditions of reinforcement, multiple-response performance, conditioned avoidance response, and conflict behavior.

In eliciting the CER, a stimulus with emotional loadings is presented while the organism is engaged in some specified activity. The degree of disruption of the activity is the measure of the strength of the emotional response. Two features are essential with this sort of experimental pardigm. First, the on-going activity must be of a stable regular nature so that any variation in it can be clearly associated with the emotional stimulus. Second, the CER must be of an intensity which will permit the resumption of the activity after the stimulus which elicits it is withdrawn.

Our CER apparatus consists essentially of a "Skinner box" and programming and recording equipment. The on-going activity is a bar-press for a food reward. The animal, which is maintained at 70% of its normal body weight, can receive a pellet of food on the average of once a minute. The exact time interval is varied so as to keep the animal responding regularly all the time. We require that his behavior should vary as little as possible. From day to day we can check the regularity by the number of responses the animal produces. We require more than this, however; we wish the animal to produce as nearly as possible the same number of responses in each successive minute of his experimental period. We can have a reasonable check on this from the cumulative graph which our apparatus automatically produces. Considerable adjustment was required before we reached this stage.

We are now introducing the emotional stimulus to our trial animals. The emotion is produced by giving the rats a mild shock to their feet while they are in the box. This shock is preceeded by an auditory stimulus which acquires the emotion-arousing properties previously mentioned. Both the duration of the sound and, more especially, the intensity of the shock are crucial to the successful reproduction of the behavior. Determining empirically the optimal combination of these and other parameters is one of the problems on which we are now working.

Dr. Frank Dalziel, Research Assistant on the project, visited Dr. H. Hunt of the University of Chicago, who has worked very extensively in this field, and acquired a great deal of unpublished information relating to the establishment and maintenance of this response. Dr. C.B. Ferster of the Medical Center, Indiana University, also supplied much useful advice, assistance and equipment.

(2). Straightaway operant response (SOR). This relatively simple response has been used advantageously to observe the speed and characteristics of locomotor movements, the effects of conflict on behavior, and to study effects of drugs on different forms of motivation. Our general plan is to standardize procedures which will permit the recording of reliable measures of the SOR when the behavior involves approach to food, avoidance of shock, and escape from water: three forms of motivation and two types of locomotion will be involved.

We have run several thousand trials in a preliminary form of the apparatus. The present runway consists of a running compartment four feet six inches long with start and goal boxes of eight inches at either end. The measure taken is the length of time required for the animal to traverse the running compartment. He is 23 hours deprived of food and is allowed to eat for a period of 10 sec. in the goal box. As is the case with all behavioral measures we will develop, our aim with this situation is to try to establish behavior of great regularity so that any drug effect will be thrown into sharp relief. This involves introducing changes in procedure at several points and observations of the effects of various situational parameters on the SOR.

Because of these changes it is somewhat difficult yet to answer the question as to how long the regular behavior takes to establish. Our present maximum estimate is two weeks, which means that it should not be too difficult to maintain a supply of trained animals for routine screening purposes. We know from the results so far that a stable level of performance can be established. We have done the necessary statistical computations for determining the internal consistency of the SOR during a day's trials and also from day to day once an asymptote for performance has been reached. The coefficients obtained are in the 0.70's,

which, we believe, can be improved by further changes in the procedure. With the assistance of Mr. M. Smith, a senior graduate student in the department, we are now working on the automation of the test situation.

In order to develop our general research designs for the study of chemical agents and our techniques for administering the agents, we are proceeding immediately with a series of agent and control runs using the present form of the SOR. We will continue to use Librium as the prototype agent in order to permit comparisons with data involving other behavior patterns.

We have also done preliminary work on the SOR involving escape from water and swimming as the mode of locomotion. The work indicated a need for certain changes in instrumentation, which are in hand.

(3). Conditioned shuttle response (CSR). We have constructed a prototype shuttle box on the general plan first employed by Mowrer and Miller. Basically this consists of a box unit divided by an obstruction over which a rat can jump. Each half of the box has an electric grid for a floor which delivers mild shocks to the rat's feet. The rat can either escape or avoid the shock, depending upon the experimental procedure, by jumping over the obstruction to the inactive grid on the other side of the unit. The intensity of the shock can be varied and is one of the major situational parameters. The apparatus may be used for observing and recording discrimination behavior, conditioned escape and avoidance responses, and conflict behavior.

Instrumentation of this experimental situation, particularly construction of equipment for precise control of shock intensity and duration, is now almost complete. Responses will be automatically recorded using photoelectric cells. A few preliminary trials have been run and we expect to have this equipment functioning on a regular schedule in the very near future.

(4). Other instrumentation for the study of rat behavior. We have begun plans for construction of an apparatus which can be used for routine measurement of food and water intake and general activity. Our aim is to combine these in a single apparatus, which will avoid some of the technical problems referred to in Report No. 1.

(5). Instrumentation for research on monkeys. Because of the interest of several faculty members in the department in the study of various behavior patterns in monkeys, we will have available apparatus which can be used in our studies of the species. We have not been able during the past six month to

devote specific attention to the adaptation of this equipment for our purposes; however, very little adaptation will be necessary. The apparatus will be semi- or fully automated; it will permit us to record and to measure behavior patterns analogous to those included in the studies using rats as subjects.

b. Related research in the Department of Psychology, Indiana University. As stated earlier, we are able to draw upon other research going on in the department, which is related to our interests in developing an adequate behavioral screening battery. It will be possible for us to use data collected to determine the reliabilities of the measures involved. Through the cooperation of the other investigators we have planned to obtain comparative data on the effects produced by administration of Librium. Availability of these data essentially extends the range of work we can expect to accomplish under the present contract.

Several of the related studies involve observations of operant responses under different conditions of reinforcement; one is designed to study an unlearned response which is very stable. One of the operant studies, under the direction of Professor J.A. Dinsmoor, has already produced measures which are very sensitive to relatively low doses of Librium. Another is being conducted by an advanced graduate student, Mr. A. Bruner; it may be used as an example of why we are interested in taking full advantage of research in the department which is related to the present project:

The essential aspect of this study is the establishment of timing behavior in rats through use of both a punishment contingency and a food reward contingency. Training is begun on a schedule of reinforcement wherein the animal must pause a specified minimum time period between bar-presses in order to receive food reinforcement. This is called the differential reinforcement of low rates (drl) and at the end of this phase, the rats are required to pause at least 18 seconds between bar-presses (drl 18). After stabilizing on this schedule, punishment (weak shock) is introduced, being given for all "premature" presses; presses that occur before the required time delay has elapsed. With extensive training under the contingencies the animals will eventually display a distribution of responses over time in which virtually no presses occur prior to the required time delay. Thus, a rather precise, temporal discrimination is obtained. The final stage involves limiting the time period after the required delay has elapsed (limited hold). As the limited hold is gradually decreased the animal has less time during which his presses may be rewarded. This may be reduced until the final situation facing the rat is as follows:

(1). A bar-press which follows a previous press by less than 18 seconds is punished.

(2). A bar-press which follows a previous press by at least

18 seconds, but no more than 21 seconds is food reinforced.

(3). A bar-press which follows a previous press by more than 21 seconds receives neither positive nor negative reinforcement but instead resets the timing device, measuring the start of the next inter-response time.

A very precise temporal discrimination is thus established. It is maintained under a reinforcement contingency whose character suggests that the competing nature of the non-bar-press versus the bar-press discriminable only by the passage of time is a rather stressful situation for the animal, one which may in fact lead to a breakdown in behavior. The applicability of pharmacological investigation, therefore, would seem to be of considerable interest, since we have a form of stable behavior (timing) which is extremely sensitive to drug influences, combined with a stressful competing response situation which might be greatly altered in regard to the animals' behavior under the effects of chemical agents.

5. Laboratory tests: human. By selecting Librium as a prototype agent for our present studies we have been able to coordinate our work with drug research on human subjects being conducted in the Department of Psychology under support from other sources. This research has been under the direction of Professor R.C. Davis.

Professor Davis' research involves the measurement of both electrophysiological and behavioral response variables. The former include: a variety of circulatory variables, respiratory variables, skin resistance, and electromyographic variables. The performance tests used are: intermittent tapping, continuous tapping, maximum speed tapping, and time estimation. Measurements are taken before and after a loud auditory stimulus. Taken by themselves, the pre-stimulus records constitute a "rest level"; the difference between post- and pre-stimulus recordings are taken as the response to the stimulus. Comparisons of rest levels and responses to the stimulus following administration of drugs and placebo are made in absolute units or in per cent change. To date five different chemical agents have been used in the research; they have produced differential effects on the measures recorded.

The data collected in this research can be analyzed for our purposes.

6. Biochemical and pharmacological studies. These studies have been conducted under the direction of Dr. Ethelda N. Sassenrath, who joined the project on September 15, 1960 under an extension to the original contract. The immediate objective of these studies is the development and standardization of procedures for use in conjunction with behavior studies in the characterization of potential psychoactive chemical agents. It is proposed to study a spectrum of known psychoactive agents with reference to their physiological and behavioral toxicities, and to trace their blood level and tissue distribution following administration under the exact conditions in which our behavioral tests are being evaluated. For what we believe to be

very convincing reasons (see Russell, R.W. Drugs as tools in behavioral research. In: Uhr, L. and Miller, J.G. Drugs and behavior. New York: Wiley, 1960. Particularly pp. 32-35.) we wish to have toxicity and metabolic data whenever possible; such data can aid greatly in the design of particular experiments and in accurate interpretation of any behavioral effects observed. It is hoped that ultimately these studies, together with selected biochemical assays of drug-treated organisms, may be of value in elucidating the modes of action of the drugs employed.

All work in this first six months has been subject to limitations in physical facilities, equipment and animal housing. However, it has been possible to complete some preliminary work of a chemical and analytical nature with the tranquilizer Librium. It has also been possible to apply some of these procedures and techniques to blood-analysis studies on human subjects participating in another project in the Psychology Department. With the recent availability of suitable animal stock and facilities for animal work, studies with Librium-treated rats are now being started.

a. Background chemical studies with Librium. Librium (7-chloro-2-methylamino-5-phenyl-3H-1, 4-benzodiazepine-4-oxide hydrochloride) was available in 25 mg. capsuls (Hoffman-LaRoche, for oral clinical administration), each of which contained inert "carrier" solids to a total weight of 200 mg. The capsul contents were not completely soluble in water or dilute HCl (at a Librium level of 100 mg%). However, both the soluble and insoluble diluents appeared to be inert in all of the chemical studies reported here. A supply of pure Librium has been made available by Hoffman-LaRoche for rat studies.

(1). Colorimetric determination of Librium. A procedure designed for the clinical determination of Librium in blood or urine was made available to us by Dr. L.O. Randall of Hoffman-LaRoche. The procedure involved drug extraction from alkaline solution into ether and back-extraction into 6N HCl; acid hydrolysis of the extracted drug (to yield 2-amino-5-chlorobenzophenone); and colorimetric determination of the liberated amine by diazotization and coupling with N-(1-napthyl)-ethylene diamine dihydrochloride (Bratton-Marshall reagent). The method had a reported limiting sensitivity of 2 µg Librium.

We found it possible to increase the sensitivity of the method four-fold by making the following procedural changes: reduction of the acid concentration in the extraction and hydrolysis steps; hydrolysis under milder conditions of temperature as well as acid concentration; reduction of the total volume during diazotization and coupling; and extension of the time allowed for color development. This increased sensitivity was judged desirable for working with the smaller blood samples anticipated in rat studies.

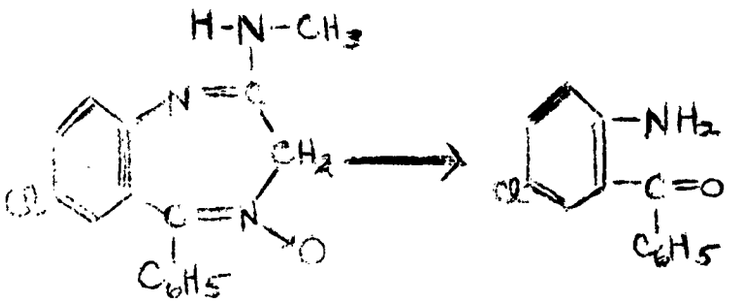
(2). Ultra violet adsorption spectra studies. Fresh solutions of Librium were observed to show a diffuse adsorption peak

in the UV region with a 245 - 250 mμ maximum. By observing changes in this characteristic UV spectrum, it was possible to demonstrate instability of the drug in acid solution at concentrations < 5 μg per ml. after storage for more than 24 hours in the cold and to characterize changes during the course of acid hydrolysis.

The observed instability of dilute solutions of Librium confirmed observations made on blood extracts of Librium-treated subjects, and indicated the necessity of analysing the blood samples immediately upon removal from the organism.

The transitory nature of the changes in the UV spectra observed during the course of acid hydrolysis indicated a progressive degradation and a range of intermediate hydrolysis products. These observed spectral changes suggested the type of metabolically-induced changes which might be looked for in the analysis of blood from Librium-treated organisms.

(3). Studies on the N-Oxide grouping. The diazotization procedure for the determination of Librium involves a hydrolysis of the ring containing the N-oxide grouping to yield an amino benzophenone derivative, as follows:



It is apparent that the assay of Librium via quantitative determination of the amino hydrolysis product does not differentiate between intact drug and drug in which the less stable 7-membered ring might have been metabolically altered. For this reason it was felt that a specific test for the N-oxide group would be of particular interest in the blood level studies with Librium. Such a procedure was developed for use with aqueous or ethereal solutions of Librium, utilizing the reported color formation by the action of acetic anhydride on N-oxide compounds (Culvenor, Rev. Pure and Applied Chem. 3: 83-114, 1953). Using this procedure it was possible to follow the acid hydrolysis of Librium with time and to demonstrate the progressive loss of the N-oxide group concurrent with the appearance of the diazotizable amine hydrolysis product.

It was not possible to apply this procedure to the human blood samples analyzed to date because of the low drug levels and the limited blood volumes available. However, rat studies using higher levels of Librium, should show whether the N-oxide group disappears faster than, or in a stoichiometric relationship with, the part of the drug molecule which is determined by the diazotization procedure.

b. Blood analysis studies on Librium-treated human subjects. These studies have involved use of the hydrolysis-diazotization and UV spectral analysis procedures.

(1). Blood level studies. Using the hydrolysis-diazotization procedure for the determination of Librium level in the blood, a study was made of human subjects on a chronic administration schedule of Librium. On a dosage of 100 mg. per day (25 mg. q.i.d. per os), two subjects attained a drug level of approximately 4 μg per ml. plasma. This level was apparently attained within three days. On cessation of treatment, blood levels of the drug dropped off to zero over a period of 5 to 7 days. Data for the two subjects analyzed are shown in Table 1. A single blood analysis on one subject who had been on continuing Librium therapy for 60 days at a dosage of 30 mg. per day (10 mg. t.i.d.) showed a blood level of 1.9 μg per ml. plasma.

It is of interest to note that, with both subjects I and VI shown in Table I, the blood levels of Librium are low after one day of therapy (ca. 0.5 μg per ml. plasma). However, other studies on these subjects show that at this time there are pronounced changes in certain physiological indices selected to show changes in the resting levels and/or responses to stress of respiratory, vascular, sweat gland, and gastrointestinal systems (R.C. Davis, unpublished data). Conversely, the effects on most of the physiological indices drop off sharply within 1 or 2 days after cessation of Librium administration, while the blood levels of the drug decrease much more slowly. At least one physiological measure shows prolonged effect long after the drug is no longer detectable in the blood. It is hoped that rat studies now being initiated may throw some light on the cause for this non-correspondence between blood level and drug effects.

(2). Ultra violet spectra of blood extracts. In the course of determining the blood levels of Librium reported in Table I, additional UV spectral analyses were run on the fresh blood extracts. This was done in an attempt to detect any metabolically-induced changes in the extracted blood which might be comparable to the type of chemically-induced changes observed during the course of hydrolysis. It was found that there was a rough correlation between the peak heights at 245 - 250 mμ and the colorimetrically determined drug levels. However, there was little evidence for a drug metabolite showing a unique adsorption peak

in the UV region.

Hydrolysis of Librium-containing blood extracts produced spectra with a single maximum in the UV region at 255 m_μ. However, this same peak had been observed with hydrolysates of blood extracts of fasted untreated control subjects. It occurred spontaneously on storage of the acid (3N HCl) blood extracts in the cold over a period of several days, as well as after hydrolysis at 100°C. in 3N HCl for 45 minutes. It was not observed with the one untreated control subject who was non-fasted. It is, then, possibly a blood component which is mobilized endogenously under the experimental conditions (all blood samples taken $>$ 4 hours after food intake), and which has solubility properties similar to Librium so that it is carried through the ether and HCl extraction.

The results of these spectral analysis studies indicated that it is possible to detect Librium levels in the blood extracts semi-quantitatively in this way. However, little additional information was gleaned by this procedure, and the spectral studies have been discontinued.

c. Research in progress. We are now proceeding with studies using rats as subjects.

(1). Basic rat toxicity studies. The Holtzman line of Sprague Dawley albino rat is now being tested for its docility during oral and intraperitoneal drug administration. Subsequent to these preliminary studies will be those aimed at establishing a criterion for determination of suitable dose levels for behavior tests. Such studies will involve the determination of LD_{50} values for each drug under our experimental conditions and with our experimental animal stock. However, prime concern will be given to development of criteria for determining the ID_{50} or "immobilizing dose," i.e. the drug level which renders the animal physically incapable of response in a behavior test. The first drugs being tested are Librium and DFP.

(2). Blood level-time curves. Rat studies are being planned to determine the blood level-time curves following acute oral administration of Librium at selected dose levels. In these studies the Librium concentration will be determined concurrently by the two aforementioned procedures, i.e., the diazotization procedure used previously with human subjects and the acetic anhydride procedure for the determination of the N-oxide group. Such studies should demonstrate any preferential elimination of the N-oxide group in the course of drug metabolism in the rat, as well as demonstrate any correspondence between blood level and behavioral effects.

Similar blood level-time curve studies will be performed

with DFP, contingent upon the establishment of suitable analytical procedures for DFP in the blood.

7. Plans for continuation of the present project. The present project is concerned with developing reliable behavioral tests for the screening of potential psychoactive chemical agents and with relating effects evidenced in the tests to data concerning certain pharmacological and biochemical actions of agents. Initial studies to implement the general program outlined in Report No. 1 have been taken during the period covered by the present report. Such studies will be continued during the next six months; they will involve additional measures of behavior and other prototype chemical agents.

Table I

Librium Blood Level Studies: Human Subjects

Subject	Treatment			Librium Level*
	Daily Dose (mg)	Days on Drug	Days off Drug	(μg/ml. plasma)
I: AR	100	1	---	0.3
		5	---	4.5
		5	2	1.75
		5	5	0
VI: M	100	1	---	0.4
		3	---	4.0
		6	---	3.6
		6	4	0.9
		6	7	0
V: H	30	60	---	1.9

* Librium determined by hydrolysis and diazotization procedure.