March 12, 1970

Dear Sirs:

Enclosed in quadruplicate is a proposal for the extension of work being conducted under Contract No. Task Order No. for the period May 1, 1970, to April 30, 1971.

The proposal provides for maintenance of the present level of effort.

Sincerely yours

Enclosures

TECHNICAL PROPOSAL

[redacted] submits this unsolicited proposal for continuation of the program on pharmacological screening of potentially useful new agents.

ABSTRACT

Each new compound will be examined by primary screening using the acute toxicity screen and locomotor activity tests in mice, as well as by the effect on physical, neurological, and behavioral status of cats. Compounds will also be studied for evidence of peripheral pharmacologic activity in anesthetized cats. Promising candidate materials will be evaluated by secondary screening, including a motivation test and a sequential response test in hooded rats. Further testing will be done with very promising materials to evaluate effects on the dominance behavior and visual discrimination of squirrel monkeys. Further studies will be performed including the mechanism of action, the effects of antagonists, and detailed analysis of effects on behavior using suitable animal species with emphasis on primates. One or two selected compounds, possessing activity of sufficient interest to warrant human clinical trial, will be subjected to preclinical subacute animal studies in order to permit clinical evaluation.

TECHNICAL PROPOSAL

[redacted] submits this unsolicited proposal for continuation of the program on pharmacological screening of potentially useful new agents.

HISTORY:

During the three years the program has been in progress, [redacted] has developed a stepwise procedure for the systematic screening of promising compounds for the detection and study of potentially useful products. Arrangements for suitable sources of compounds have been made through the contracting agency.

The initial efforts were to develop and evaluate primary and secondary screening tests, using known pharmacologic agents, and to select tests that yielded maximum information without duplicating test results. Ten unknown compounds supplied by the contracting agency were evaluated during the first year.

During the second year, approximately 50 new compounds were screened by the primary and secondary test procedures, and several potentially useful compounds were detected. An advanced screening procedure, which uses squirrel monkeys in a dominance behavior test, was developed, and initial test data were obtained.

One hundred forty-two additional compounds have been received this year and approximately 120 will have been screened in the contract year. Arrangements were made for additional sources to supply new compounds. The potentially useful compounds detected earlier in the program have been evaluated in the advanced screening tests. A special test for weak sedative compounds was evaluated and is utilized for study of very interesting materials. Additionally, mechanism of action studies were begun.

A behavioral scientist [redacted] was added to the staff. He has further refined the advanced behavioral testing procedures. An automated apparatus for testing visual discrimination of squirrel monkeys has been received and assembled and is being used to study selected compounds.

A pharmacologist [redacted] was added to the staff to conduct pharmacodynamic screening and mechanism of action studies. The effects of 18 compounds on blood pressure, heart rate, and respiration, as well as the interactions of these compounds with neurohumoral agents, were studied in anesthetized cats.

A natural products chemist prepared a list of oriental plant products to which have been attributed medicinal properties. Extracts of several of these materials have been prepared and tested for biological activity. The isolates which show activity are under further study.

One compound, which was found to possess potent psycho-pharmacological activity suggestive of a major tranquilizing agent, has been subjected to detailed investigation. The dose-response profile of this compound was studied in the behavioral tests, including motivational and sequential response paradigms in rats and the dominace behavior test in squirrel monkeys. Some similarity to [redacted] was noted. Samples of the compound were submitted for further evaluation by Dr. [redacted] elsewhere.

PROPOSAL FOR FOURTH YEAR:

Each compound will be examined by primary screening methods in mice and cats as described below.

I. PRIMARY SCREENING PHASE

1. Acute Toxicity Screen - Mice

This procedure represents the first step in eventual acceptance or rejection of a compound. The screen is designed to eliminate from consideration compounds which do not possess sufficient biological activity, and to indicate types of promising activity. Criteria used in the selection of a compound include:

(a) Ratio of lethal dose to effective dose (safety factor)
(b) Speed of onset of pharmacologic signs
(c) Duration of action
(d) Type of action
(e) Completeness of recovery from the effects
(f) Degree of severity of signs observable

The effects sought are pharmacologic signs which are readily reversible in a progressive series of tests. Compounds are administered intravenously, and toxic signs are noted by gross observation or by manipulation and are recorded from among those listed in a List of Reaction Signs and Standard Terms for Toxicity and Symptomology Reporting. If the LD_{50}/MED_{50} ratio is 10 or greater, the compound is tested by intravenous injection in cats and administered in behavior tests for secondary screening.

From previous experience, it has been found that with trained observers, the mouse toxicity screen detects significant

reaction signs (a preferred term to toxic sign) for compounds known to be mentally or physically incapacitating to man. The test is performed in a "partially blind" manner; i.e., the observer is not informed of the structures of the compound to be screened, but is responsible for preparing solutions and dilutions, and injecting the mice. Technicians usually work in pairs, one person recording the data and, whenever necessary, helping to observe the mice, while the other technician serves as the regular observer. From time to time, known or standard compounds are introduced into the routine screening as unknowns to check the reliability and reproducibility of the screening technique.

Preparation of Compound for Injections:

In the absence of solubility information, very small, unweighed amounts of compound are tried in the following solvents and in the order listed until a suitable one is found. Heat may be used to aid solution.

Observations -

(1) Total Time and Intervals: After injection, animals are observed individually for reactive signs at 3, 15, 30, and 60 minutes, hourly thereafter until the end of the work day, and finally at 24 hours (or daily thereafter until signs disappear).

(2) Scope: An attempt is made to record all apparent reactions of control (saline treated) animals studied in parallel.

(3) General Technique: Each treated mouse is first observed for any reaction signs which might appear without handling the mouse. Immediately following this, each treated mouse is observed during a routine series of manipulative tests. Controls are also put through manipulative tests if they show any solvent effects. Manipulative tests include: placing reflex, position sense, rotorod test, pupillary light reflex, and eye examination. A list of reaction signs is appended.

(4) Maintenance of Animals Overnight: Mice are housed individually when in such poor condition that they might be expected to die overnight among grouped mice. The doses producing death are repeated with mice housed in separate cages. The results from this repeat test are used in estimating lethality.

2. Locomotor Activity in Mice

The spontaneous locomotor activity test measures the drive of an animal to move. This test is recognized as measuring a basic parameter for the screening of potential tranquilizers and sedatives (Jacobsen 1964) and excitants (Chen 1964). The apparatus used for this test, the

For interpretation of the data, special attention is given the following parameters:

(a) Dosage levels at which maximal, minimal, or no effect is seen.

(b) Time of onset of drug effect at each dosage level.

(c) Duration of a change in locomotor activity of treatment groups as compared to that of control groups.

REFERENCES:

Jacobsen, E., Tranquillizers and Sedatives, Chapter 10, Evaluation of Drug Activities, Vol. I, ed. by Laurence, D. R. and Bacharach, A. L., pp 215-237, Academic Press, N.Y., 1964

Chen, G., Antidepressives, Analeptics, and Appetite Suppressants, Chapter 11, Evaluation of Drug Activities, Vol. I, pp 239-260, Academic Press, N.Y., 1964

3. Physical and Neurological Examination with Behavioral Observations of Cats Treated with Candidate Compounds

In order to assess the overall physical, neurological, and behavioral effects of candidate compounds, a systemic examination of physical signs, sensory, motor and reflex reactions, and behavioral reactions to the observer is conducted in intact cats (McGrath, 1960, Norton and deBeer, 1956, and Norton, 1969). Each animal is examined before receiving drug, and periodically thereafter.

The examination consists of observation of heart rate, respiration rate, body temperature, pupillary diameter (constricted or dilated), pupillary response to light, sensory response using a sharp needle to scratch or poke the skin (superficial) or pinching the toe pads (deep), motor activity (walking), spinal reflexes (flexor reflex, knee jerk, extensor thrust, scratch reflex, crossed extensor reflex, spinal visceral reflex), and attitude and postural reactions (attitude reflexes, tonic neck placing reactions, and hopping reactions). The behavior of each animal and its reaction to the observer are noted during the examination period. Drugs are administered intravenously (on a weight basis) in a dosage of 0.1 of the LD_{50} level in mice. Cats are examined in most instances at approximate intervals of 0.5, 1.0, 2.0, 4.0, 6.0, and 21 hours after compound administration.

Unusual observations are presented in the tables which accompany the detailed reports. Although only positive significant effects are usually noted, the presence of a normal sign may be emphasized in order to indicate that an unusual effect was especially sought. Thus when dilated pupils are recorded, the pupillary light reflex is especially noted. Where both dilatation of pupil and paralysis of the light reflex occur, a parasympatholytic effect may be assumed to be present. The hopping reaction seems to be an especially sensitive one for testing proprioception. Therefore, this test is individually recorded. In some cases, sedation or overactivity and hyperexcitability are noted. When the animal becomes hyperexcitable, it sometimes is impossible to do a complete examination. However, such observations which can be made are noted in these instances.

REFERENCES:

McGrath, J. T., Neurologic Examination of the Dog, 2nd Ed. Lea and Febiger, Phila., 1960

Norton, S. and deBeer, E. I., Effects of Drugs on the Behavioral Patterns of Cats. Annals of the New York Academy of Sciences

II. SECONDARY SCREENING PHASE

1. Motivation Test

This test is used for more detailed study of promising drugs, and is conducted according to the method of Barry and Miller (1965). Hooded rats are trained to run from a start box down a straight alleyway to a goal box. The response is measured under three motivational conditions: food approach, shock avoidance, and shock escape. Separate groups of hooded rats, approximately equally distributed by weight and age are initially randomly assigned to groups, and trained to approximately equal performance. Thus the motivational conditions are highly comparable (same response, approximately equal performance) yet independent of each other (separate groups).

Six rats are trained to each motivation. Each of the rats is run through six trials daily for five days per week. Start and run times are recorded electronically to the nearest 0.01 second. The dosage schedule is as follows: On Monday, no injections are administered, but the rats run through the trials to "warm up" for the week. On Tuesday, saline injections are administered and "control readings" obtained. On Wednesday and Thursday, drug injections are administered intravenously to two rats of each group, and saline to the remaining four, and trial runs are then conducted with all rats after an elapsed time period previously determined to be that of probable peak drug effect.

Compounds will be further evaluated by the Sequential Response Method in hooded rats (Polidora, 1963). (See reference page 13.)

2. Sequential Response Behavior Test

The sequential response behavioral test (Polidora, V. M., Jour. Exper. Anal. Behav. 6, 271-277, 1963) is a method useful for the study of detrimental drug effects on complex behavior using hooded rats as experimental subjects. The test compartment is cylindrical in shape, and contains at the periphery, four response pedals separated by 90° angles. Above the response pedals are a signal light and a fountain to yield liquid rewards after a correct sequence of pedals is pressed by the experimental subject. Subjects are maintained at a stable response level by 23-hour water deprivation, one-half hour ad lib water, and frequent practice sessions. Baseline data control are obtained by administration of saline solution, in place of drug, and obtaining characteristic response and reward rates at that time.

REFERENCES:

Barry, H., and Miller, N. E., Comparison of Drug Effects on Approach, Avoidance, and escape Motivation, Jour. Compar. Physiol. Psychol. 59, 18-24, 1965

III. TERTIARY SCREENING PHASE

1. Social Behavior in the Squirrel Monkey

The effect of very promising drugs will be evaluated on social behavior of squirrel monkeys using the method of Plotnick (Jour. Comp. physiol. Psychol. 66 (2), 369-377, 1968). The apparatus consists of two boxes with plexiglass sides connecccted by a tunnel, which is separated from the boxes by guillotine doors. The floors of each box and the tunnel contain parallel rods, which may be electrified. Food pellets can be administered at the end of each box.

Groups of squirrel monkeys are trained to respond to a cue (light, for negative reinforcement, sound for positive

reinforcement) by running through the tunnel to the opposite compartment. Observations are made of running order and aggressive interactions, to determine the hierarchy of dominance-submission. Food deprivation is maintained to furnish the drive for positive food reinforcement. The effects of candidate drugs on aggressive and submissive behavior, will be determined.

2. Visual Discrimination Test

Squirrel monkeys on a schedule of water deprivation will be trained for shock avoidance and water rewards to a criterion of performance to choose between two simultaneously presented visual discriminanda, tentatively a circle and an ellipse. The discriminanda will be chosen to provide difficult visual cues.

The underlying hypothesis is that very subtle drug-induced effects on discriminatory abilities will result in significant changes from the baseline pattern of performance. The data derived from the experiment will provide information on response latency times, or "tendency to respond," and in the per cent of incorrect responses. Data on total responses will reveal the effects of a compound on general activity.

Brief Description of Training - Testing Procedure for Free-Operant VDT by Squirrel Monkeys.

A. Training

(1) Monkey is acclimated to test apparatus, being allowed free movement and manipulation.

(2) Both visual display (IDD) cells are connected, but no pattern is shown. The monkey is 20-hour liquid deprived. Any time the push plate located over either cell is pressed, a liquid reward follows.

(3) After a pressing pattern is established, the positive discriminandum is presented on one IDD display. The monkey is then only rewarded for pressing this push plate.

(4) After this response is established, a brief mild shock is given if the blank IDD cell is pressed or a after a latency period (approximately 10 seconds) passes with neither cell being pressed. The shock after the latency period might be stronger and of a longer duration than the incorrect response shock. At this same time, a clicker is presented two seconds before and during the stimulus presentation. The single stimulus is randomized as to the side on which it appears.

(5) After stabilization of responses with the clicker and random single stimulus presentation, the second (incorrect) stimulus object is presented simultaneously with the correct, conditioned display. At first, the correct one is rewarded and a shock occurs only if the latency period passes and no choice is made. Then, the incorrect response is also shocked, but milder than that following a latency period with no choice.

(6) After a set criterion of percentage of correct responses and a baseline latency time is determined, testing begins.

B. Testing Procedures

(1) Two monkeys, one monkey trained to one of the stimuli (circle) and the other monkey trained to the other stimulus (ellipse), are used for each drug test. The dosage levels are determined on an untrained monkey prior to testing.

(2) The clicker is activated and continued, the stimuli are presented simultaneously two seconds later, and the clock is started to count latency of response time.

(3) If the monkey responds correctly, it is rewarded, the response is recorded, and a 30-second delay ensues before the next trial. If the monkey responds incorrectly, it receives a mild shock and the trial ends (to begin again 30 seconds later) and an incorrect response is recorded. If the monkey does not respond in the latency period, it receives a severe shock which is recorded and the trial ends.

(4) This procedure is continued for a prescribed number of trials until the session is terminated.

C. Data

(1) The data recorded will include response latency times, in the form of a chart and a histogram.

(2) A percentage of incorrect responses will be obtaine by dividing the total incorrect responses plus

(3) The number of incorrect responses and latency of the responses will be compared to determine whether the monkey is responding or not.

(4) The number of inter-trial responses, which do not count in the data will be tabulated as an overall index of pressing frequency.

(5) The number of right or left responses will be tabulated, aside from correct or incorrect, to ensure that a response pattern centering on one side or the other does not occur.

3. Preclinical Toxicology

It is anticipated that one or two compounds will proceed to the point during the next year where clinical evaluation will become necessary. In order to satisfy the requirements for preclinical toxicology, the following protocols have been prepared in accordance with established Food and Drug Administration guidelines for such studies:

I. Four-Week Repeated Administration to Rats
II. Four-Week Repeated Administration to Dogs or Monkeys

I. REPEATED ORAL ADMINISTRATION TO RATS FOR FOUR WEEKS

ANIMALS: Eighty albino rats obtained at weaning from the housed individually in temperature-controlled quarters, and acclimated to laboratory conditions for one week will be placed on experiment as follows:

Group	Number per Group	Dosage Level*
I	10 Males, 10 Females	Control
II	10 Males, 10 Females	High
III	10 Males, 10 Females	Intermediate
IV	10 Males, 10 Females	Low

* Actual dose levels to be established

COMPOUND ADMINISTRATION: By incorporation in the diet by mechanical mixer with levels adjusted to maintain a relatively constant intake of test material in terms of mg/kg/day.

DURATION: Four weeks.

CLINICAL OBSERVATIONS: General observations daily; body weights, food consumption, and physical examination weekly. Ophthalmic examinations will be conducted on all animals at termination.

HEMATOLOGY: Hemograms consisting of hemoglobin, microhematocrit, coagulation time, thrombocyte counts, and total and differential leucocyte counts will be determined on 5 males and 5 females each from Groups I and II at termination.

CLINICAL CHEMISTRY: Blood glucose, prothrombin time, and serum glutamic pyruvic transaminase will be determined on 5 males and 5 females each from Groups I and II at at termination.

NECROPSY: To be performed on any animal dying or at the point of death and all survivors sacrificed at termination.

The following organs will be weighed: heart, lung, liver, kidneys, spleen, gonads, adrenal, thyroid, prostate or uterus, and pituitary. Portions of these organs plus portions of the following tissues will be grossly examined and preserved in 10 per cent formalin: duodenum, intercostal muscle, urinary bladder, pancreas, mesenteric lymph node, mammae, bone marrow, stomach, and brain. In addition, eyes will be taken, grossly observed, and preserved in Zenker's fixative.

HISTOPATHOLOGY: The above named tissues (approximately 18 per rat) will be examined from any animal dying when the tissues are in good condition and from 5 males and 5 females each from Groups I and II sacrificed at termination. In addition, eight selected tissues will be examined from 5 males and 5 females each from Groups III and IV sacrificed at termination. Additional tissues will be examined from lower levels if indicated by results from Group II.

II. REPEATED ORAL ADMINISTRATION TO DOGS FOR FOUR WEEKS

ANIMALS: Sixteen purebred beagle dogs, six to eight months, of age, individually housed in temperature-controlled quarters, properly treated for intestinal parasites, immunized against rabies, distemper, hepatitis, and leptospirosis, and acclimated to laboratory conditions for three weeks will be placed on experiment as follows:

Group	Number per Group	Dosage Level*
I	2 Males, 2 Females	Control
II	2 Males, 2 Females	High
III	2 Males, 2 Females	Intermediate
IV	2 Males, 2 Females	Low

* Actual dose levels to be established

COMPOUND ADMINISTRATION: By gelatin capsule, once daily, seven days per week, with empty gelatin capsules to controls.

DURATION: Four weeks.

CLINICAL OBSERVATIONS: General observations consisting of behavior, food intake, stool consistency, etc., daily; body weights and detailed physical examination weekly. In addition, heart rate and blood pressure will be determined and electrocardiograms and ophthalmic examinations will be conducted on all animals initially and at termination.

HEMATOLOGY: Hemograms consisting of hemoglobin, hematocrit, sedimentation rate, coagulation time, thrombocyte counts,

and total and differential leucocyte counts will be determined on all animals twice initially and at termination.

CLINICAL CHEMISTRY: Blood urea nitrogen, serum alkaline phosphatase, blood glucose, prothrombin time, serum glutamic pyruvic transaminase, and serum glutamic oxalacetic transaminase will be determined on all animals twice initially and at termination.

URINALYSES: Qualitative urinalyses consisting of general appearance, pH, specific gravity, albumin, glucose, and microscopic examination of urinary sediment will be determined on cage-collected samples from all animals initially and at termination.

NECROPSY: To be performed on any animal dying or at the point of death and all survivors sacrificed at termination.

The following organs will be weighed: heart, lungs, liver, kidneys, spleen, thyroid, adrenal, prostate or uterus, gonads, pituitary, and brain. Portions of these organs plus portions of the following tissues will be grossly examined and preserved in 10 per cent formalin: nerve, esophagus, duodenum, jejunum, ileum, colon, cecum, stomach, pancreas, parotid salivary gland, thymus, trachea, gall bladder, intercostal muscle, urinary bladder, mesenteric lymph node, femoral bone marrow, spinal cord, mammae, and abdominal skin. In addition, eyes will be taken, grossly observed, and preserved in Zenker's fixative.

HISTOPATHOLOGY: The above named tissues (approximately 32 per animal) will be examined from any animal dying when the tissues are in good condition and from all control and high level animals. In addition, 13 selected tissues will be examined for each of the remaining animals. Additional tissues will be examined from lower levels if indicated by results from Group II.

4. Drug Classification Studies

Suitable studies will be conducted to determine class of drug action, mechanism of action, and antidotes of promising test materials.

Such tests in rats and other species will include effect of drugs on threshold to produce convulsions with metrazol or electric shock, ability to produce catalepsy, potentiation or blockage of drug effect by neurohumoral agents and potentiation or blockage of known active materials by test compounds. Discussion of the applicability of these tests is given in several chapters of Animal Behavior and Drug Action, ed. By Steinberg, H., Little Brown & Co., 1964, especially the discussion by Janssen (p. 392).

5. Advanced Behavioral Analysis on Nonhuman Primates

Method development using primates as test subjects will continue for the purpose of application of suitable tests for a complete description of behavioral and central nervous system effects of promising drugs.

COST ESTIMATE

TECHNICAL PROPOSAL - FOURTH YEAR

PERSONNEL:

Classification	Hours	Rate Per Hour	Total Cost

COST BREAKDOWN

FOURTH YEAR

Month	Per Cent Expenditure of Total	Accomplishment
1	8.3	Primary, Secondary, & Testing Phases
2	8.3	Primary, Secondary, & Testing Phases
3	8.3	Primary, Secondary, & Testing Phases
4	8.3	Primary, Secondary, & Testing Phases
5	8.3	Primary, Secondary, & Testing Phases
6	8.3	Primary, Secondary, & Testing Phases
7	8.3	Primary, Secondary, & Testing Phases
8	8.3	Primary, Secondary, & Testing Phases
9	8.3	Primary, Secondary, & Testing Phases
10	8.3	Primary, Secondary, & Testing Phases
11	8.3	Primary, Secondary, & Testing Phases
12	8.3	Primary, Secondary, & Testing Phases