

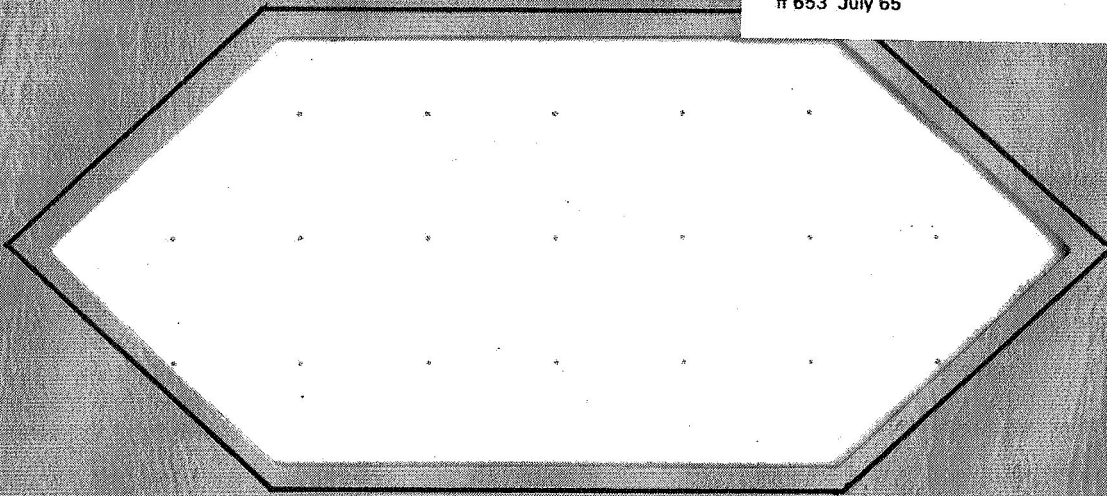
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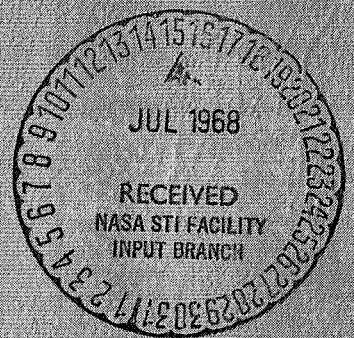
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**INSTITUTE OF CHEMICAL BIOLOGY**

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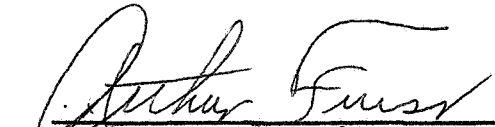
BRAIN AMINO ACIDS AND BIOGENIC AMINES  
UNDER VARIOUS ATMOSPHERIC MIXTURES

Arthur Furst and H. B. Chermiside

Project Period

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Submitted by:

  
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Director

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### Appendices:

1. Chermiside, H.B. and Furst, A.: An Atmosphere Exposure Chamber for Small Animals, publication draft for Proceedings of the Western Pharmacology Society.
2. Chermiside, H.B. and Ledin, G.; Some Statistical Techniques for the Programma 101, I.C.B. Working Paper.

## INTRODUCTION AND SUMMARY

This report covers the period May 1, 1967 through January 31, 1968. Some data from earlier reports is re-examined.

The major objective of this project is the possible effect on brain biochemistry (with emphasis on free amino acid pools) in rodents exposed to artificial atmospheres. After exposure periods of varying lengths in different gaseous compositions, the animals are sacrificed and the free amino acid pools of the animals' brains are measured.

The methodology has been discussed in previous reports. Some modifications of earlier techniques are discussed herein. We report on an unsuccessful program to develop an analysis for serotonin in one lobe of rats' brains, and comment on some possibilities for farther work on this technique.

During this period we have constructed and used a new exposure chamber. A publication regarding this aspect of the work is appended. We have continued our regular analyses of free amino acids in the brain using the Beckman 120-C analyzer; considerable time still must be spent on instrumental problems. We have initiated a new series of experiments which, though self-contained in nature, have yielded data which are compatible with earlier results. We have developed satisfactory statistical procedures for analysis of the data using a small desk computer: the Programma 101. An Appendix contains this information. We have used these programs to analyze both old and new data, and some results are reported herein. We have planned

explicitly the remaining experimental work on this project, which will be completed by July 31, 1968. The final report will contain full methodological and experimental details. We expect publications to result from the data.

Evidence to date suggests that the following variables contribute to changes in the amounts of specific amino acids in the brains of rats: a) the age of the animal; b) the strain of the animal; c) exposure to 100% oxygen; and d) the length of this exposure. The data leading to these conclusions are discussed. Incomplete data indicates that the nature of the "inert" component of a gaseous mixture also affects the amino acid content in the brain; this will be more fully discussed in the final report.

### AMINO ACID ANALYZER METHODOLOGY

During the report period, 242 analyses were performed on the Beckman 120-C amino acid analyzer. Of these, approximately one half were required for calibration. The instrument was inoperative at various times due to breakage and malfunction; re-standardization was required after each such episode. A number of analyses were made in an attempt to identify some unknown or uncertain peaks.

We were able to take advantage of this inordinate amount of standardization time to modify slightly our original instrumental technique reported in NASA publication #N66-26235. By altering the parameters of buffer pH, time of buffer change, and time of temperature change, we have been able to operate both columns of this instrument with the same program. This helps prevent operator error when changing the programmed parameters. With an additional colorimeter these changes would allow a 100% increase in analyses performed in a given time.

We are presently using the procedure recommended by the manufacturer with the exception of the parameters mentioned.

In these cases we are using the following:

Buffer change time, both columns:	140 min.
Temp. change time, both columns:	90 min.
Total time of analysis, both	280 min.

Buffers	3.2 <sup>o</sup> pH	Na normality
A	3.23 ± 0.01	0.20
B	4.26 ± 0.02	0.20
C	4.25 ± 0.02	0.38
D	5.36 ± 0.01	0.35

## EXPOSURE SYSTEM

A new more flexible exposure system has been developed. The system may be used as a sealed or dynamic system and has pressure capabilities above and below atmospheric. Major items of interest in the system are the chamber, the oxygen demand valve and the gas scrubbing loop.

The new chamber itself, made from a piece of 12" sewer pipe, will accommodate 6 rats conveniently for periods up to 72 hours without the necessity of opening. The oxygen demand valve was constructed by modifying a government surplus aviators bail-out bottle and is quite inexpensive. The gas scrubbing loop utilizes a cold trap in liquid nitrogen to remove metabolic wastes and contaminants of the atmosphere; it appears that this type of gas scrubbing removes toxic materials not ordinarily disposed by the usual carbon dioxide and water absorbants.

A paper on this system, including also the old exposure chamber reported in an earlier report (NASA publication #N67-15883) was presented in January at the meeting of the Western Pharmacological Society. The publication text of this paper is included in this report as Appendix I. (When available, reprints will be mailed.) All construction and operation details are discussed in this paper.

## BIOGENIC AMINE ANALYSIS

Our attempts to analyze the biogenic amines in only one-half of the brain of a rat proved fruitless. During this report period we have experimented only on the analysis for serotonin. We have not been able to develop an analytic technique which is sufficiently sensitive for our purposes.

The goal of our serotonin analysis was to determine differences in the serotonin content in one-half of the rat brain as a result of our exposures. For this volume of tissue (0.5 - 0.8 g), the spectrofluorophotometer appeared to be the only practical instrument. The instrument itself, an Aminco Bowman Model 4-8106, has been set up and standardized. We have demonstrated that its response is linear, and in discussion with other laboratories using this model we have determined that the response is typical.

We have modified the instrument slightly to increase the sensitivity. This has been done by providing a single-throw switch which can be activated to bypass the 2.7 K ohm resistor (R 13) on the + side of the meter movement in the photo multiplier microphotometer. [This and subsequent part numbers refer to the schematics in the instructions provided by the manufacturer.] This modification increases the sensitivity of the meter by a factor of 3 to 5. It does however, make the instrument extremely sensitive to line voltage fluctuations. We do not therefore activate this circuit unless absolutely necessary. Incidentally, we have modified the rectifier in the power supply of



This meter in the interest of increased reliability and ease of service. The original rectifier (rectifier 1) has been replaced by two Sarxtarzian F-8 diodes protected with two 10 ohm resistors in series with the load.

The serotonin extraction procedure used and reported previously (NASA Publication X66-11693) is based on the differential solubility extractions of Bodgansky and of Weissbach. Continued work with this procedure did not result in any increase of sensitivity to the level we needed. Therefore, we have followed the recent literature of serotonin analyses and have made several attempts at other extractions and at various techniques of enhancing fluorescence in order to increase the sensitivity. Although none have been successful, we will describe them.

Maickel and Miller [Anal. Chem., 38 1937 (1966)] recently published a class reaction for enhancing the fluorescence of indole derivatives by treating them with o-phthalaldehyde (OPT). We have attempted to replicate this work with limited success, i.e. the fluorescence of the OPT product as compared to the blank at a concentration of 5  $\mu$ g/ml was 83.33; at a concentration of 0.5 mg/ml this ratio was 9.67. This does not, however, approach the results reported by Maickel and Miller who have indicated that they obtained a ratio of 695 for 1 mg/ml. We followed their published procedure and checked the purity of all chemicals used and have found no reason for our failure to replicate their work. We have been in communication with Dr. Maickel and have received from him a proposed extraction and analysis technique. We have not used

this in its entirety due to the failure to replicate the indole class reaction. We feel that phthalic anhydride may work as well or better than OPT for such a reaction. We have not yet checked this technique.

Reports by Weiss [Anal. Biochem. 18, 14 (1967)] and by Addie and Hughes [Anal. Biochem. 11, 395 (1965)] suggest that by placing a polarizer in the fluorescent beam emitted by the sample cell one can increase the ratio between the fluorescence peak and the background noise. We attempted this using a polarizer from a local camera shop. We do not feel that the results of this were successful. We have not yet attempted the same work using the Kodak Pola-screen recommended in these publications.

Weiss has also reported an extraction which seems promising [loc. cit.; also Anal. Biochem. 18, 369, (1967)]. We have tried this in a "dry run" and found that the extraction procedure is somewhat complex, but plausible for use in this type of work. No fluorometric assay of the extract has yet been performed due to the aforementioned dissatisfaction with assay technique.

We have examined and rejected the extraction steps of Quay [Anal. Biochem. 5, 51 (1963)], who reports that this extraction produces a 30% recovery of 5-hydroxytryptophan (5HTP) in addition to a 92% recovery of serotonin. In work done in this laboratory under another sponsor we have found that 5HTP and serotonin have similar fluorometric spectra. The results of this are summarized in the following table:

TABLE I: Fluorescence data for 5-hydroxytryptophane and serotonin

SOLVENT	5 HTP			SEROTONIN		
	Activation*	Emission*	Strength**	Activation*	Emission*	Strength**
H <sub>2</sub> O	300	340	1.77	300	340	20.7
pH 10.1	300	350	1.11	300	350	7.8
pH 6.5	290	350	2.55	300	350	0.72
2N H <sub>2</sub> SO <sub>4</sub>	290	340	0.42	300	350	1.17

\* mμ - uncorrected

\*\* relative scale, arbitrary units

Because of this similarity of response, we feel that the extraction reported by Quay would introduce more contamination of data than is acceptable.

We shall continue as a minor effort to develop an extraction and assay technique for serotonin. We do not however, expect to be able to apply this to the same tissue samples from which we have already made amino acids determinations. It is probable therefore, that serotonin analysis of the brain tissues of exposed animals will not be available for the final report on this project. We have been in communication on a personal level with other workers in nearby labs who are attempting serotonin analyses. They also appear to be having similar problems in obtaining an extraction and assay technique sufficiently sensitive for analysis of similar low levels of serotonin concentration.

EXPERIMENTS COMPLETED

The experiments completed to date have provided data and conclusions regarding how the free amino acid pools of rats' brains are related to the age and strain of the rats and to the length of exposure to 100% oxygen. The results of these experiments will be combined with later work and re-analyzed for a more positive evaluation of these effects and of other influences.

Previously reported work included values of the free amino acid pools from control animals and some oxygen-exposed animals of both Long Evans and Sprague Dawley strains. To these data we have added new groups of animals as summarized in Table II.

TABLE II: Experimental Groups Completed This Report Period

<u>Group</u>	<u>Strain</u>	<u>Age Days</u>	<u>Exposure Gas</u>	<u>Exposure Length</u>
LE C 1 c	LE	150	Control	Control
SD C 1 a *	SD	110	Control	Control
LE O <sub>2</sub> 100-18 *	LE	120	100% O <sub>2</sub>	18 hr
LE He20-18 *	LE	100	20% He, 80% O <sub>2</sub>	18 hr
LE He20-14	LE	110	20% He, 80% O <sub>2</sub>	41 hr

\* These groups enlarged in this period

A total of 35 animals were used for these studies. In addition, analyses were made on 9 animals at the request of Dr. Henry Leon of NASA Ames Laboratories. These animals were from a feeding experiment, and the data on brain amino acids from this group are not included in our totals.

The new exposure results were evaluated with previously obtained data; the summaries are reported under Experimental Results to Date.

EXPERIMENTS INITIATED

The completion of our experimental program has been planned. Some of the individual experiments have been initiated, in that the exposures have been made and the brains removed, frozen in liquid nitrogen, and stored. The following groups will complete the program:

TABLE III: Experimental Groups for Completion of Program

<u>Group</u>	<u>Strain</u>	<u>Age Days</u>	<u>Exposure Gas</u>	<u>Exposure Length</u>
LE C 1 a *	LE	110	Control	Control
LE C 1 c *	LE	150	Control	Control
LE C 1 d	LE	235	Control	Control
LE He20-72	LE	200	20% He, 80% O <sub>2</sub>	72 hr
LE A 20-72	LE	205	20% A, 80% O <sub>2</sub>	72 hr
LE Ne20-72	LE	210	20% Ne, 80% O <sub>2</sub>	72 hr
LE A 20-22	LE	110	20% A, 80% O <sub>2</sub>	22 hr
LE A 20-40	LE	105	20% A, 80% O <sub>2</sub>	40 hr
LE O <sub>2</sub> 100-20	LE	105	100% O <sub>2</sub>	20 hr

\* Additional data to be gathered on these groups

The data from these groups will enable us to analyze for the comparative effects of various "inert" atmospheric components at various lengths of exposure. (We regret that the expense of neon will prevent our use of exposing more than one group; the 72 hour exposure length was selected as the most likely to show an effect if one exists.) The final report on the project will contain the analyses of these data within themselves and a comparison with previously acquired data.

## STATISTICAL TECHNIQUES

The computational procedures used on this project have changed several times. This has been due largely to changes in the computers available for this project. Originally, we had access to an IBM 1620 at no cost. Once this privilege was lost we changed over to the use of an Olivetti Programma 101 desk top computer. The University has recently acquired an IBM 1130. Attempts were made to modify the 1620 programs for use on the 1130; however, due to new installation problems, the use of this system has not been satisfactory. Therefore, we have returned to the Olivetti Programma. Although this computer is considerably slower than a larger one, time is saved overall because data may be analyzed in the laboratory directly from worksheets by untrained personnel. We have developed several computational systems for use on this computer including calculations of Students "t" statistics, simple analysis of variance, and two-way analysis of variance. An ICB working paper describing all the programs we used on this project is included as Appendix 2. The programs and worksheets for these computational systems are available from this laboratory.

As previously reported, we compare data from groups of animals with that from an appropriate, previously analyzed control group using Student's "t" test. Groups which are expansions of previously analyzed control groups are so compared to determine if, for any reason, there is a significant difference in the amino acid pools as analyzed. Such an occurrence would indicate that there was difference in the procedures used; this is not the case.

Experimental groups are, of course, compared to the relevant control group. The same method is used. However, the use of Student's "t" test is valid only for the comparison of two groups. Therefore we have developed simple and 2-way analysis of variance procedures for comparisons among several groups.

The results reported below have been obtained from five analyses of variance comparisons, and have then been collected for presentation. They have not been further analyzed, as more information is being gathered now.

The following analyses have been performed for this report:

TABLE IV: Analyses Discussed In This Report

<u>Test</u>	<u>Method</u>	<u>Groups</u>	<u>Effects Exhibited</u>
1	2-way A.V.	LE C 1 a , LE C 1 b , LE O <sub>2</sub> 100-18, LE O <sub>2</sub> 100-24	age, exposed-or-not to O <sub>2</sub>
2	2-way A.V.	SD C 1 a , LE C 1 a , SD O <sub>2</sub> 100-18, LE O <sub>2</sub> 100-18	strain, exposed-or-not to O <sub>2</sub>
3	2-way A.V.	LE C 1 a , SD C 1 a , LE C 1 b , SD C 1 b	Age, strain
4	Simp. AVV.	LE C 1 a , LE O <sub>2</sub> 100-18 LE O <sub>2</sub> 100-28	Length of exposure to O <sub>2</sub> in LE rats
5	Simp. A.V.	SD C 1 a , SD O <sub>2</sub> 100-18 SD O <sub>2</sub> 100-50	Length of exposure to O <sub>2</sub> in SD rats

It should be noted that for the 2-way analysis of variance mathematical model we use, equal numbers of cases must be provided within each group. In order to equalize N's, we cast out data from the more populous groups following a random method. Therefore, the group averages reported for groups sub-

mitted to this analysis are not the same as those reported for the group as a whole. However, it is considerably beyond the capacity of our computational equipment to do non-orthogonal 2-way analysis of variance.

The results of these analyses are discussed under Experimental Results to Date.



## EXPERIMENTAL RESULTS TO DATE

The analyses of experimental results reported here are interim results only. These analyses were made for two reasons: to evaluate the work so far in order to determine the most fruitful future work, and to evaluate the statistical procedures we had planned to utilize.

The analyses have examined the effects of three variables on the free amino acid content of rats' brains: age of the animal, strain of the animal, and effect of exposure to 100% oxygen.

As no overall analysis of the perimental program can be made until all data have been gathered, we present here only the results of the interim analyses. At this point we have selected the 0.10 level of significance as worthy of attention. The results are presented in the three following tables; only significant results are presented. The data are expressed in terms of the probability that the observed occurrence is a random variation, thus the smaller the number, the greater the probability that the experimental effect caused the occurrence.

Although interaction effects were observed in tests 1, 2, and 3, these are not discussed; again it appears prudent to await the completion of the experimental program.

TABLE V: Amino Acids For Which Concentration in Rats' Brains Are Significantly Changed By Exposure To 100% O<sub>2</sub> (Combined Data)

<u>Amino Acid</u>	Significance Level Observed			
	<u>Test 4</u>	<u>Test 5</u>	<u>Test 2</u>	<u>Test 1</u>
Glycerophosphoethanolamine		.05		
Phosphoethanolamine	.025	.05		
Taurine	.001	.001		
Urea	.005			.01
Unknown #1	.05			
Unknown #3		.01		
Aspartic Acid	.001	.001	.025	.05
Threonine		.001	.005	
Serine	.05			
Glutamine (Asparagine?)	.001	.001		.005
Glutamic Acid	.001	.001	.10	.005
Cystathionine		.01		
Methionine		.001	.025	
Unknown #5		.001	.005	
Isoleucine		.025		
Leucine	.10			
Tyrosine	.005			
Phenylalanine	.025		.10	
<u>beta-Alanine</u>	.001			
<u>gamma-Aminobutyric Acid</u>		.001	.001	
Ornithine	.025		.025	.01
Ethanolamine	.10			
Ammonia *	.01	.025		.05
Carnosine	.10	.10		
Tryptophan			.025	

\* Probably this data is contaminated by instrumental artifacts

TABLE VI: Amino Acids For Which Concentration In Rats' Brains Differ With Age Between 50 and 120 Days

<u>Amino Acid</u>	Significance Level Observed	
	<u>Test 1</u>	<u>Test 3</u>
Phosphoserine	.05	
Glycerophosphoethanolamine	.01	
Phosphoethanolamine	.01	.025
Taurine		.001
Urea	.001	.001
Unknown #1		.005
Aspartic Acid	.001	
Serine	.005	
Glutamine (Asparagine?)		.001
Glutamic Acid		.001
Glycine	.001	
Alanine	.001	.05
<u>Alpha</u> -Aminobutyric Acid	.05	
Methionine		.001
Unknown #5	.05	.001
Isoleucine	.001	.01
Leucine	.001	
Tyrosine	.001	.10
Phenylalanine	.001	.001
<u>Beta</u> -Alanine	.001	
<u>Gama</u> -Aminobutyric Acid	.05	
Ornithine	.01	
Ethanolamine	.	.05
Ammonia *	.01	.001
Histidine	.005	
Tryptophane	.05	
Argenine	.05	

\* Probably this data is contaminated by instrumental artifacts

TABLE VII: Amino Acids For Which Concentrations In Rats' Brains Differ With Stain Between Long Evans and Sprague Dawley Animals

<u>Amino Acid</u>	Significance Level Observed	
	<u>Test 2</u>	<u>Test 3</u>
Glycerophosphoethanolamine		.10
Phosphoethanolamine		.025
Taurine		.001
Urea		.001
Unknown # 1	.10	
Aspartic Acid	.10	.001
Threonine	.025	
Glutamine (Asparagine?)		.001
Glutamic Acid		.001
Valine	.025	
Methionine	.01	.025
Unknown #5	.01	.005
Isoleucine		.005
Tyrosine	.025	.01
Phenylalanine		.10
<u>Beta-Alanine</u>		.10
<u>Beta-Aminoisobutyric Acid</u>		.05
<u>Gama-Aminobutyric Acid</u>	.001	
Ethanolamine	.025	
Histidine		.05
Tryptophan	.025	
Argenine		.01

**APPENDIX 1**

**An Atmosphere Exposure Chamber for Small Animals**

**Herbert B. Chermiside & Arthur Furst**

**IN PRESS: Proceedings of the Western Pharmacology Society**

AN ATMOSPHERE EXPOSURE CHAMBER FOR SMALL ANIMALS

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ABSTRACT

A flexible, inexpensive system has been designed for exposing small animals to various gaseous mixtures and pressures. Construction details for dynamic and recirculating operations are presented. A simple gas scrubbing system for removing toxic metabolic byproducts is described.

A study of the effects of various atmospheric mixtures on the free amino acid content of the brains of rats is being conducted. These studies required an exposure system with considerable flexibility. It was necessary to expose groups of 5 to 10 rats to various mixtures of gases, some of which included expensive inert gases. It was also necessary to conduct experiments above and below ambient pressure.

No commercially available system met all our requirements; those which could be easily modified were too expensive. The exposure systems described in the literature ranged from the simple Bell jars, glass aquaria and desiccators (1,2,3), to a very complex system with individual capsules for each animal (4). Again, equipment with the flexibility needed for our uses was not economically built. This paper describes the system designed and built to satisfy our requirements.

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\* Supported by National Aeronautics & Space Administration grant NGR -05- -029-001. Further reproduction is authorized to satisfy the needs of the United States Government.

This system is designed in a modular fashion; a number of subsystems can be used as needed to provide the particular exposure conditions desired. The block diagram in Figure 1 shows the various subsystems in their relationships to the exposure chamber.

## CONSTRUCTION AND FUNCTION OF SUBSYSTEMS

### Exposure Chamber 1

Chamber 1 was designed for exposures at ambient pressure with dynamic gas flow. It was constructed from two 5 gallon reagent shipping canisters. The top was removed from one canister and both the top and bottom from the other. A sketch with major dimensions is presented in Figure 2. Hose nipples, legs, floor supports, and bolt fittings were braized to the canisters. The "front end" fittings were duplicated on both canisters. The canisters may be joined, or one half only used as a half-sized chamber. Two removable floors, full sized and half sized, were constructed from expanded steel. The front cover was fabricated from 1/2" lucite. The completed unit was painted with a household type catalyzed epoxy paint.

This chamber is only nominally airtight; therefore, it is operated slightly above ambient pressure. The increment is obtained by flowing the exhaust through a head of 1" to 2" H<sub>2</sub>O. This small pressure increase above ambient prevents contamination of the exposure atmosphere by environmental diffusion. After placing animals in this chamber the cover is fitted loosely and the chamber is purged at a rate of 1 to 3 chamber volumes per minute for 3 to 5 minutes. Thereafter the input flow is reduced to approximately 1/10 chamber volume per minute.

### Exposure Chamber 2

Chamber 2 was designed for sealed exposures with a recirculating flow through a gas scrubbing loop. It may be used at

pressures above or below ambient. A sketch of this chamber with major dimensions is shown in Figure 3. It was constructed from 12" I.D. welded mild steel pipe, with wall thickness of 1/4". The turret was a short piece of 4" I.D. pipe of similar thickness. Flanges, legs and cover plates were fabricated from 1/4" steel plate. All joints were beveled and arc welded. The main unit and cover plates were sandblasted and painted with an industrial grade catalyzed epoxy paint and its appropriate primer.<sup>1</sup>

Two additional end plates were fabricated from 1" lucite. Steel washers, the same diameter as the flanges and 1/8" thick, were fixed to the lucite end plates with epoxy cement in order to equalize stresses when the cover plates were bolted up. Gaskets were cut from 3/8" neoprene sheet. The floor was made from expanded steel. The turret cover plate was used for all input and output fixtures. These consisted of one 7-pin cannon plug and 6 bulkhead fittings to accept 1/4" NPT gas and tubing fittings. Gas flow inside the chamber was controlled by proper orientation of elbows made from stainless steel automotive brake line tubing. A similar piece of tubing was made to serve as a water point supplied by an external reservoir. All fittings were sealed to the turret cover with "plastic rubber". It was necessary to seal all other threaded fittings with pipe joining compounds each time they were assembled.

When chamber 2 is functioning in a sealed system there are two inputs. After the animals are introduced to the chamber, the cover is placed loosely on the chamber. It is then purged with gas from the input system for 3 to 5 minutes at 1 to 3 chamber volumes per minute. A gas scrubbing loop is also in operation at this time. The cover plate is then sealed and the input and putput main valves are operated to bring the chamber

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<sup>1</sup>Mobil Chemical Co., Maintenance and Marine Coatings Division



to the desired pressure. A secondary input valve joining the chamber and the oxygen supply is then opened so that oxygen may be obtained through the demand valve. During the exposure the circulating system is operated at a rate of 1/10 to 1/5 chamber volumes per minute.

#### Demand Valve

A demand valve was constructed by modifying a government surplus bail out oxygen regulator.<sup>2</sup> The unit purchased included an oxygen bottle and a first stage preset regulator; these were discarded. The oxygen input micron filter and valve unit was removed and discarded. This part and a bellows are under a plate with a screw adjusting point. The four spacer washers in the bellows were discarded. The "suit pressure" circuit, under an adjacent plate was rendered inoperative. The cover for the sensing diaphragm was removed and machined as follows: 2mm was removed from the inner end of the screw adjusting post; 1 mm was removed from the area of the inside of the cover around the screw adjusting post. The cover was drilled and tapped for a gas fitting which was installed with an epoxy resin cement as reinforcement. Similar cement was used to seal the original pressure sensing hole in the cover. Adding this fitting and sealing the holes is not needed if the entire valve is at the reference pressure. The 3 levers attached to the main valve and operated by the diaphragm were bent slightly. This step is a cut-and-dry operation; it will determine the sensitivity of the valve. The valve was set to supply at a pressure differential of 1" H<sub>2</sub>O. The entire assembly was attached to a standard gas regulator.

When the system is operated with recirculating flow using the gas scrubbing loop, oxygen in the chamber is metabolized by the animals. This creates a small drop in pressure which is sensed

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<sup>2</sup>Bendix Aviation Corp., Eclipse-Pioneer Division. The unit modified was a regulator, oxygen, pressure demand, bail out, type F-1, stock #5509-50C3619.

by the demand flow valve and the pressure is returned to the pre-set exposure pressure with oxygen. The  $P_{O_2}$  will fluctuate  $\pm 1$ " of water from the  $P_{O_2}$  of the original mixture.

#### Gas Scrubbing Loop

The gas scrubbing loop is shown in block diagram in Figure 4. The first stage is a separable vacuum trap 6 cm diameter and 50 cm long. The inner tube is 2.5 cm diameter and 40 cm long. This trap is placed in a 25 liter wide mouth liquid nitrogen (LN) refrigerator.<sup>3</sup> A bypass with appropriate valves is provided so that the trap may be disconnected from the circuit for cleaning without interrupting gas flow. Provision is also made for a gas analyzer to be connected in parallel with this trap. The second stage of this loop is a gas washing bottle filled with concentrated  $H_2SO_4$ . This is followed by a stage containing KOH pellets or 50% KOH solution. A heavy duty aquarium pump<sup>4</sup> and a gas flow meter complete this circuit.

The gas scrubbing loop was designed primarily to remove carbon dioxide and water, metabolic waste products from the animals, from the chamber atmosphere. The vacuum trap is placed in the LN refrigerator in such a manner that it does not quite contact the LN. Although the atmosphere above the LN in the refrigerator is sufficiently cold to freeze out of the gases in the trap water and carbon dioxide, it is not cold enough to freeze or liquify components of the exposure gas. Thus the composition of the exposure atmosphere remains constant. Periodically the vacuum trap must be bypassed and cleaned of frozen wastes. The chemical absorbants are provided as a back-up system and operate primarily during the period that the vacuum trap is being cleaned.

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<sup>3</sup>Linde type LNR-25B

<sup>4</sup>Conde Co., Sherril, New York, Model #0

## Miscellaneous Components

The valves, gauges, and controls for gas flow are all commercially available equipment selected for the specific exposure conditions desired. For operation below 2 atmospheres pressure, connections are made with thick wall rubber tubing. However, provisions have been made to use copper or stainless steel tubing at higher pressures. All connective fittings are 1/4" NPT automotive brake system or welding fittings. All gases are obtained commercially in high pressure bottles.

## ASSEMBLY OF SYSTEM FOR VARIOUS EXPOSURES

### Dynamic Flow, Ambient Pressure

For this exposure, chamber 1 is used. If the input has more than one component gas these are mixed on a volume-to-volume basis in a gas mixer.<sup>6</sup> The output is vented through a small head of water to a fume hood.

### Dynamic Flow, Non-ambient Pressure

For these exposure conditions chamber 2 is used. For pressure above ambient a metering valve is placed in the exhaust flow. Flow through this valve and the pressure of the input may be adjusted to provide the exposure pressure desired. For less than ambient pressure a low pressure source is connected to the exhaust metering valve and another metering valve is installed between the input mixer and the chamber. The desired pressure may be obtained by proper adjustment to these two valves.

### Recirculating Flow, Ambient Pressure

Chamber 2, the gas scrubbing loop, and the demand oxygen supply provide these conditions. The chamber is purged, filled and then sealed from the mixing input system. The only exhaust is an emergency dump valve.

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<sup>6</sup>Matheson Model 665

## Recirculating Flow, Non-ambient Pressure

The same components provide the basis for this exposure. An additional chamber with a volume of about 1 liter is required for the demand oxygen supply. This chamber is connected with an open circuit to the fitting on the pressure sensing side of the diaphragm of the demand valve. It is also connected through a valve to the exposure chamber. After the exposure chamber reaches the desired pressure this valve is closed. The smaller chamber serves as a reference for the demand valve to "remember" the desired pressure. After being purged with the exposure mixture, the exposure chamber is sealed. Pressure from the supply tanks or vacuum from the low pressure source is used to bring the chamber to the desired pressure.

## DISCUSSION

Dynamic flow systems have been used at ambient and greater than ambient pressure. Mixtures of oxygen and inert gases or 100% oxygen have been used. As no provision has been made for cleaning the exposure chamber while retaining the exposure conditions, no exposures have been longer than 72 hours.

It has been reported that differing responses in experimental animals are found with dynamic and with recirculating systems (5,6,7) and it has been suggested that this difference is due to toxic metabolic waste products or their oxidation products which may accumulate in the atmosphere of the recirculating system (8). We believe that the LN trap as a gas cleaner obviates this problem. We have observed, when using the chemical system only for several hours, that upon opening the chamber there is a strong odor like that of very dirty animal quarters. However, when the LN trap is used for a similar period, the odor in the chamber is very faint, and when the trap is cleaned the odor is strong in the waste ice and dry ice. The animals appear to suffer less distress when the LN trap is used. For these reasons we believe that the toxins mentioned above are removed by the LN trap. We have not followed this up with chemical analyses of the waste materials.

Necropsies were performed on rats exposed for 72 hours in the sealed system using the LN trap. The mixture was helium with 20% oxygen at 1 atmosphere. Gross observation of all organs showed no abnormalities. In particular, the lungs appeared perfectly normal and contained no fluids. In contrast, the lungs of animals similarly exposed with only the chemical purifier often showed signs of edema.

Another advantage of the LN trap was strictly fortuitous: by controlling the flow rate through the recirculating system we can cool the chamber to compensate for metabolic heat generated by the animals. In a dynamic system this cooling effect comes from the expansion of the supply gases. With both techniques, the temperature has ranged between 22 - 26°C, an acceptably normal range for rats (7). Therefore we have not had to continue development of special temperature controls.

This comparatively inexpensive system has applications in toxicology, anesthesiology, exobiology - in practically any study calling for a non-ambient atmosphere. We expect to continue expanding the flexibility of this exposure system.

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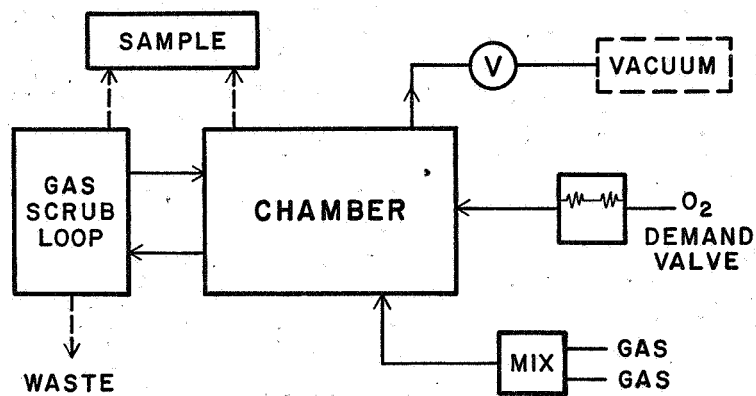


FIG. 1. BLOCK DIAGRAM OF SYSTEM.

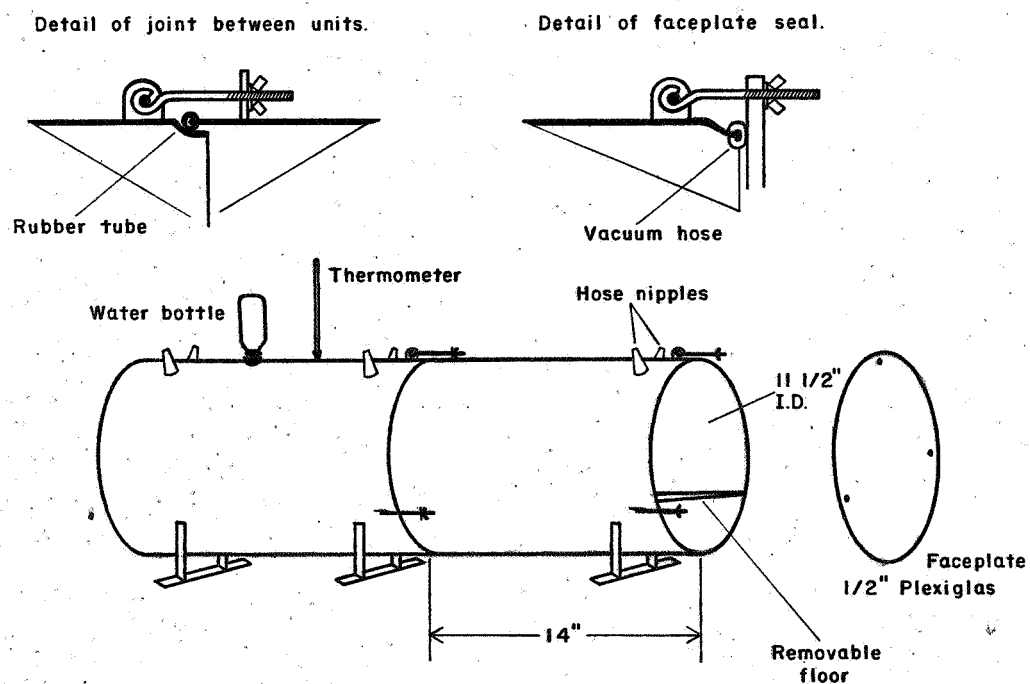


FIG. 2. EXPOSURE CHAMBER I.

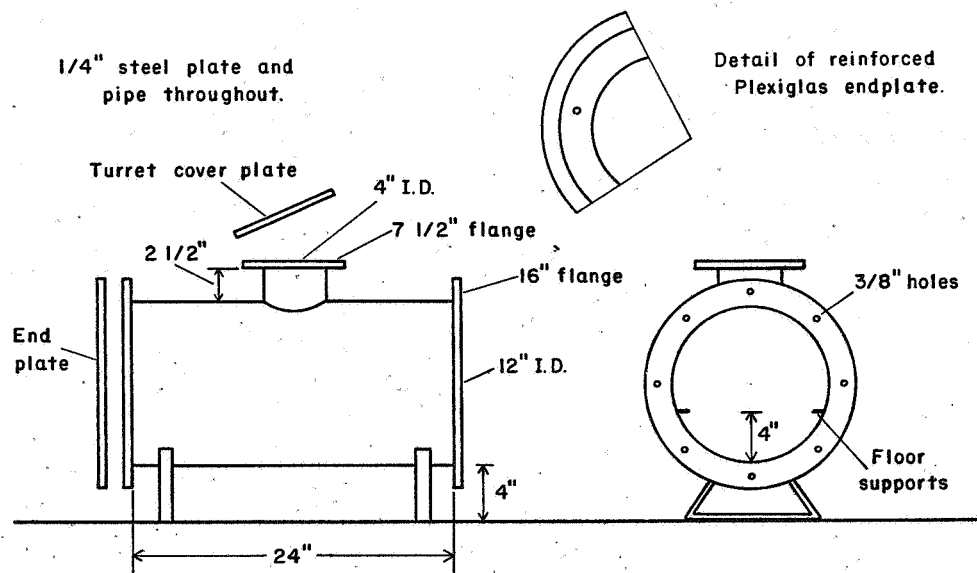


FIG. 3. EXPOSURE CHAMBER 2.

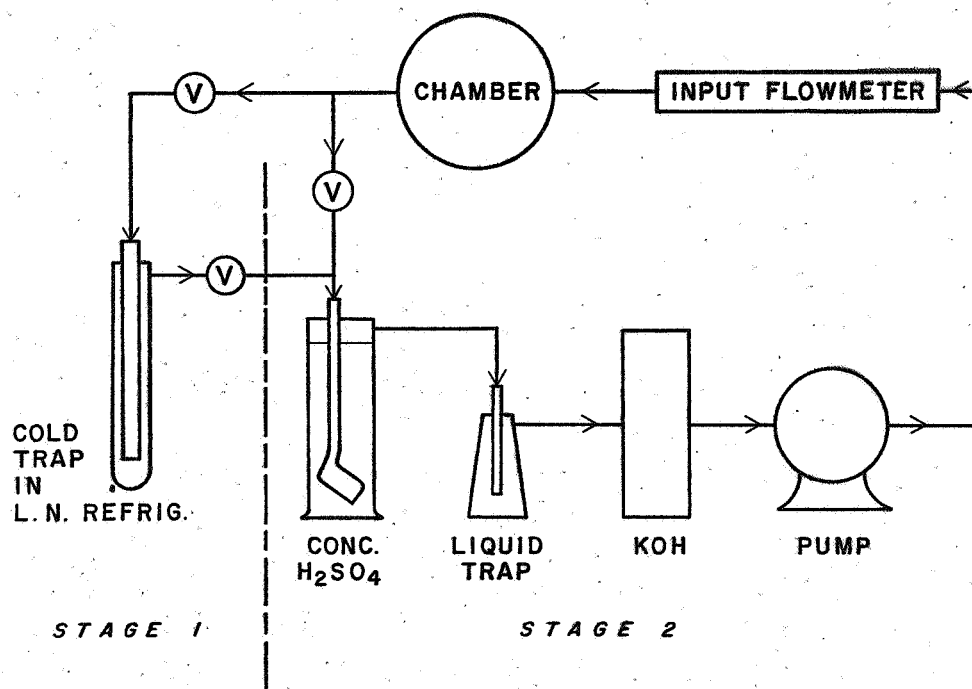


FIG. 4. FLOW DIAGRAM OF GAS SCRUBBING LOOP.



APPENDIX II

Some Statistical Techniques for

The Programma 101

Herbert B. Chermiside & George Ledin, Jr.

ICB WORKING PAPER

SOME STATISTICAL TECHNIQUES FOR

THE PROGRAMMA 101

Herbert B. Chermside & George Ledin, Jr.

[ICB Working Paper]

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2. General Operating instructions: Programma 101
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7. Analysis of Variance, Two-way

**SOME STATISTICAL TECHNIQUES FOR THE PROGRAMMA 101****Herbert B. Chermiside & George Ledin, Jr.****[I.C.B. working paper]****INTRODUCTION:**

This paper is a collection of instructions for the use of the Programma 101 in certain statistical evaluations of experimental data gathered in the laboratories of the Institute. These instructions refer to programs already prepared and on hand.

We have attempted to write the instructions in such a manner that no training will be needed to operate the Programma if the instructions are followed precisely. Each write-up has a short discussion of the mathematics involved, but we recommend that if anyone has questions regarding the applicability of any technique to his data and experimental design, that he discuss the matter with a statistician.

The first article contains general instructions for the use of the Programma. The articles following are write-ups of specific statistical and computational routines which have been developed and used. Magnetically recorded programs for these routines are available. Several of the routines

are designed to be used with special worksheets, examples of which are included in the write-up.

These articles are written somewhat anthropomorphically with the hope that this style will communicate the essence of the computer's operation without getting the reader bogged down in technical language. We have adopted the convention of using underlined full capitals for the names of the controls on the computer.

**GENERAL OPERATING INSTRUCTIONS: Programma 101****The Computer:**

The Programma 101 is a desk-top electronic computer with a keyboard input and a printed tape output. It will add, subtract, multiply, divide and take square root directly. It can be programmed to do sequences of these operations, and to make certain logical decisions. It handles only numerical data. It has a maximum of five memory registers, each of which contains numbers of up to 22 digits, plus or minus sign, and decimal point; these may be split into 10 registers which have half the capacity. There are techniques for storing more smaller numbers, but these techniques are complex and should be left to an experienced programmer to use. Instructions are recorded on magnetic cards which, when passed through the computer, "teach" it what to do for a specific computational routine.

In general, to operate the computer one must do the following:

- 1) Turn it on and clear it of previous information.
- 2) Program it with instructions -- usually with prepared magnetic cards.
- 3) Input data in the proper sequence, sometimes following this with a command to start calculations.
- 4) Stand back and let it do your work -- the answers will be printed on paper tape.
- 5) Some programs are quite long; for these it will be necessary to put intermediate answers on a worksheet for later re-inputting.

### Turning On and Clearing:

The ON-OFF switch is a push-switch on the front edge of the keyboard. You can hear the computer's mechanism when it is on. The GEN. RESET key in the upper left of the keyboard clears the computer - makes it "forget" everything and readies it for further use. On the upper right of the face of the computer there is a red light; when this glows, there has been some error in the instructions or operating conditions and the computer will stop, or cease to continue with the routine. Whenever this light goes on (as it always does when the computer is turned on) one must press GEN. RESET and start anew, or press an operation key (specified in the write-ups where applicable) and re-input data.

### Programming:

Although the computer can be programmed manually at any time, it is recommended that only prepared programs be used. These programs are recorded on magnetic cards. These cards will each hold two programs; only one is recorded by the computer at any one time.

Turn the computer ON and clear it with GEN. RESET. Make sure that RECORD PR. and PRINT PR. are OFF. These switches are two-position push-to-operate switches which are ON when in IN position and Off when in OUT position. It is suggested that both switches be operated to make sure they are OFF; do not trust that they look to be in the out position.

Insert the magnetic card with the printed face toward

you. The computer will pull it in and pass it through, picking up the information on the way. The magnetic cards have two programs on them. The computer "reads" the program on the lower half of the card -- the one the name of which you can read right side up as you put the card in.

#### Decimals:

To the right of the keyboard is a DECIMAL WHEEL. This should be set for the desired number of places to the right of the decimal point according to the write-ups. To enter decimals on the keyboard, enter directly using the . key. In the case of a number less than 1, the zero to the left of the decimal MUST be entered, e.g. for a value 0.032, the entire number must be entered; never enter .032.

#### Entering Constants in Memory:

There are several memory banks. Constants may be entered in up to ten of these as called for in specific write-ups. These memory banks may be split. Thus, a program may call for the use of, say, memory D and memory "E-split" (sometimes written "e" or "E/").

When instructions call for entering a value into a memory bank, use the following procedure:

Enter the value on the keyboard.

Press the key for the correct register; if a split register is called for, also press the SPLIT key.

Press the "go to M" key -- M.



Entering Data:

The program write-ups call for entering data. This is done in accordance with the specific instructions, but in general the following technique is used:

Enter value on numerical keyboard.

Press START key -- the largest key on the keyboard.

Operation Keys:

The keys V, W, Y and Z are operation keys. They cause the computer to execute an operation, to jump to another part of the program, or to perform other operations, depending on the program. These keys should be used as described in the write-ups. It is customary to use V as the initial command in programs written in I.C.B. Therefore, when incorrect data has been entered, one can usually start the computation over by pressing V and re-inputting the data.

Clearing Keyboard:

When a wrong datum has been entered on the numerical keyboard, but before the START key has been pressed, that entire number can be erased from the keyboard by pressing the CLEAR key. Re-input the number correctly. Note that when a number is entered with START the computer prints this number on the tape. At this point it is too late to use CLEAR; start the entire computation anew.

Average Values Computations:

A Program and work sheet have been developed for the computation of a measure of central tendency and the dispersion of data.

This program computes mean, standard deviation and standard error of the mean for any series of numbers. Mean is calculated according to the formula:

$$\bar{X} = \frac{\sum x_i}{N} \quad ;$$

standard deviation is calculated according to the formula:

$$s.d. = \sqrt{\frac{\sum x_i^2 - N\bar{X}^2}{N-1}} \quad ;$$

standard error of the mean is calculated according to the formula:

$$s.e. = \frac{s.d.}{\sqrt{N}} \quad ;$$

This program also counts the number of entries and prints N. Further, it prints the sum of all entries. These values are conveniently retained if needed for some other computations.

Use program #1. Clear computer, insert program card, set DECIMAL WHEEL as desired (usually one more place than the desired answer and the answer should be rounded before recording). Press V. Enter  $X_i$ , press S; do this for each value of  $X_i$ . When all values have been entered, press W. Program will print the following in order: N,  $\sum x_i$ ,  $\bar{X}$ , s.d., s.e.

The use of the worksheet on page 3.2 is self evident. It is recommended that the worksheet be used if further computations are made using any of the output data of this program.



## Reduction of Data From Amino Acid Analyzer

### General:

The output of the Beckman 120-C Amino Acid Analyzer consists of a series of peaks on optical density scale graph paper. The area under the peaks is proportional to the amount of amino acid present. For most purposes, this area can be estimated with sufficient accuracy by calculating the area of a rectangle with a height (H) equal to the height of the peak and with a width (W) equal to the width of the peak at mid-height. Each peak in an analysis must be compared to an internal standard, in order to account for instrumental variation between analyses. Because the response of the instrument to each amino acid is slightly different, if one wishes quantitative values for the amounts of amino acids, each standardized answer must be corrected with a factor comparing the response for the desired amino acid with that for the internal standard.

### Worksheets and Calculation Sequence:

The Amino Acid analysis Worksheet on pages 4.6 & 4.7 provides on its face blanks for the following information used in the data reduction:

brain weight (or any pertinent tissue weight)

composition and amount of standard

H, W, and Constant for standard on normal and attenuated scales (C,  $C_{alt}$ ).

On the back side are positions for the following:

Brain weight

C and  $C_{alt}$  for standard

H and  $H_{alt}$  for each peak

W and  $W_{alt}$  for each peak

Volume of sample made from tissue sample ( $V_{tot}$ )

Volume of sample applied to analyzer column ( $V_{col}$ )

nM/g or  $\mu$ M/g - the answer after computation

Height (H) should be recorded in absorbance units

from the scale on the chart paper. Width at the midpoint (W) is calculated by the following method, usually by hand (no program is provided):

Measure:	gross height
Subtract:	baseline
Get as result:	Height (H) (transfer to worksheet)
Divide H by:	2
Get as result:	1/2 H
Add:	Baseline
Get as result:	Position on chart of half-height

Measure the width of the peak at the half-height point.

This is usually measured in "dots": the chart printer spaces its points regularly along the horizontal axis, and is the most accurate measure of W. Note that in "counting dots" it is the spaces between dots that are being counted, so start by counting the first one as "dot zero".

With the data now on the worksheet, the standard constants, the amount of amino acid in standard units, and the absolute amounts of amino acid can be calculated using prepared programs and the Programma 101.

Calculation of Constant:

The internal standard provides H and W values from which a constant is calculated for each chromatogram. The formula for calculation is:  $\frac{H.W.}{\mu M}$

Use Program C, clear computer, insert program card. Set DECIMAL WHEEL at 2. Press V to start program. Enter H, press S. Enter W, press S. Enter  $\mu M$ , press S. The computer automatically calculates and prints C. Round answer to one place and record.

Calculation of Amino Acid Amount in Standard (Nor-leucine) units.

The amount of amino acid present in a specific brain analyzed is calculated in reference to the Nor-leucine Standard and is measured in "Nor-leucine units". The formula for this calculation is:

$$\mu M/g = \frac{H.W.V_{tot}}{C.g.V_{col}}$$

where:

H = height in chart units

W = width in dots

C - constant from standard (Nor-leucine) peak for this analysis

g = grams brain or other tissue for this analysis

$V_{tot}$  = total volume of sample made from this brain (2.0ml is usual)

$V_{col}$  = volume of sample applied to column for this analysis (0.5 ml is usual)

Use program "M/g". Clear computer, insert program card, set DECIMAL WHEEL equal to 4. Enter value for g in memory D,  $V_{tot}$  in E, and  $V_{col}$  in F. Press V. Enter H, press S. Enter W, press S. Enter C, press S. Computer automatically calculates and prints answer in  $\mu\text{M}$  per gram of sample weight. Round this answer to 3 decimals and record.

Note: If g,  $V_{tot}$ , and  $V_{col}$  are inserted with the correct decimals and H, W and C without decimals, the answer will be printed in nM per g. In this case, set decimal wheel to zero.

#### Conversion of Standardized Amino Acid Amounts to Absolute Amounts:

A correction factor (Cf) can be calculated which will enable one to calculate the absolute amount of amino acid from standardized amounts; this factor must be calculated for each amino acid. Data is drawn from calibration analyses. The formula used is:

$$Cf = \frac{C_{AA}}{C_{std}} = \frac{H_{AA} \cdot W_{AA} \cdot \mu\text{M}_{std}}{H_{std} \cdot W_{std} \cdot \mu\text{M}_{AA}}$$

The program "Cf" is written to use the third expression, in order to input information directly from Amino Acid Analysis worksheets in which known amounts of amino acid rather than tissue extracts provided the sample. This calculation should be made at least five times for each amino acid and an average value taken.

Use program "Cf". Clear computer, insert program card, set DECIMAL WHEEL at 4 (round answer to 3 places). Press V. Enter  $H_{AA}$ , press S; enter  $W_{AA}$ , press S; enter  $H_{std}$ , press S; enter  $W_{std}$ , press S; enter  $\mu M_{AA}$ , press S; enter  $\mu M_{std}$ , press S. Computer will print Cf and return to the beginning, ready to accept another batch of data without further clearing.

To obtain absolute values for any amino acid, merely multiply the value in standard units by this correction factor.







### Computation of Students "t" Statistic:

Student's "t" is the name of a statistic useful for testing the significance of a possible difference between two small groups of data (usually,  $N < 30$ ); for example, data from control and experimental groups. Mathematically, this statistic ~~assumes~~<sup>ASSUMES</sup> that the data within each group is normally distributed (i.e., follows the "bell curve"). There is no truly reliable way of knowing that this condition is met, although there are tests of the closeness of a set of data to this curve which can be supplied by a statistician. In practice, however, it has been found that this requirement is sufficiently satisfied if the variations within the data from a group are due to biological variation (e.g., several animals are measured on one parameter) or if they are due to random sampling from a homogeneous or normally distributed population (e.g. several readings of the same datum). If there are doubts about the applicability of these rules of thumb, a statistician should be consulted. This is especially so when there is reason to believe that the data undergoing this comparison come from diverse populations, in which case student's "t" should be replaced with Cochran's  $t^*$  or Behren's test. However, for most cases, Student's "t" will be sufficient. This statistic is computed according to the formula:

$$t = \frac{|\bar{X}_1 - \bar{X}_2|}{\sqrt{\frac{S_1^2(N-1) + S_2^2(N_2-1)}{N_1 + N_2 - 2}}}$$

with degrees of freedom equal to:  $(N_1 + N_2 - 2)$ .

Before computing this statistic, the mean, standard deviation and N of each group must be computed with the averages program. This data should be entered on the "t" worksheet shown on page 5.3.

Use program "t". Clear computer, insert program card, set DECIMAL WHEEL at 3 (most tables of "t" are printed to three decimal places). Press V. Enter  $N_1$ , press S. Enter  $\bar{X}_1$ , press S. Enter  $sd_1$ , press S. Enter  $N_2$ , press S. Enter  $\bar{X}_2$ , press S. Enter  $Sd_2$ , press S. Computer automatically calculates and prints in order: degrees of freedom,  $S_{\text{combined}}$  and "t".

Record these values. Using the calculated degrees of freedom and "t", one may refer to standard tables for the probability associated with these values.



Simple Analysis of Variance:

Simple analysis of variance is a technique for testing the significance of the possible differences among several groups of data on a single parameter and, specifically, discovering the source of significant variation. (Student's "t" statistic is the special case of this test when applied to only two groups.) In this test, the means and the variances of the data in the groups are compared to determine if there is, in effect, a common contributing factor to the observed variances which is not a result of sampling, biological, or other expectable, normally distributed variation. If this factor exists, it should, in a properly designed experiment, be due to the experimental variable. It is suggested that a statistician be consulted before using this type of analysis; although a "powerful" test (i.e., it is quite sensitive to certain types of variables, specifically the ones one wishes to measure), it is an easy test to misapply. This is due to the multiplicity of mathematical models which exist for this test; those models differ in the assumptions made regarding the sources of variance in the data.

Our simple analysis of variance computational routine uses program "simple A.V." and the "simple A.V. worksheet" on page 6.4. The input data comes from completed "Average Values Worksheets" for each of the groups examined. It will handle several groups, with many replications within any group. The total number of groups and replications within

any group. The total number of groups and replications is determined by the capacity of the computer and depends in part on the size of the numbers entered.

Note: If the data to be handled overflows the capacity of the computer, an approximate answer can be obtained by reducing the size of each entry with a constant which provides four significant figures for the largest entry. A judgement must be made concerning the acceptability of the inaccuracy introduced with such a technique; once again, consult the statistician.

This program is designed on a fixed effects model: the variations between data within any given group may be normally distributed (e.g., the result of biological variation or sampling variation); however, the differences in values between groups must be due to a nonrandom factor (i.e. this variation might be caused by experimental variables or by variables due to the selection of the items in the samples, e.g. differing ages of animals).

From raw data this program calculates the sum of squares between groups, the sum of squares total, the sum of squares within groups, the F-ratio, and numbers needed to compute the degrees of freedom. The degrees of freedom are computed by hand on the worksheet, it being a simple computation. The formulae are on page 6.3.

Use program "simple A.V.". Clear computer, insert program card, set DECIMAL WHEEL 2 or 3, depending upon the table of probabilities which will be used. Press V. Start-

ing with the first group, enter each data point  $X_{ij}$  and press S. After all the data in the group have been entered thus, press W. Go to the next group and enter similarly. After all groups have been entered (and W has been pressed for the last group), press Y. The machine will compute and print:  $SS_b$ ,  $SS_t$ ,  $SS_w$ ,  $N$ ,  $m$ , and  $F$ . To repeat this analysis or do the next simple analysis of variance the computer must be cleared and the magnetic card run through again; or else memory units C, c/, D, D/, and E must be cleared by pressing for each of those memory units its key and then the asterisk key.

Source	SS	df	MS	F
between groups	$\sum_j \frac{(\sum_i X_{ij})^2}{n_j} - \frac{(\sum_{ji} X_{ij})^2}{N}$	$m - 1$	$\frac{SS \text{ between}}{m - 1}$	$\frac{MS \text{ between}}{MS \text{ within}}$
within groups	$\sum_{ji} X_{ij}^2 - \sum_j \frac{(\sum_i X_{ij})^2}{n_j}$	$N - m$	$\frac{SS \text{ within}}{N - m}$	
totals	$\sum_{ji} X_{ij}^2 - \frac{(\sum_{ji} X_{ij})^2}{N}$	$N - 1$		



## WORK SHEET: SIMPLE ANALYSIS OF VARIANCE, FIXED EFFECTS

Constant Factors:

variable factors:

Source of Data: Group 1  
 Group 2  
 Group 3  
 Group 4  
 Group 5  
 Group 6  
 Group 7  
 Group 8  
 Group 9  
 Group 10

Instructions: Using program "Simple Analysis of Variance, Fixed Factors" systematically input each piece of data. Use the method described on the program envelope. Transfer the results to this sheet at the places indicated. Calculate df as indicated. Look up in standard tables the probability level of the F value calculated.

SUMMARY OF RESULTS

Source	SS	d.f.	F	P
Between treatments	$C\Diamond =$	$m-1 =$	$A\Diamond =$	
Total	$A\Diamond =$	****	****	****
Within treatments	$A\Diamond =$	$N-m =$	****	****

N =  $E\Diamond =$ m =  $D\Diamond =$ 

COMMENTS:

Date: \_\_\_\_\_

Calculator: \_\_\_\_\_

### Two-Way Analysis of Variance:

(It is suggested that the introduction to simple analysis of variance be read before continuing.)

Two-way analysis of variance is a technique for testing the significance of the possible differences among several groups of data on two parameters at the same time. It is an extension of the technique of simple analysis of variance into two analytic dimensions. The same cautions apply to the use of this technique as to simple analysis of variance. However, in oversimplified terms, this technique is used to test for the effects of two experimental variables and for the interaction of these two variables.

The system of programs and worksheet developed for this analysis follows the mathematical model of fixed effects between groups. It uses the formulae listed on page 7.3. Input data comes from completed "Average worksheets" for each of the groups examined. Again, the capacity of the computer may be taxed by too large an amount of data; in case of overflow consider correcting as described under simple analysis of variance.

With the fixed effects model there may be, within any one group, replications which vary, randomly; for example, a number of different animals would be examined and a random variation due to biological variation would exist within each group. However, between the groups, the various values which either of the two major parameters may take are determined by the experimenter; for example, there may be several groups

differing in ages and differing in exposure time. Due to the length of calculations involved the computation of this statistic is performed with five separate programs, the first four of which provide intermediate answers. The output of the fifth program gives the degrees of freedom, the mean squares for rows, columns, and interaction effects, and for the error term (variance within cells), and the appropriate F ratios.

Source	SS	df	MS	F
Rows	$\frac{\sum_k (\sum_j X_{ijk})^2}{cn} - \frac{(\sum_{jki} X_{ijk})^2}{N}$	r - 1	$\frac{SS \text{ rows}}{r - 1}$	$\frac{MS \text{ rows}}{MS \text{ within}}$
Columns	$\frac{\sum_j (\sum_{ki} X_{ijk})^2}{rn} - \frac{(\sum_{jki} X_{ijk})^2}{N}$	c - 1	$\frac{SS \text{ col.}}{c - 1}$	$\frac{MS \text{ col.}}{MS \text{ within}}$
Inter-action	$\frac{\sum_{jk} (\sum_i X_{ijk})^2}{n} - \frac{\sum_k (\sum_{ji} X_{ijk})^2}{cn} - \frac{\sum_j (\sum_{ki} X_{ijk})^2}{rn} + \frac{(\sum_{jki} X_{ijk})^2}{N}$	(r - 1)(c - 1)	$\frac{SS \text{ int.}}{(r - 1)(c - 1)}$	$\frac{MS \text{ int.}}{MS \text{ within}}$
within cells	$\sum_{jki} X_{ijk}^2 - \frac{\sum_{jk} (\sum_i X_{ijk})^2}{n}$	rc(n - 1)	$\frac{SS \text{ within}}{rc(n - 1)}$	*****
Total	$\sum_{jki} X_{ijk}^2 - \frac{(\sum_{jki} X_{ijk})^2}{N}$	(rcn) - 1 or N - 1	*****	*****

Computation of Two-Way Analysis of Variance:

For each group the "average values" worksheet should be completed. Some data from this is entered into Steps 1 and 2 of the two-way analysis of variance worksheet. The "two-way AV" worksheet on page 7.7 provides a matrix which will accept up to <sup>five</sup>~~five~~ different groups on each of two dimensions. Fill in the headings of the matrix with the various values of the two different parameters. Within each cell of the matrix, place the values, from the "average values" worksheets, of the sum of x's ( $\Sigma x$ ) for the groups under the matrix heading relating to that cell. When the matrix is completed, fill in the number of rows and the number of columns (r & c).

STEP 2

$N_{total}$  and  $SS_{total}$  must be computed as intermediate values and for further use. For this use Program #2. Clear computer, insert program card, set DECIMAL WHEEL for any applicable value less than 8. Press V. Enter each  $X_{ijk}$ , press S. (These  $X_{ijk}$  are the raw data points from all of the group averages worksheets; i.e., every reading. The order in which these are entered makes no difference so long as all data points are entered once only.) When all are entered press W. The machine automatically computes and prints  $N_{total}$  and  $SS_{total}$ . These values are entered on the two-way worksheet.

Interim sums of squares must be calculated using program 3. This program must be operated twice!!! The values  $X_{ij}$  are the values which have been entered into the matrix

in Step 1. Use Program 3. Clear computer, insert program card, set DECIMAL WHEEL to any number less than 8. Press V. Enter  $X_{ij}$ , press S; enter all  $X_{ij}$  thus, reading across rows in the matrix. At the end of the row press W and go to the next row. When done (after pressing W for the last row) press Y. The program prints three intermediate sum of squares. These should be entered in Step 3 of the two-way A-V worksheet at value 1, value 2 and value 4. Repeat this entire operation entering all  $X_{ij}$  reading down columns, pressing W at the end of each column. The machine will compute three values as before. Enter these in order as values 1, 3 and 4. (If the values for 1 and 4 differ in the two operations of program number 3 an error has been made and the program should be done over.)

From data now entered on the two-way A-V worksheet four sums of squares may be computed using Program #4. Clear the computer, insert program card. Set DECIMAL WHEEL to any value less than 8. Press V to start program. From Step 1 on the worksheet enter R, press S; enter C, press S. From Step 3 on the worksheet enter value 1, press S; enter value 2, press S; enter value 3, press S; enter value 4, press S. From Step 2 on the worksheet enter  $SS_{total}$ , press S; enter  $N_{total}$ , press S. The machine will now compute automatically and print out in order these sums of squares:  $SS_{rows}$ ,  $SS_{columns}$ ,  $SS_{interaction}$ ,  $SS_{within}$ . Record these values in Step 4 of the worksheet.

From the data now available the degrees of freedom, mean squares, and F-ratios for the two-way analysis of variance may be computed using Program 5. Clear the computer, insert program cards. Set DECIMAL WHEEL at any value between 2 and 8. From Step 1 enter R, press S; enter C, press S. From Step 4 enter  $SS_{\text{rows}}$ , press S; enter  $SS_{\text{columns}}$ , press S; enter  $SS_{\text{interaction}}$ , press S; enter  $SS_{\text{within}}$ , press S. From Step 2 enter  $N_{\text{total}}$  press S. The machine will compute and print out in order the following values:  $df_{\text{R}}$ ,  $df_{\text{C}}$ ,  $df_{\text{I}}$ ,  $df_{\text{W}}$ ; mean square<sub>R</sub>, mean square<sub>C</sub>, mean square<sub>interaction</sub>, mean square<sub>W</sub>;  $F_{\text{R}}$ ,  $F_{\text{C}}$  and  $F_{\text{I}}$ . (For this and all other programs the worksheet is marked with the symbol which is printed beside the output answer.) These answers should be transferred to the worksheet. Use standard tables for finding the probability of the F ratio calculated.

1. Fill out matrix below; transfer EX values from Group Averages Worksheets.

Columns Conditions

Rows conditions					

# rows = r = \_\_\_\_\_  
 # columns = c = \_\_\_\_\_

2. Using all data from Group Averages Worksheets and Program #2, calculate:

N total =  $c \diamond$  = \_\_\_\_\_

SS total =  $A \diamond$  = \_\_\_\_\_

3. Using data from matrix and Program #3 operated twice, calculate interim values:

① =  $A \diamond$  = \_\_\_\_\_

② =  $c \diamond$  = \_\_\_\_\_ [entries made across rows]

③ =  $c \diamond$  = \_\_\_\_\_ [entries made down columns]

④ =  $D \diamond$  = \_\_\_\_\_

4. Using data from steps 1 & 3 and Program #4, calculate sums of squares:

SS rows =  $C \diamond$  = \_\_\_\_\_

SS columns =  $d \diamond$  = \_\_\_\_\_

SS interaction =  $E \diamond$  = \_\_\_\_\_

SS within =  $f \diamond$  = \_\_\_\_\_

5. Using data from steps 1, 2 & 4 and Program #5, calculate df,  $S^2$  and F and enter:

	df	mean square	F	P<
Rows	$b \diamond$ = _____	$c \diamond$ = _____	$c \diamond$ = _____	_____
Columns	$B \diamond$ = _____	$C \diamond$ = _____	$C \diamond$ = _____	_____
Interaction	$A \diamond$ = _____	$d \diamond$ = _____	$d \diamond$ = _____	_____
Within Cells	$e \diamond$ = _____	$D \diamond$ = _____		_____

6. Using standard tables of the distribution of the F ratio, determine and enter the probability of each F above.

Rows variable: \_\_\_\_\_

DATE: \_\_\_\_\_

Columns variable: \_\_\_\_\_

CALCULATOR: \_\_\_\_\_

Constant Factors: \_\_\_\_\_